3) 向敦史、橋本有弘
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Age-related changes of directly isolated satellite cells by genome-wide gene expression analysis
第 31 回日本分子生物学会年会・第 81 回日本生化学会大会合同大会
神戸、2008 年 12 月 9 日ー11 日

6) 橋本有弘
骨格筋幹細胞を用いた腹圧性尿失禁に対する再生治療法開発のための基礎研究
第 11 階 UTP シンポジウム
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Specification of fusion-competent areas of plasma membrane in myogenic cells
EMBO Conference: The molecular and cellular mechanisms regulating skeletal muscle development and regeneration
スペイン、Sant Feliu de Guixols, 2008 年 9 月 23 日ー10 月 1 日

G. 知的財産権の出願・登録状況
なし

厚生労働科学研究費補助金（長寿科学総合研究事業）
分担研究報告書
高齢者腹圧性尿失禁に対する括約筋機能再生治療：
移植用筋細胞の品質管理システムの開発と筋増殖・肥大因子のスクリーニング
研究分担者　上住（池本）円　国立長寿医療センター　細胞再生研究室長

研究要旨

前立腺全摘出手術時に摘出した高齢者の腹直筋あるいは錐体筋より分離、培養された骨格筋幹細胞を、CD56 及び骨・腎臓・肝臓型アルカリフォスファターゼ（BKL-ALP）を指標とし、フローサイトメーターを用いて品質評価する方法を検討した。その結果、CD56 および BKL-ALP の発現は、移植用筋細胞の「質」を移植直前に判定するための良い指標となることが確認できた。この成果は、移植細胞による治療効果および安全性を担保するために必要な『移植細胞の品質管理システム』の開発に寄与するものと考えられる。

A.　研究目的

自己骨格筋幹細胞を用いた腹圧性尿失禁に対する括約筋機能再生治療を実現するためには、移植用筋細胞の性質を確認し、治療効果および安全性を担保するための『細胞品質管理システム』を確立することが必要である。本分担研究の目的は、フローサイトメーターと筋細胞の増殖・分化能を反映するマーカーを用いて、（1）移植に適した筋細胞と適さない細胞を識別する方法、また（2）移植に適した細胞のみを、生きたまま分離する方法、を確立することにある。本分担研究の成果は、内在性筋幹細胞の増殖・分化能力の維持あるいは亢進に関わるシグナル分子を探索するために有用な実験系を提供する。

B.　研究方法

ヒト筋組織から分離、培養した骨格筋幹細胞の抗体染色

研究分担者の橋本らによってヒト筋組織から分離後、培養によって増幅された筋幹細胞を、0.05% trypsin/EDTA を用いて dish から剥がし、細胞懸濁液を作成した。これを毎分 1,000 回転で 5 分間遠心し、上清を除去した後、2%ウシ胎児血清を含む PBS 100 μl で細胞を懸濁した。ここに、APC (Allophycocyanin) ラベルされた BKL-ALP 抗体を 100 μl 加え、4 ℃、暗所で 30 分反応後、さらに PE (phycoerythrin) ラベルされた CD56 抗体を 20 μl 加え、10 分間反応させた。洗浄後、2%ウシ胎児血清を含む PBS 1 ml で細胞を懸濁し、40 μm のナイロンメッシュフィルターに通してフローサイトメーター用チューブに回収した。

フローサイトメーターによる解析

抗体反応後の細胞懸濁液を定法に従ってフローサイトメーター (BD FACSCalibur)にて展開し、CD56 と BKL-ALP 抗体による細胞の分離パターンを解析した。

さらに、各分画の筋細胞の増殖・分化能力を調べるため、セルソーター（JSAN）を用いて各分画の細胞をソーティングし、I 型コラーゲンコート dish に播種して培養を行った。

増殖用の細胞培養液としては、Primary Myocytes Growth Medium (pmGM, Wada et al., 2002)を用い、分化誘導には、Primary Myocytes Differentiation Medium (pmDM, Wada et al., 2002)を用いた。また、気相は、「二酸化炭素：空気＝1：9」とし、培養温度は 36.6-36.8℃を保つように設定した。

（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

ヒト筋組織から分離、培養された筋幹細胞をCD56とALPを指標としてフローサイトメーターで展開すると、3 分画に分かれることがわかった。CD56、 ALP とも陽性の細胞が約 60%と最も多く、次いで両マーカーとも陰性の細胞が約 25%、CD56 陰性かつ ALP 陽性細胞の割合が約 15%であった。また、 CD56 陽性かつ ALP 陰性の細胞はほとんど検出されなかった。

各分画の細胞をソーティングし、培養により、それぞれの筋分化能を比較すると、CD56、ALP とも陽性の細胞が高い筋分化能を示したのに対し、CD56 陰性かつ ALP 陽性細胞の分化能は低いことが示された 。

これらの結果から、CD56 の発現自体が筋細胞の高い分化能力を反映する可能性が示唆され、このマーカーを用いた、移植用筋細胞の品質評価方法が、移植後の治療効果および安全性を担保するための品質管理システムの開発に有用であると考えられる。

D. 考察

昨年、我々はヒト筋組織から直接セルソーターを用いて CD56 陽性細胞を単離し、この細胞画分に筋幹細胞が濃縮されていることを報告した。このことから CD56 抗体が高い能力を保持した筋細胞をセレクションするための良いマーカーになると考えられる。

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どうかを、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。本年度、我々は、高齢者筋組織から分離、培養された筋細胞から CD56 と BKL-ALP を指標として、高い筋分化能を保持した細胞を識別する方法を確立した。これにより、移植前にその細胞の治療効果および安全性を予測することが可能になると期待される。また、生きたまま細胞を解析できることから、質の悪い細胞が多い場合には、質の良い細胞だけをソーティングし、移植に供することも選択肢の一つとなる。

一方、培養後の筋細胞では、ほとんどの細胞で BKL-ALP の発現が認められるが、培養前の単離直後の筋幹細胞が ALP を発現しているかどうかは不明である。また、CD56 陰性の細胞が培養によって増加する可能性についても検討する必要がある。分離直後のヒト筋幹細胞を、培養した細胞と比較することによってこの点を明らかにし、筋細胞を高い増殖・分化能力を保ったまま、培養下で増幅させるための条件を確立することが重要である。

E. 結論

高齢者の筋組織から分離後、培養によって増幅された筋細胞から、CD56 と BKL-ALP を指標として、高い筋分化能力を保持した細胞を識別する評価方法を確立した。この成果を発展させることにより、自己筋細胞移植の

治療効果および安全性を担保する品質管理システムを確立することが期待できる。

F. 研究発表

1. 論文発表

なし

2. 学会発表

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2) Ikemoto-Uezumi M, Uezumi A, Tsuchida K, Fukada S and Hashimoto N. Age-related changes of directly isolated muscle satellite cells by genome-wide gene expression analysis. 第31回日本分子生物学会年会・第81回日本生化学会大会合同大会, 2008年12月9日～12日, 神戸

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）
分担研究報告書

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

筋細胞移植を高齢者腹圧性尿失禁の有効な治療法として確立するために、筋芽細胞の効率的な筋線維への分化を促進する支援技術として、基底膜の主成分である IV 型コラーゲンの筋管形成促進効果について検討した。Duchenne 型筋ジストロイフィー症患者由来の生検標本での病理組織学的所見並びに実験動物を用いた実験的筋再生過程での所見から、筋線維を取り巻く基底膜の主成分である IV 型コラーゲンが、筋芽細胞の筋管形成を促進する事が示唆されてきた。0.2% bupivacaine 投与による成熟ラット腓腹筋壊死後の筋再生モデルにおいて、0.2% bupivacaine と同時にコラーゲン三重鎖形成とコラーゲン合成を妨げる 2, 2'-dipyridyl を同時投与すると、筋管形成時に観察される、筋管周囲の肥厚した IV 型コラーゲンの代わりに、菲薄な IV 型コラーゲンが認められるのみであり、筋再生は著しく遅延する。また、マウスの初代培養筋芽細胞を、増殖培地下に IV 型コラーゲンゲル上で培養すると、培養開始 48 時間後には自発的収縮を示すよじれた紐状の細長い構造を形成する。この構造物を電子顕微鏡で観察すると、多くの細胞で良く発達した粗面小胞体が観察され、一部の細胞突起はアクチン細線維を豊富に持ち、収縮構造の形成が示唆された。IV 型コラーゲンは筋管形成時に収縮蛋白の発現を促進する効果を持ち、基底膜の他の成分とともに、筋管形成を促進することが示唆された。

A. 研究目的

筋細胞移植が高齢者腹圧性尿失禁に対する括約筋機能再生治療技術として有効であるためには、移植した筋芽細胞が効率的に筋線維に分化できることが条件となる。そうした移植筋細胞の分化を促進する条件を決定することができれば、治療法としての筋細胞移植技術の有効性を高めることができる。特に、こうした治療の対象となる高齢者の場合、筋再生能力が加齢とともに劣化しており、筋芽細胞の自家移植を治療手段として用いる場合には、こうした移植の周辺技術が治療効果を高めることが期待できる。

我々はこれまでに、Duchenne 型筋ジストロイフィー症患者由来の生検標本での病理組織学的所見並びに実験動物を用いた実験的筋再生過程での所見から、筋線維を取り巻く基底膜の主成分である IV 型コラーゲンが、筋芽細胞の筋管形成を促進する事を示唆する所見を得ているので、この点をさらに、*in vivo* 筋再生モデルにおいてコラーゲン形成阻害剤が筋再生に与える影響、ならびに IV 型コラーゲンゲル上で培養した筋芽細胞の電顕的観察所見に基づいて検討する。

B. 研究方法

1. 成熟ラット腓腹筋に 0.2%bupivacaine を投与して筋壊死をおこさせた後の筋再生過程を調べる。この動物を対照とし、bupivacaine と同時に、コラーゲン三重鎖形成とコラーゲン合成を妨げる 2, 2'-dipyridyl を同時投与した動物での筋再生過程を比較検討する。経時的に採取した腓腹筋から未固定凍結切片を作成し、ヘマトキシリン・エオジン染色で再生過程を組織学的に検討する。また、抗 IV 型コラーゲン抗体を用いて、IV 型コラーゲンの発現を免疫組織学的に検討する。
2. マウスの初代培養筋芽細胞を、増殖培地下に IV 型コラーゲンゲル上で培養し、自発的収縮を示すよじれた紐状の構造が形成された段階で Karnovsky 液で固定し、電顕観察する。

（倫理面への配慮）

本研究では倫理面で特に配慮すべき問題点はない。

C. 研究結果

1. ラット腓腹筋に bupivacaine を投与して筋壊死をおこさせた後の筋再生過程を調べると、投与後 48-72 時間の、集積してきた筋芽細胞が筋管形成を始める時期に、著しく肥厚し、波打った形状の IV 型コラーゲンが認められる（図 1）。この時期は、筋芽細胞が融合し、筋管形成を開始する時期に当たっている。投与 7 日後の、筋管形成が終了した時点では IV 型コラーゲンの厚さには対照群との差が認められない。
2. ラット腓腹筋に bupivacaine と同時に 2, 2'-dipyridyl を投与後 3 日目には、bupivacaine のみを投与した対照動物での著しく肥厚した IV 型コラーゲンに対し、菲薄な IV 型コラーゲンが認められるのみであり（図 2）、筋再生も著しく阻害されている（図 3）。2, 2'-dipyridyl の筋再生遅延効果は投与後 2 種間でも観察された。
3. マウスの初代培養筋芽細胞を、増殖培地下に IV 型コラーゲンゲル上で培養すると、培養開始 48 時間後には自発的収縮を示すよじれた紐状の細長い構造を形成する。対照とした I 型コラーゲン上ではこうした構造は認められない。このような状態の、IV 型コラーゲンゲル上で培養した細胞を電子顕微鏡で観察すると、多くの細胞で良く発達した粗面小胞体が観察された。一部の細胞突起はアクチン細線維を豊富に持ち、収縮構造の形成が示唆されたが、筋小胞体、横紋構造は認められなかった。

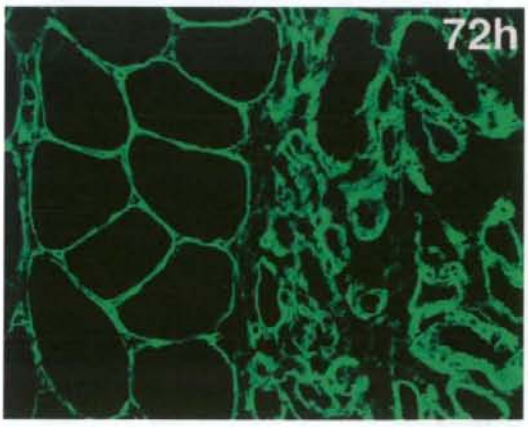

図 1：bupivacaine 投与 3 日後の腓腹筋における IV 型コラーゲンの発現。緑色蛍光は抗 IV 型コラーゲン抗体による免疫活性を示す。向かって左側が bupivacaine が浸透していない正常筋線維を、右側が bupivacaine 投与によって筋壊死が起こった後、再生過程にある筋線維を示す。正常筋周囲の薄い IV 型コラーゲンに対し、再生筋周囲には著しく肥厚し、波打った形状の IV 型コラーゲンが認められる

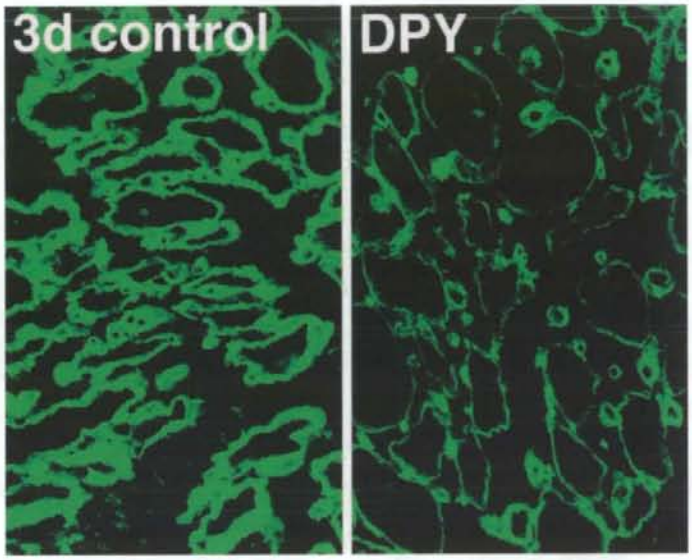

図 2：IV 型コラーゲンの発現に及ぼす 2, 2'-dipyridyl の影響。向かって左側が bupivacaine 単独投与後 3 日目の再生筋周囲に認められる IV 型コラーゲン免疫活性を、右側が bupivacaine と同時に 2, 2'-dipyridyl を投与して 3 日目の再生筋周囲に認められる IV 型コラーゲン免疫活性を示す。後者では IV 型コラーゲンの発現は著しく減弱している。

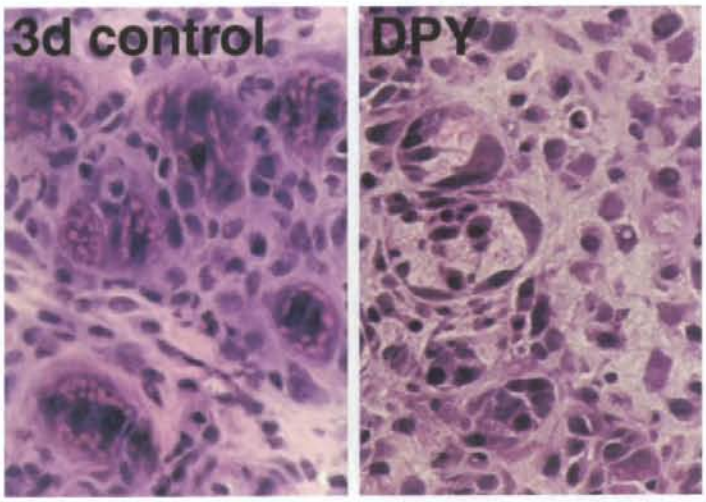

図 3：筋再生に及ぼす 2, 2'-dipyridyl の影響。向かって左側が bupivacaine 単独投与後 3 日目の所見で、筋芽細胞が融合して形成される初期の筋管が認められる。右側は bupivacaine と同時に 2, 2'-dipyridyl を投与して 3 日目の所見で、筋芽細胞の融合像はほとんど認められず、筋再生過程が著しく阻害されていることがわかる。

D. 考察

Duchenne 型筋ジストロイフィー症患者由来の生検標本を検索すると、再生筋周囲の基底膜に肥厚した IV 型コラーゲンが認められる。また、ラット腓腹筋に bupivacaine を投与して筋壊死をおこさせた後の筋再生過程を調べると、集積してきた筋芽細胞が筋管形成を始める投与後 48-72 時間の時期に一致して一過性に、著しく肥厚し、波打った形状の IV 型コラーゲンが認められる。これらの所見から、IV 型コラーゲンが筋管形成を促進する可能性が考えられた。

一方で、bupivacaine と同時に、コラーゲン三重鎖形成とコラーゲン合成を妨げる 2, 2'-dipyridyl を同時投与した動物では、投与後 3 日目には、筋管周囲に認められる IV 型コラーゲンは、bupivacaine のみを投与した対照動物に比べて極めて薄く、筋管形成および筋再生は著しく阻害されていた。これらの所見は、個体レベルでの筋再生過程では、筋管形成期に一致して著しく肥厚する基底膜の IV 型コラーゲンは、筋管形成に促進的に作用する可能性を強く示唆している。

IV 型コラーゲンゲル上で培養した筋芽細胞が形成する、自発的収縮活動を示す紐状の構造の電顕的観察では、良く発達した粗面小胞体と、一部の細胞突起で豊富なアクチン細線維形成が認められ、収縮構造の形成が示唆された。ただし、筋小胞体、横紋構造は認められず、*in vitro* 環境での IV 型コラーゲンゲルの効果は、主として収縮蛋白の発現促進にあることが示唆された。IV 型コラーゲンは基底膜成分として存在しており、*in vivo* では基底膜の他の成分との共同作用によって、収縮蛋白の発現筋管形成を促進しているものと思われる。

E. 結論

基底膜の主成分である IV 型コラーゲンは、筋再生過程で筋芽細胞が筋管形成する時期に一致して著しく肥厚し、筋管形成時の収縮蛋白の発現を促進する効果を持つ事が示唆された。

F. 研究発表

1. 論文発表

なし

2. 学会発表

Song S-Y., Hashimoto N., Kato C., and Adachi E. Functional significance of thick type IV collagen around regenerating muscles in myotube formation.第 31 回に本神経科学大会 2008 年 7 月 9 日～11 日　東京 (Proceedings in Neuroscience Res 61 Suppl 1, S58, O2-F08)

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

研究成果の刊行に関する一覧表

1. Urahama Y, Ohsaki Y, Fujita Y, Maruyama S, Yuzawa Y, Matsuo S, Fujimoto T. Lipid droplet-associated proteins protect renal tubular cells from fatty acid-induced apoptosis. Am J Pathol. 173(5):1286-94. 2008

2. 後藤百万、山本徳則　腎臓・膀胱微小循環と再生　unite 2008　35-36, 2008

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Cardiovascular, Pulmonary and Renal Pathology

Lipid Droplet-Associated Proteins Protect Renal Tubular Cells from Fatty Acid-Induced Apoptosis

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Proteinuria is a major cause of tubulointerstitial kidney damage, and free fatty acids bound to albumin are thought to play an important role in its pathogenesis. However, the mechanism whereby proteinuria causes tubulointerstitial damage to the kidney is unclear. Using primary human renal proximal tubular cells, we observed that albumin replete with fatty acids (rBSA) and defatted albumin (dBSA) complexed with linoleic acid (LA) induced significantly more apoptosis than did defatted albumin alone. Oxidative stress was partially involved in apoptotic induction by LA/dBSA but not by rBSA. Administration of fatty acid-bound BSA increased the number of lipid droplets (LDs) and the LD-associated proteins, adipocyte differentiation-related protein and TIP47. LDs are organelles that store esterified fatty acids, and the LD-associated proteins are presumed to facilitate LD formation. Knockdown of adipocyte differentiation-related protein or TIP47 by RNA interference enhanced induction of apoptosis by both rBSA and LA/dBSA. Apoptotic induction was observed similarly when either rBSA or LA/dBSA was applied to only the apical surfaces of polarized LLC-PK1 cells. The present results suggest that LDs and LD-associated proteins have protective effects against apoptosis induced by fatty acid-bound albumin by sequestering free fatty acids. Therapeutic manipulation of these LD-associated proteins could aid in the amelioration of nephritic diseases. *(Am J Pathol 2008, 173:1286–1294; DOI: 10.2353/ajpath.2008.080137)*

Functional impairment of the kidney during glomerulonephritis correlates better with the degree of tubulointerstitial atrophy than glomerular alteration.[1] Many studies have shown that proteinuria is a major cause of damage in the renal tubules and interstitium.[2–4] Albumin is the predominant protein in the urine of nephritic patients. *In vitro* studies have shown that albumin affects intracellular signaling pathways in proximal tubular epithelial cells,[5,6] induces them to generate various chemoattractants and extracellular matrices,[7,8] and changes the balance between cell proliferation and cell death.[9,10]

The mechanism whereby proteinuria causes tubulointerstitial damage is unclear. Although many studies have concluded that albumin itself is important for the development of the pathological changes, other studies have inferred that free fatty acids (FFAs) bound to albumin play critical roles.[9–11] In mice, FFA-bound albumin caused more severe tubulointerstitial damage, including cortical apoptosis, than albumin depleted of FFA.[12,13] Also, in cultured proximal tubular cells (PTCs), FFA-bound albumin induced apoptosis by activating peroxisome proliferator activated receptor (PPAR)-γ.[11] These results suggest that FFAs are involved in the pathogenesis of tubulointerstitial damage.

FFAs are potentially harmful to cellular functions, but cells readily esterify them to form triacylglycerol and cholesterol esters. The esters are then stored in lipid droplets (LDs), consisting of a globular mass of lipid esters surrounded by a phospholipid monolayer.[14,15] In fact, administration of FFAs increases the number and size of LDs in many kinds of cells in culture, including PTCs.[16] Recent studies revealed that a number of proteins are associated with LDs, and that their expression is increased on FFA loading. Furthermore, engagement of LDs in intracellular lipid trafficking, lipid metabolism, sig-

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nal transduction, and other cellular functions has been suggested.[15,17–19]

In view of the novel functions attributed to LDs and LD-associated proteins, we aimed to study whether their manipulation could modify the effect of FFAs on PTCs. For this purpose, we used primary human PTCs and two PTC lines and confirmed that albumin replete with FFAs as well as defatted albumin complexed with long-chain FFAs induced a higher degree of apoptosis than defatted albumin alone. Using this experimental system, we found that reduction of LD-associated proteins, ie, adipose differentiation-related protein (ADRP) and TIP47, by RNA interference increased apoptosis induced by FFA-bound albumins. The result suggests that manipulation of LD-associated proteins could be a potential target of therapeutic intervention for nephrotic diseases.

Materials and Methods

Reagents

Rabbit anti-human TIP47 antibody was raised as described.[20] Mouse anti-ADRP antibody (Progen; Richlands BC, Queensland, Australia), and secondary antibodies (Jackson ImmunoResearch, West Grove, PA; Invitrogen, Carlsbad, CA; Pierce Chemical, Rockford, IL) were obtained commercially. Bovine serum albumin replete with FFAs (rBSA, catalog no. A9306; Sigma Chemical, St. Louis, MO; and endotoxin <0.1 ng/mg, catalog no. 013-15104; Wako Pure Chemical, Osaka, Japan), and essentially FFA-free BSA (dBSA, catalog no. 017-15141; Wako) were used. Oleic acids (OA, Sigma), linoleic acid (LA, Sigma), and docosahexaenoic acid (DHA, Sigma) were vigorously mixed with dBSA in phosphate-buffered saline at a molar ratio of 6:1,[21] filter-sterilized, and added to culture media at the final fatty acid concentration of 400 μmol/L (OA, LA) or 100 μmol/L (DHA). This concentration of FFA was used because the molar ratio of FFA to albumin could reach up to 8.59,[22] and the albumin concentration in the proximal tubular lumen could be as high as 2.9 mg/ml, or 43 μmol/L.[23] In the current experimental condition, the BSA concentration was 4.4 mg/ml when the fatty acid was used at 400 μmol/L. Vitamin E and desferrioxamine were purchased from Sigma. The FFA content of the rBSA preparation was analyzed by gas chromatography by Mitsubishi Kagaku BCL Inc. (Tokyo, Japan).

Cell Culture

Human primary PTCs (RPTECs; Cambrex, Walkersville, MD), showing characteristics of PTCs *in vivo*, were cultivated in renal epithelial cell basal medium (REBM, Cambrex) supplemented with REGM SingleQuots (0.5 μl/ml hydrocortisone, 10 pg/ml hEGF, 0.5 μg/ml epinephrine, 6.5 pg/ml triiodothyronine, 10 μg/ml transferrin, 5 μg/ml insulin, 50 μg/ml gentamicin, 50 pg/ml amphotericin B, and 0.5% fetal bovine serum). HK-2 cells, an immortalized human PTC line, were obtained from American Type Culture Collection (Rockville, MD) and were cultured in K1 medium. The K1 medium contained Ham's F-12/Dulbecco's modified Eagle's medium (1:1, Sigma), 12.5 mmol/L HEPES, 10% heat-inactivated fetal bovine serum (JRH Biosciences Inc., Lenexa, KS), and select hormones as described.[24] LLC-PK1 cells, a pig PTC line, were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan), and grown in 199 medium (AppiChem, Darmstadt, Germany) supplemented with 10% fetal bovine serum. LLC-PK1 cells were grown in Transwell chambers (Corning, Corning, NY), and used for experiments 3 days after reaching confluence. All of the cells were kept at 37°C in 5% CO_2/95% air.

Immunofluorescence Microscopy and Data Analysis

Cells were fixed with 3% formaldehyde and 0.05% glutaraldehyde for 30 minutes, permeabilized with 0.01% digitonin for 30 minutes, and treated with 3% BSA before immunolabeling for ADRP and TIP47. LDs were stained with BODIPY493/503 (Invitrogen). Images were obtained with an Axiovert fluorescence microscope equipped with Apotome (Carl Zeiss, Oberkochen, Germany) and analyzed with Image J software (National Institutes of Health, Bethesda, MD) as described.[18] Contrast and brightness of micrographs were adjusted by Adobe Photoshop 7.0 for data presentation.

RNA Interference and cDNA Transfection

For RNA interference (RNAi), siGENOME duplexes (Dharmacon Inc., Lafayette, CO) were used to knock down the expression of TIP47 and ADRP. A control RNA duplex, siControl nontargeting siRNA, was also obtained from Dharmacon. RNAi was conducted by electroporation using the Gene Pulser 2 system (Bio-Rad, Hercules, CA).[25] RPTECs (1.5×10^6) were electroporated (600 V, exponential decay 300 ms, 1 pulse) with 10 μg of siRNA in 400 μl of siPORT siRNA electroporation buffer (Ambion, Austin, TX) using 2-mm electroporation cuvettes. Cells were analyzed 3 days after RNAi.

Human ADRP and TIP47 cDNAs were cloned by polymerase chain reaction and inserted into the pcDNA3.1 vector (Invitrogen). The plasmid vectors were introduced into the cells by Lipofectamine2000 (Invitrogen), and the cells were analyzed 3 days later. The transfection protocol was optimized by using pEGFP-C1 vector (Clontech, Mountain View, CA), and the efficiency was estimated as ~60%.

Western Blotting

Total cell lysates were prepared in a sodium dodecyl sulfate-sample buffer, an equal amount of protein (30 μg) was electrophoresed in 15% acrylamide gels, and the protein was transferred to nitrocellulose membranes. The blots were incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. The reaction was detected with the SuperSignal West dura extended duration substrate (Pierce).

Apoptosis and Cell Viability Assays and Thiobarbituric Acid Reactive Substance (TBARS) Assay

DNA fragmentation was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) using an ApoAlert DNA fragmentation kit (Clontech). Nuclei were counterstained with 4,6-diamidino-2-phenylindole. More than five areas, each containing 30 to 170 cells, were randomly selected for each sample under identical microscopic settings. The ratio of positive cells was counted and given in percentages.

Activated caspases were detected by the CaspACE FITC-VAD-FMK *in situ* marker (Promega, Madison, WI) that was added directly to living cells and incubated for 20 minutes. The cells were then fixed and observed with a microscope. For flow cytometric analysis, cells were detached from the substrate by a trypsin-ethylenediaminetetraacetic acid solution after fixation and all of the cells were subjected to analysis in a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ). Fluorescence was excited at 488 nm and measured at 530 nm. For each experiment, control samples obtained from cells cultured in the complete medium containing 10% FCS were analyzed along with the experimental groups, and a threshold was set so that 1% of the control cells were classified as apoptotic. Cell viability was determined with the Cell Counting Kit 8 (Wako). The amount of TBARS including lipid hydroperoxides, which increase as a result of oxidative stress, was measured by the TBARS assay kit (Cayman Chemical, Ann Arbor, MI).

Statistical Analysis

Where appropriate, experimental data were analyzed by unpaired *t*-tests, assuming unequal variance. A P value of <0.05 was considered statistically significant.

Results

FFA-Bound Albumin Induces Apoptosis in Cultured PTCs

We first tested whether FFAs induce apoptosis of cultured human PTCs (RPTECs). Because albumin itself could induce apoptosis, we compared the effect of FFA-free BSA versus the same concentration of FFA-bound BSA using two different combinations: first, dBSA that was depleted of FFAs was compared with the same concentration (4.4 mg/ml) of dBSA prebound with linoleic acid (18:2) or oleic acid (18:1) (LA/dBSA, OA/BSA; FFA concentration, 400 μmol/L); second, dBSA and rBSA at 30 mg/ml were compared. Gas chromatographic analysis revealed that the medium with 30 mg/ml of rBSA contained ~380 μmol/L FFAs, among which 56.6% (w/w) were saturated, ie, stearate, palmitate, and myristate, and the rest were unsaturated, ie, linoleate and oleate. An albumin concentration of 30 mg/ml is higher than that is found in the normal proximal tubule lumen *in vivo*, but was necessary to reproduce the FFA concentration seen in the nephrotic renal tubule.[11] The ratio of apoptotic cells was examined by TUNEL staining (Figure 1, A and B) and fluorescence microscopic analysis of the caspase marker (FITC-VAD-FMK) (Figure 1, C and D). Both assays showed that LA/dBSA and rBSA induced a significant increase in apoptosis compared to dBSA at the same

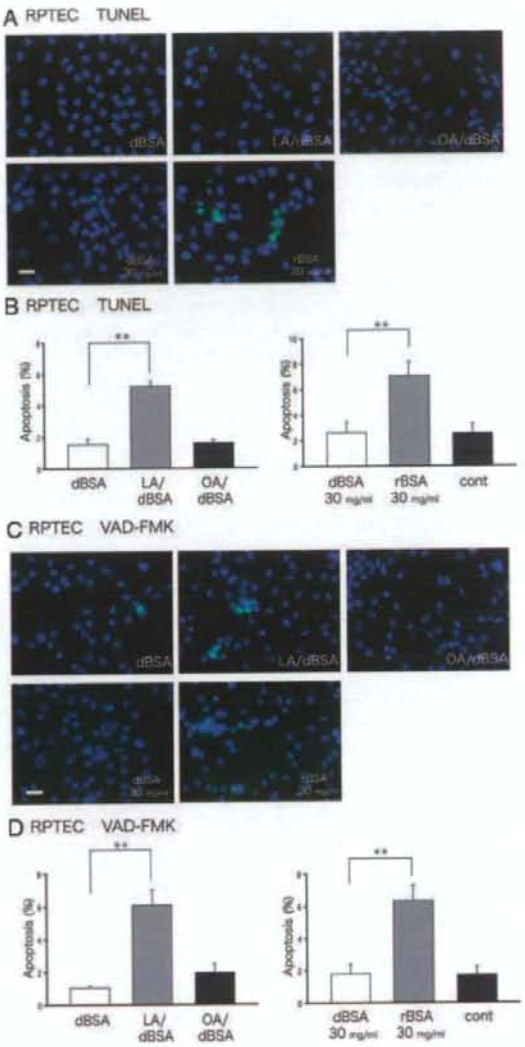

Figure 1. Fatty acids bound to BSA increase apoptosis in human PTCs (RPTECs). Human PTCs, cultured in medium containing 0.5% FCS, were treated with five different combinations of BSA and FFAs for 24 hours: i) dBSA (4.4 mg/ml); ii) 400 μmol/L LA/dBSA (BSA concentration, 4.4 mg/ml); iii) 400 μmol/L OA/BSA (BSA concentration, 4.4 mg/ml); iv) dBSA (30 mg/ml); and v) rBSA (30 mg/ml; FFA concentration, 380 μmol/L). **A** and **B**: TUNEL assay; **C** and **D**: FITC-VAD-FMK assay. **A** and **C**: Photographs show representative areas. **B** and **D**: The number of positive cells were counted in eight random areas in three independent experiments and averaged (mean ± SD, $^{**}P < 0.01$). In controls, no protein was added to the culture medium. Both assays showed that LA/dBSA and rBSA significantly increased apoptosis compared to dBSA at the same concentration. Scale bars = 10 μm.

concentration. OA/BSA caused a slight increase in apoptosis, but the difference was not statistically significant. The result indicates that, irrespective of whether BSA alone can induce apoptosis, FFAs bound with BSA can exert additional effects to induce apoptosis of human PTCs, and that the amount of FFAs included in the normal rBSA preparation was sufficient for the effect. This may explain the origin of oval fat bodies in the urine, which are likely to be cell debris after fatty degeneration.[26] The observed apoptosis did not occur because of lipopolysaccharide contaminating BSAs,[27] because rBSA with little endotoxin contamination (<0.1 ng/mg, A9306; Sigma) caused apoptosis in the present experiment.

The effect of FFAs was also examined using human HK-2 cells, which have been used as a model of PTCs in many studies.[28–30] An increase in the apoptotic ratio (Figure 2B) and a decrease in the surviving cell ratio (Figure 2A) were observed by FFA treatments. The relatively low apoptotic ratio in HK-2 cells was probably because they are transformed cells. Nonetheless, the result was essentially the same as that obtained in human PTCs. We further examined the dose effect of rBSA on apoptotic induction. As shown in Figure 2C, the apoptotic ratio increased in a dose-dependent manner, and at any concentration the effect was larger than dBSA at the same concentration.

We also examined the effect of FFAs that was applied only to the apical surface of the polarized PTC using LLC-PK1 cultured on a Transwell filter support. This experiment was done because PTC *in vivo* is the simple epithelium with a tight intercellular barrier and FFA-bound albumin bathes the apical surface alone in nephrosis. Formation of an impermeable monolayer was confirmed both by the *trans*-epithelial resistance measurement and by immunofluorescence microscopy of ZO-1 (data not shown). When various BSA preparations were added to the apical chamber, rBSA and LA/dBSA, but not OA/dBSA, increased the apoptotic ratio than dBSA in both TUNEL and FITC-VAD-FMK assays (Supplementary Figure S1, see *http://ajp.amjpathol.org*). This result showed that exposure of the apical surface of polarized epithelial cells to FFAs is sufficient to induce apoptosis.

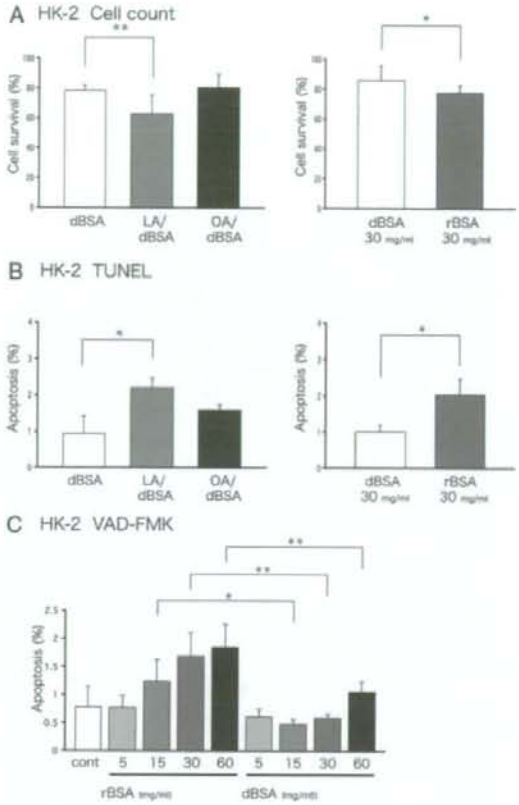

Figure 2. Apoptosis in HK-2 cells. **A:** HK-2 cells, preincubated for 24 hours in serum-free medium, were treated with dBSA (4.4 mg/ml), LA/dBSA, OA/dBSA (FFAs, 400 μmol/L; BSA, 4.4 mg/ml), dBSA (30 mg/ml), or rBSA (30 mg/ml; FFA concentration, 185 μmol/L) for another 24 hours. Cell survival was analyzed with the Cell Counting Kit 8. The number of surviving cells are shown as a ratio to that of control cells kept in 10% FCS. The results of nine samples obtained in three independent experiments were averaged (mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$). **B:** HK-2 cells treated in the same manner as described in **A** were analyzed by TUNEL assay. The number of positive cells was counted in eight random areas in three independent experiments and averaged (mean ± SD, $^{*}P < 0.05$). **C:** HK-2 cells were preincubated for 24 hours with 0.5% lipoprotein-deficient serum (LPDS), treated with dBSA or rBSA (5, 15, 30, 60 mg/ml) for another 24 hours, stained by FITC-VAD-FMK, and analyzed by flow cytometry. Control cells were kept in 0.5% LPDS without any further additions. Results obtained in three independent experiments were averaged (mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$).

FFA-Bound Albumin Increases LDs, ADRP, and TIP47 in Human PTCs

In many cell types including PTCs,[16] FFAs were shown to increase the total volume of LDs and expression of LD-associated proteins. We examined whether similar increases occur in human PTCs using the above treatments that induced apoptosis. In human PTCs treated with dBSA alone, either at 4.4 mg/ml or 30 mg/ml, LDs were hardly visible by BODIPY493/503 staining, and labeling for ADRP or TIP47 was negligible (Figure 3A). After treatment with LA/dBSA, OA/BSA, or rBSA, LDs were clearly observed and were positively labeled for ADRP and TIP47. Quantification of the labeling intensity in randomly taken fluorescence micrographs showed that both ADRP and TIP47 labeling in LDs increased after FFA-bound albumin treatment (Figure 3, B and C). A very similar result was obtained in HK-2 cells and after the DHA/dBSA treatment (data not shown). TIP47 can exist stably both as soluble and LD-bound forms, and is rapidly recruited from the cytosol to LDs when FFAs are administered.[31,32] On the other hand, ADRP exists in LDs constitutively and is a more reliable marker for estimating the amount of LDs. Consistent with this, Western blotting showed that the total expression of ADRP was greater in cells treated with rBSA than in those treated with dBSA, whereas the expression of TIP47 was not significantly different in the two samples (Figure 3D). An increase of ADRP in LA/dBSA- and OA/dBSA-treated cells was also confirmed by Western blotting (data not shown).

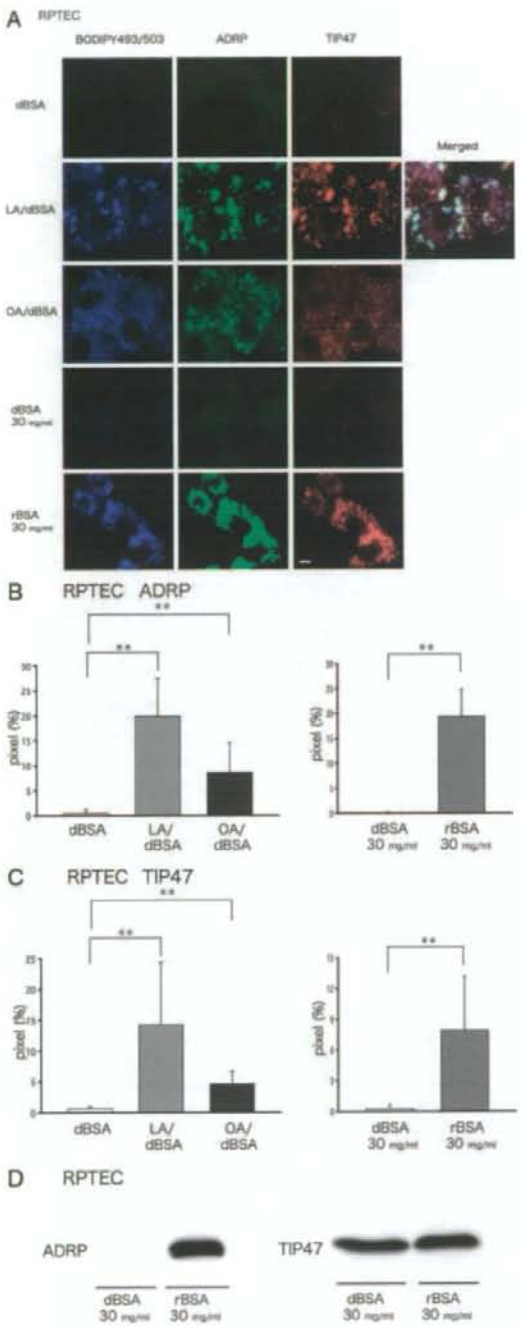

Figure 3. LDs and LD-associated proteins in human PTCs (RPTECs) increase significantly on treatment with FFAs bound to BSA. **A:** Human PTCs were treated with dBSA (4.4 mg/ml), LA/dBSA, OA/dBSA (FFAs, 400 μmol/L; BSA, 4.4 mg/ml), dBSA (30 mg/ml), or rBSA (30 mg/ml) for 24 hours as described in Figure 1. LDs were stained by BODIPY493/503 (green), whereas ADRP (blue) and TIP47 (red) were labeled with antibodies. A merged picture showed clearly that ADRP and TIP47 co-localize in the same LDs. Treatment with LA/dBSA, OA/dBSA, or rBSA significantly increased the labeling intensity of LDs, ADRP, and TIP47. **B** and **C:** The labeling intensity of ADRP (**B**) and TIP47 (**C**) by immunofluorescence microscopy was quantified (mean ± SD). Results obtained in three independent experiments were averaged. Eight areas were randomly chosen and photographed at the same setting. The ordinate is the percentage of pixels that shows the labeling more intense than a threshold (mean ± SD, $^{**}P < 0.01$) **D:** Western blotting of ADRP and TIP47 using the total cell lysate. Treatment with rBSA (30 mg/ml) drastically increased the expression of ADRP, whereas the influence on TIP47 expression was minimal. Equal amounts (30 μg) of protein were loaded in each lane. Scale bar = 5 μm.

Antioxidants Suppress FFA-Induced Apoptosis to Various Extents

Polyunsaturated FFAs (PUFAs) are substrates of iron-dependent peroxidation reactions that give rise to lipid peroxides, or reactive lipid oxygen species, and increase oxidant stress.[33,34] To explore whether lipid peroxidation is involved in FFA-induced apoptosis, we examined the effect of antioxidants: vitamin E (120 μmol/L) and desferrioxamine (100 μmol/L), a specific chelator for iron that inhibits iron-dependent lipid peroxidation,[35] were used. After preincubation with vitamin E or desferrioxamine for 1 hour, human PTCs were treated with LA/dBSA or rBSA for another 24 hours in the continued presence of antioxidant. Apoptosis induced by LA/dBSA was suppressed by incubation with either vitamin E or desferrioxamine (Figure 4A), but was still significantly more than that caused by dBSA alone (Figure 4B). In contrast, apoptosis induced by rBSA was not affected by the presence of antioxidants (Figure 4C). Similar results were obtained in HK-2 cells by flow cytometry after FITC-VAD-FMK staining (data not shown). Interestingly, apoptosis caused by DHA/dBSA in HK-2 cells was completely suppressed by antioxidants (Supplementary Figure S2, see *http://ajp.amjpathol.org*). Consistent with these results, the TBARS assay showed that DHA/dBSA is the strongest oxidative stress, whereas LA/dBSA is significantly weaker than DHA/dBSA, and rBSA was comparable to dBSA (Supplementary Figure S2C, see *http://ajp.amjpathol.org*). The results corroborate that lipid peroxidation is the major cause of apoptotic induction by polyunsaturated (24:6) DHA/dBSA, whereas it is only partially involved in the induction by LA/dBSA containing two unsaturated bonds (18:2) and is not related to the induction by rBSA primarily consisting of saturated fatty acids.

Knockdown of ADRP and TIP47 Augments FFA-Induced Apoptosis

The experiments described above show that treatment with FFA-bound albumin increases LD formation and apoptosis in PTCs. By storing excess FFAs as lipid esters, LDs are thought to protect cells against the toxicity of FFAs.[36] This led to speculation that manipulation of LD formation may influence the outcome of FFA loading. To test this hypothesis in PTCs, we knocked down expression of ADRP and TIP47 by RNAi and examined the effect on FFA-induced apoptosis.

The RNAi procedure using electroporation significantly decreased the expression of both ADRP and TIP47 in human primary PTCs, although the effect was consistently more pronounced for TIP47 than for ADRP (Figure

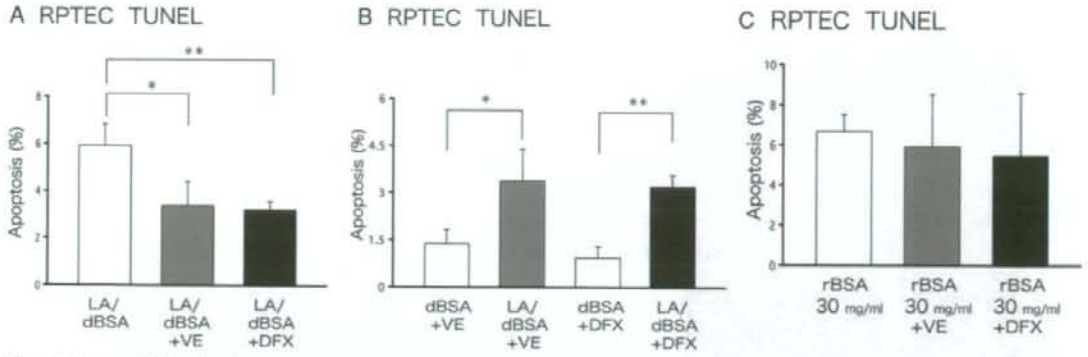

Figure 4. Increased FFA-induced apoptosis persists in the presence of antioxidants. Human PTCs were preincubated with 120 μmol/L vitamin E (VE) or 100 μmol/L desferrioxamine (DFX) for 1 hour, and were then incubated with LA/dBSA (LA, 400 μmol/L; BSA, 4.4 mg/ml) or rBSA (30 mg/ml) for 24 hours. Apoptosis was measured by the TUNEL assay. **A:** Vitamin E and desferrioxamine significantly decreased apoptosis induced by LA/dBSA. **B:** Even in the presence of vitamin E or desferrioxamine, LA/dBSA caused a higher ratio of apoptotic cells than dBSA alone. **C:** Apoptosis induced by rBSA was not significantly reduced by vitamin E or desferrioxamine. Results obtained in three independent experiments were averaged (mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$).

5A). Two days after RNAi, the cells were treated with LA/dBSA or rBSA, and the apoptotic ratio was examined 24 hours later by the TUNEL assay. The apoptotic ratio was compared between cells transfected with random control siRNA and those treated with siRNAs specific for ADRP or TIP47. Knockdown of either ADRP or TIP47 significantly increased the ratio of apoptotic cells that were induced by both LA/dBSA (Figure 5B) and rBSA (Figure 5C). A similar result was obtained by quantifying the nuclear condensation after the 4,6-diamidino-2-phenylindole staining (Supplementary Figure S3, see *http://ajp.amjpathol.org*). A basal level of apoptosis observed in the presence of dBSA was not changed by the RNAi procedure. A similar result was obtained in HK-2 cells that were subjected to RNAi and examined by flow cytometry after FITC-VAD-FMK staining (data not shown). These results suggest that endogenous ADRP and TIP47 may protect cells from apoptosis by augmenting storage of FFAs as lipid esters in LDs.

Discussion

In the present study, we first showed that dBSA bound with LA or rBSA induced more apoptosis than dBSA at the same concentration using three different cell preparations: human primary PTCs and two immortalized cell lines derived from PTCs, HK-2 and LLC-PK1. Second, we demonstrated that knockdown of either ADRP or TIP47 aggravated FFA-induced apoptosis, suggesting a protective role of those LD-associated proteins in PTCs.

Concerning the first point, the result of LLC-PK1 cells is important because they retain the apico-basolateral polarization when cultured on semipermeable supports and form tight junctions to demarcate the apical and basal fluid compartments. During nephrosis, a drastic increase in protein concentration in the renal tubular fluid causes various changes in the epithelial cells, whereas the basolateral environment should remain primarily unaffected at least during the initial stage. This condition could be produced *in vitro* by adding BSA to only the apical compartment of polarized LLC-PK1 cells. In the present experiment, apoptosis was similarly induced in nonpolarized human PTCs and HK-2 cells that were exposed to FFA-bound BSAs on all surfaces. But in other experimental conditions, loss of polarization could cause changes in cellular reactions. Conflicting results have been reported concerning the effect of BSA on cultured renal tubular cells,[7,9–11] and we speculate that some of these discrepancies could be related to lack of cellular polarization in the experimental systems.

FFAs can induce apoptosis through different pathways, and the pro-apoptotic potency of each FFA may vary depending on the cell types.[36] In the present study, rBSA and LA/dBSA caused more apoptosis than OA/dBSA in PTCs. Because the induction of apoptosis by DHA/dBSA in HK-2 cells was completely suppressed by either the antioxidant vitamin E or the iron-chelator desferrioxamine, oxidative stress appears to be the major cause of apoptosis in this case, as previously suggested.[37] However, apoptosis caused by LA/dBSA was only partially suppressed by these reagents, and apoptosis caused by rBSA, primarily bound with saturated FFAs, was not affected at all. This result suggests that oxidative stress is a major effect that PUFAs exert on cells, but that FFAs with less unsaturation induce apoptosis by other mechanisms. That is, if human serum albumin is primarily bound with saturated FFAs as rBSA, antioxidants would not be effective to block apoptosis in PTCs of the nephrotic kidney.

In many cases including the present study, OA has been shown to be less toxic than saturated FFAs or PUFAs.[38] It may be partly because monounsaturated OA may not generate strong oxidative stress and is not a precursor for ceramide synthesis.[38] Additionally, OA may counterbalance its toxicity by promoting LD formation, thereby reducing the FFA concentration.[36] This supposition can be extended to other FFAs, and we speculated that the proapoptotic potency of each FFA can be determined by the balance between its toxicity and ability to induce LD formation. From this viewpoint, we hypothesized that reduction of the LD-associated proteins, ADRP and TIP47, would compromise the capacity of cells to

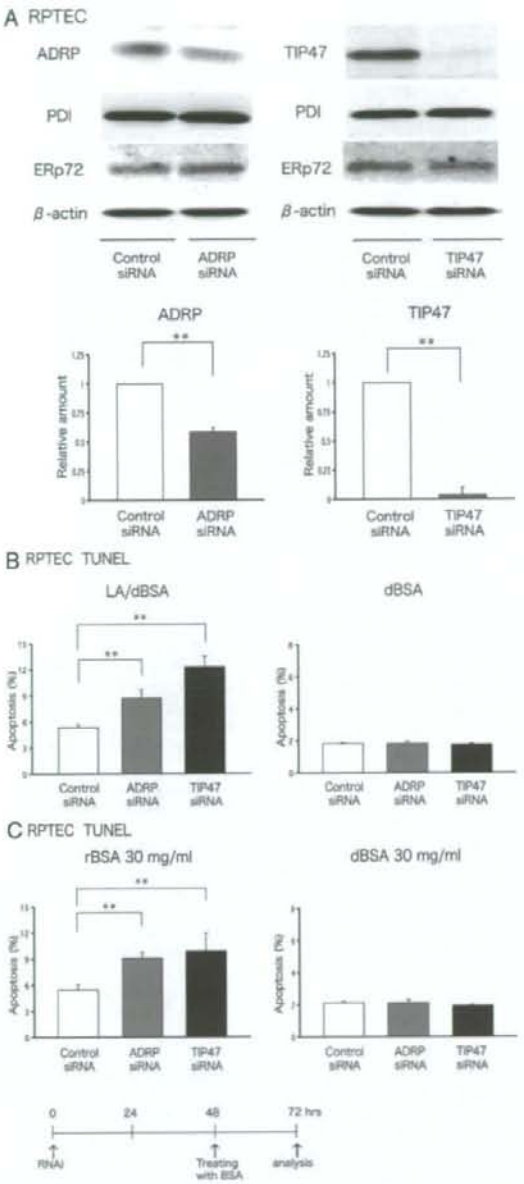

Figure 5. Knockdown of ADRP or TIP47 augments FFA-induced apoptosis in human PTCs (RPTECs). **A:** Western blotting showed that expression of ADRP and TIP47 in human PTCs was reduced significantly by RNAi. Equal amounts (30 μg) of total cell lysate were electrophoresed and probed with antibodies to ADRP, TIP47, PDI, ERp72, and β-actin. The RNAi of ADRP and TIP47 reduced expression of respective protein significantly, but did not affect other proteins. Results obtained from three independent experiments were averaged and shown in the bar graph (mean ± SD). **B** and **C:** Human PTCs were transfected with either random, ADRP, or TIP47 siRNA, and then challenged with LA/dBSA, rBSA, or dBSA. Apoptosis was measured by the TUNEL assay. The result is shown as the average of four independent experiments (mean ± SD, $^{**}P < 0.01$). **B:** Knockdown of either ADRP or TIP47 increases apoptosis induced by LA/dBSA (LA, 400 μmol/L; BSA, 4.4 mg/ml), whereas it does not influence the basal level of apoptosis observed in the presence of dBSA (4.4 mg/ml). **C:** The knockdown also increased apoptosis by 30 mg/ml of rBSA, but it did not affect the apoptotic ratio in cells treated by the same concentration of dBSA.

store lipid esters and increase apoptosis on administration of FFAs. As expected, when either ADRP or TIP47 was down-regulated by RNAi, apoptosis induced by LA/dBSA or rBSA was significantly enhanced. The effect on apoptosis was more consistently observed with TIP47 RNAi than with ADRP RNAi. This difference could result from potential critical roles played by TIP47 in sequestering FFAs to LDs, but it may simply be explained by the fact that knockdown of TIP47 was more efficient than ADRP.

ADRP has been presumed to be involved in the generation of LDs.[21,39,40] Consistent with this supposition, ADRP-null mice show a drastic reduction of LDs and lipid esters in the liver.[41] Perilipin, a LD-associated protein expressed in adipocytes and steroidogenic cells, prevents cytosolic hormone-sensitive lipases from acting on lipid esters in LDs.[42,43] ADRP may also have a similar protective role against lipases,[44] and may also reduce the cytotoxic effect of FFAs by functioning in LDs in an undefined manner. On the other hand, the function of TIP47 with regards to LDs is unclear,[18,31,45] whereas its involvement in recycling of the mannose-6-phosphate receptor from endosomes to the *trans*-Golgi network has been reported.[46] TIP47 shows significant similarity to ADRP both in amino acid sequence and three-dimensional structure[45,47] and is likely to have a related function in some aspect of lipid metabolism. Even though the molecular mechanism is not clear, we speculate that reduction of ADRP and TIP47 compromised the ability of cells to sequester FFAs as lipid esters and thus led to an increase in FFA-induced apoptosis in PTCs (Figure 6).

We expected that cDNA transfection of ADRP or TIP47 would provide a protective effect against FFA-induced apoptosis, but it did not cause a significant change in HK-2 cells (data not shown). We speculate that this negative result was primarily attributable to the low level of protein overexpression after cDNA transfection (data not shown). Expression of ADRP is posttranslationally regulated through polyubiquitylation and proteasomal degradation.[48,49] Without binding to LDs, they are likely to be short-lived, and thus an introduction of cDNA did not lead to a significant increase of protein. The regulatory mechanism of TIP47 protein expression is not known, but the lack of a significant increase in expression after cDNA transfection suggests a similar mechanism. Additionally, different LD-related proteins may cooperate with each other, such that they may need to be simultaneously overexpressed to be truly functional. That is, if expression of ADRP, TIP47, and some other proteins can be physiologically increased, they may give rise to an increase in protective function. In this context, it is noteworthy that PPAR ligands inhibited progression of diabetic nephropathy[50–52] and experimental glomerulonephritis.[9,53] This phenotype may be the result of an increase of not only ADRP but also other LD-related proteins.[15,54,55]

The present study shows that FFAs bound to BSA induce apoptosis in PTCs, and that reduction of ADRP or TIP47 further increases the ratio of apoptotic cells. This result does not exclude the possibility that high concentrations of urinary proteins alone can affect renal tubular cells in the nephrotic kidney. Most importantly, this study

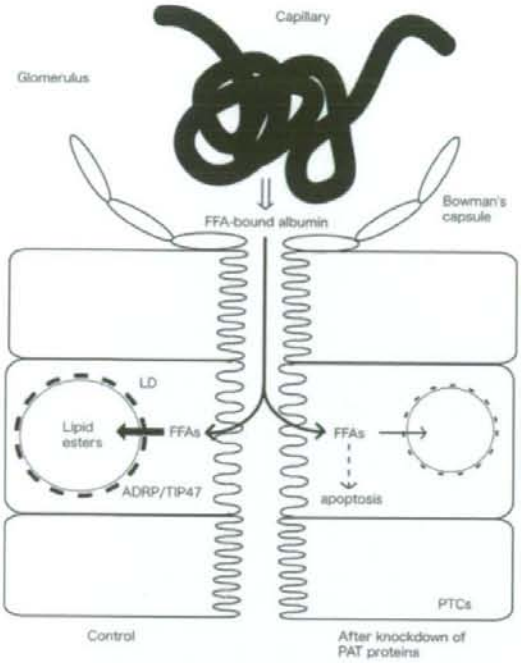

Figure 6. Based on the results of the present study, we hypothesize that LD and LD-associated proteins function in PTCs of the nephrotic kidney as described in the figure. Albumin that reached the tubular lumen presents FFAs to PTCs in an unregulated manner. FFAs may induce apoptosis in PTCs, but LDs can sequester FFAs as lipid esters and thereby protect cells from their toxicity. When the LD-associated proteins were reduced, FFA-sequestering ability of LDs may be compromised, and the resultant increase of FFAs in the cytosol may induce an increase in apoptosis.

demonstrates for the first time that LD-associated proteins play some protective role against FFA-induced cytotoxicity in PTCs. Furthermore, it reveals that the cellular toxicity of FFAs is heterogeneous and not linearly correlated with the number of double bonds. The result suggests that the prognosis for nephrosis may vary depending on the expression level of LD-associated proteins and composition of FFAs bound to serum proteins. It also suggests that LD-associated proteins and their regulatory mechanisms may be used as targets of therapeutic intervention for nephrotic diseases. We hope that the role of LD-associated proteins in PTCs will be confirmed in animal experiments and translated to clinical applications in the near future.

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医療／薬品

腎臓・膀胱 微小循環と再生

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病態外科学　泌尿器科

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一言アピール

尿道括約筋不全による腹圧性尿失禁は、女性骨盤底機能障害、前立腺癌に対する根治的前立腺摘除術後、および神経因性膀胱においてみられ、その治療需要は極めて大きいにもかかわらず、有効な治療法が確立されていない。我々は、自己採取脂肪由来幹細胞を用いた尿道括約筋再生による臨床治療応用を目指している。
また、腎移植のドナー不足の問題点を解消するために、腎微小循環から尿細管上皮細胞再生にホーカスを当て、尿中落下細胞から培養した尿細管上皮前駆細胞を中心とした包括的臨床腎臓再生技術を開発している。

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研究のキーワード

尿失禁、尿中落下細胞、尿細管上皮前駆細胞、SP 細胞、末梢血幹細胞、脂肪由来幹細胞

保有技術・機器

【保有技術】・毛細血管血流時空間解析
【主な機器】生体顕微鏡、腎臓拡大内視鏡

主な特許・論文・著書

【主な特許】・特願 2006-216234　低血清培養による脂肪由来幹細胞治療（尿失禁、腎障害、創傷治癒、骨粗鬆症、下肢虚血）
・特願 2006-114152　尿中落下細胞からの尿細管前駆細胞の培養と腎障害治療
・特願 2003-375321　末梢血幹細胞による腎機能障害治療
・特開 2003-329934　生体顕微鏡における光学経路

【主な論文】・Kondo A, Isobe Y, Kimura K, Kamihira O, Matsuura O, Gotoh M, Ozawa H: Efficacy, safety and hospital costs of tension-free vaginal tape and pubovaginal sling in the surgical treatment of stress incontinence. *Obstet Gynaecol Res*, 32:539-544, 2006
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研究 01　尿失禁の再生治療

概要

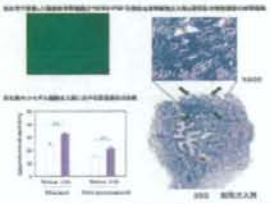
腹圧性尿失禁は、女性骨盤底機能障害、前立腺癌に対する根治的前立腺摘除術、神経因性膀胱などにおける尿道括約筋障害によってみられ、潜在患者は極めて多いにもかかわらず、適切な治療が得られず、患者の QOL 低下を引き起こしていることが多い。現在有効な薬物治療はなく、女性における括約筋不全による腹圧性尿失禁に対しては TVT スリングなどの外科的治療が広く行われているが、根治的前立腺摘除術後や神経因性膀胱に対しては有効な治療法がなく、新しい治療の開発が待たれている。尿道括約筋再生治療は、原因疾患にかかわらず括約筋障害にもとづく腹圧性尿失禁に対して有望な治療方法である。さらに、自己脂肪由来幹細胞は採取が容易であるとともに、その括約筋への注入は経尿道的内視鏡下に容易に行うことができ、脂肪由来幹細胞による括約筋再生治療は、低侵襲で実現可能性が高く、また治療による QOL 改善効果へのインパクトが極めて大きいものである。

この研究の新規性・独創性

低血清培養による小型でヒト、ラットの比較的均一な脂肪由来幹細胞の培養に成功した。麻酔下に除神経した尿失禁モデルラットの膀胱頚部に局所注入した２週間後、尿道抵抗を反映する leak point pressure（膀胱に生理食塩水を注入し尿が漏れる時の膀胱内圧を測定する）がコントロール群に比して有意に高い、すなわち尿失失禁を改善せしめる所見を得た。その部位の病理組織では尿道周囲にコラーゲン繊維を反映するマッソン染色される細胞塊のこぶが形成され、尿道内圧が上昇した一つの原因と考えられた。この研究は特許出願しており、名古屋大学独自のものである。

産学連携を目指した応用研究

尿失禁の新しい治療として将来が期待される。尿失禁薬剤を開発している薬品会社の共同開発を求める。



研究 02　腎臓の再生治療

概要

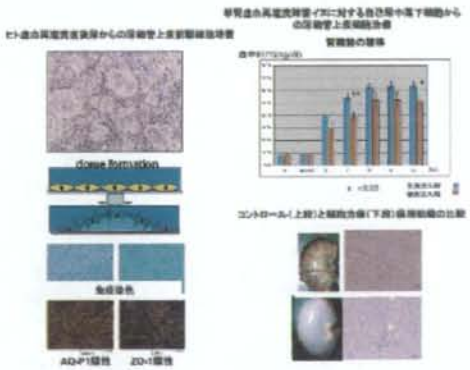
腎再生のマテリアルとして尿細管上皮細胞の再生を中心に 1) 尿中落下細胞からの尿細管上皮細胞（腎組織幹細胞）の培養治療、2) 低血清培養による脂肪由来幹細胞 3) 末梢血幹細胞 4)HUVEC を用いて腎障害を軽減することを明らかにした。また独自に開発した生体顕微鏡でその微小循環環境を解明した。

この研究の新規性・独創性

1) については、臨床において生体腎移植、死体腎移植そして腎血管を一時的に遮断して行う腎部分切除の血流再開直後の尿から落下細胞を採取、ドーム形成を有する尿細管上皮前駆細胞（腎組織幹細胞）の一次培養に初めて成功した。その細胞は 0.33% と高率にも SP 細胞を含んでいた。また、単腎イヌ虚血再灌流障害モデルにこの細胞を皮膜下投与を行い腎保護作用を明かにし、細胞治療の可能性を示唆した。この研究は上皮性細胞治療で 2) は間葉系細胞治療であり、1） 2）を組み合わせた上皮―間葉系治療も今後行う予定である。
1-3 は特許出願しており、名古屋大学独自のものである。
腎機能障害の新しい治療として将来が期待される。

産学連携を目指した応用研究

腎機能障害の新しい治療として将来が期待される。透析装置または腎保護作用の薬剤を開発している企業または薬品会社の共同開発企業を求める。



これまでの研究テーマ

- 膀胱微小循環障害の解明と治療薬剤の開発
- 腎微小循環障害の解明とその治療薬剤の開発
- 腎微小循環の酸化ストレスとバイオマーカの開発