厚生労働科学研究費補助金
(ヒトゲノム・再生医療等研究事業)

ユビキチンシステムの多機能性を活用した脳神経系加齢性病態の克服に関する研究
(H17ーゲノムー009)

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総 括 研 究 報 告 書

ユビキチンシステムの多機能性を活用した脳神経系加齢性病態の克服
(H17−ゲノム−009)

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本研究では、ユビキチンシステムが不用蛋白質の分解系として機能するだけでなく、多数の蛋白質の活性制御に関与し様々な生命現象に深く係わるという多機能性を活用し、脳神経系の老化ならびに老化がもたらす痴呆などの病態について、ユビキチンシステム、特に脱ユビキチン化酵素から見た克服法を開発する。なかでも UCH-L1、UCH-L3 は酵素として作用する以外にそれぞれユビキチンキャリア蛋白、ジユビキチンキャリア蛋白として機能しアポトーシス関連因子、細胞生存因子を統合的に制御することで神経細胞の生存と死に密接に関わっていることが研究代表者の和田らにより明らかにされてきた(Nat Genet, 1999; Hum Mol Genet, 2003; Am J Pathol 2004 など)。また、UCH-L1 は酸化ストレスによりそれ自身が酸化され神経細胞機能低下を引き起こすことが判明している。このように研究代表者は脱ユビキチン化酵素の生物学的研究で先駆的成果を収めてきたが、本提案ではこれらの成果をさらに発展させ、脱ユビキチン化酵素の機能変化がもたらす神経細胞老化の初期変化を解明するとともに、脱ユビキチン化酵素による老化関連蛋白質制御の分子的実体を明らかにする。研究開始の本年度においては UCH-L1、が酵素活性非依存的に神経系前駆細胞の形態制御を行い、神経発生を調節していること、UCH-L3 が欠損したマウスではミトコンドリアの変化を伴う神経細胞死が増加することを見出し、両者は神経細胞の機能と生存に関して重要な調節因子であることを示した。また中性子小角散乱法を活用し UCH-L1 の水溶液中における蛋白構造を世界で初めて決定した。

A. 研究目的

本研究では、現時点では有効な予防診断法の乏しい脳の加齢変化に対してより信頼性と確度の高い生物学的検出法を提供し、さらには神経細胞老化がもたらす痴呆などの病態の修復法を開拓し、その実施を通して健全な社会を実現することに貢献することを目標とする。その達成にむけて今回はこれまで研究代表者が精力的に研究を続けてきたユビキチンシステムに焦点を当て、脱ユビキチン化酵素、UCH-L1 と UCH-L3 を機軸にした神経細胞老化の分子メカニズムの解明と脱ユビキチン化酵素の機能モニタリングによる神経系老化の評価系の構築をめざす。

研究開始の本年度は、UCH-L1、UCH-L3 を題材、UCH-L1 が神経発生や神経新生の制御に深く関わること、UCH-L3 が細胞活性を制御しその機能障害は神経細胞死を誘導する可能性の高いことを見いだした。また、中性子小角散乱法という異分野技術を活用し UCH-L1 の水溶液中における構造を決定した。

B. 研究方法

(1) 神経前駆細胞・神経細胞におけるUCH-L1の機能解析

免疫組織化学的手法を用いて、マウス胎仔脳におけるUCH-L1の発現解析を行った。どの様な細胞にUCH-L1が発現しているかを調べるため、未分化細胞のマーカーである nestin 抗体と初期神経マー

カーであるTuJ1抗体それぞれとUCH-L1抗体を用いた二重染色を、神経分化の盛んな胎生14日目とアストロサイトの分化が開始するといわれている胎生16日目で行った。あわせて、先のGPCRを対象にした解析で用いた神経前駆細胞の培養系を用い、神経前駆細胞を分化させた時どのような細胞にUCH-L1が発現しているか、マーカーとの共染色を行うことにより解析した。さらに、UCH-L1のどの様な活性が神経分化において重要であるか調べるために、野生型UCH-L1と変異型UCH-L1を神経前駆細胞に遺伝子導入し解析を行った。また、同腹のgadマウスとヘテロマウスから神経前駆細胞を採取培養し比較実験を行った。

(2)UCH-L1の水溶液中の構造解析

ヒトUCH-L1蛋白質の大腸菌発現系を構築した。大腸菌発現系からニッケルアフィニティーならびにイオン交換の2段階の液体クロマトグラフィーによりUCH-L1蛋白質の精製を行い、中性子小角散乱法による構造計測用の試料を調製した。高エネルギー加速器研究機構のサイクロトロン施設において冷中性子線(波長＝0.5-16.1Å)をUCH-L1蛋白質溶液に対し照射し小角領域の散乱を検出する事でサブナノメートルレベルの構造を決定した。これと同時に円二色偏光法によりUCH-L1蛋白質2次構造計測も行った。さらにパーキンソン病等の神経変性疾患の発症に関与するヒトUCH-L1の変異に関しても同様に精製蛋白質を調製して中性子線小角散乱法によって構造決定を行った。

(3)神経細胞におけるUCH-L3の機能解析

UCH-L3遺伝子欠損マウスは生後数週間で網膜変性をきたす。その機序を解明することにより、神経細胞である視細胞においてUCH-L3が果たす役割を検討した。免疫組織化学的手法を用いて野生型マウスにおけるUCH-L3の網膜内局在を調べた後、生後0日、10日、3週、6週、8週、12週齢のUCH-L3遺伝子欠損マウスの網膜各層の厚さとTUNEL陽性細胞数の経時的変化を測定し野生型マウスと比較した。また、電子顕微鏡を用いて変性網膜の微細構造を観察し、視細胞内節のミトコンドリアの形態学的変化を評価する為、ミトコンドリアの面積に対するクリステの面積比を算出した。さらに各種アポトーシス及び酸化ストレス関連タンパク質の網膜内各層における発現の変化を調べた。

(倫理面への配慮)

動物を使用する研究計画はすべて国立精神・神経センター神経研究所動物実験倫理問題検討委員会で審議され承認を受けた。実際の動物使用に当たっては国の法律・指針並びに米国NIHの基準を守り動物が受ける苦痛を最小限に留めた。ヒト標本を用いた研究は実施しなかった。

C. 研究結果

(1)神経発生・新生におけるUCH-L1の役割解明

マウス胎仔脳を用いた発現解析により、UCH-L1は分化した神経細胞だけでなく未分化な神経前駆細胞にも発現していることが示された。終脳における神経分化はE11に始まり、E14頃にピークとなり、E16以降減少することが知られている。E14においては、グリア細胞は存在せずその分化も始まっていないが、E16にはグリア細胞の分化が始まると考えられている。nestin、TuJ1とも各領域の発現量はE14とE16で変化がなかった。これに対して、UCH-L1の免疫反応性は、E14では神経細胞の存在するCP(cortical plate)より未分化神経前駆細胞の存在するVZ(ventricular zone)において強く、逆にE16ではVZよりもCPにおいて免疫反応性が強かった。神経前駆細胞の培養系を用いた解析からもUCH-L1は、TuJ1陽性の神経細胞だけでなくnestin陽性の神経前駆細胞にも発現していることが示された。さらに、nestin陽性細胞は分化時期特異的に、UCH-L1が発現している細胞としていない細胞が存在し、それらの細胞を分類すると、UCH-L1が発現しているnestin陽性細胞は神経突起が短く単極性の細胞が有意に多いことが示された。このことから、UCH-L1は神経前駆細胞の形態を変化させ神経分化を促進

している可能性が考えられた。そこで、野生型UCH-L1 を神経前駆細胞に遺伝子導入する実験を行ったところ、UCH-L1 導入細胞では nestin 陽性突起の長さが有意に短くなった。この UCH-L1 の活性には水解酵素活性は要求されなかった。また、UCH-L1 の発現していない gad マウスにおける神経前駆細胞の神経突起の長さは、同腹のヘテロマウスから採取培養した細胞の神経突起の長さよりも有意に長かった。

(2) UCH-L1 の水溶液中の構造解析

水溶液中の UCH-L1 蛋白質に対して冷中性子を照射して小角領域の散乱強度を測定した。散乱角に対する散乱強度プロットを作製し数学的モデルとのフィッティング解析を行ったところ、UCH-L1 蛋白質は水溶液中において球状蛋白質の 2 量体構造をとっている事が明らかになった。さらに詳細な数学的モデルとのフィッティング解析によって UCH-L1 蛋白質は長径 4.3 nm、短径 2.9 nm の回転楕円体の 2 量体構造をとっている事が明らかになった。また、円二色偏光法による解析も同時に行い UCH-L1 蛋白質の 2 次構造の計測を行った。その結果、UCH-L1 蛋白質内の 2 次構造は α ヘリックス 43%、の β シート 24%、β ターン 7%、ランダムコイル 26%であった。さらにパーキンソン病等の神経変性疾患の発症に関与する変異を有する I93M 変位型ならびに S18Y 多型 UCH-L1 蛋白質に関しても中性子小角散乱法で解析を行った。その結果、I93M 変位型 UCH-L1 蛋白質は長径 4.3 nm、短径 2.0nm の扁平な回転楕円体の 2 量体構造をとり、S18Y 多型 UCH-L1 蛋白質は長径と短径がほぼ等しい球状の 2 量体構造をとっている事が明らかになった。また同時に行った円二色偏光法による解析からも変異に伴った β ターン構造の有意な変化を見出すことが出来た。これらの研究からパーキンソン病等の神経変性疾患の発症に関係するアミノ酸変異がもたらす UCH-L1 蛋白質のサブナノメーターレベルの構造変化を中性子小角散乱法によって明らかにすることが出来た。

(3) 神経細胞における UCH-L3 の機能解析

UCH-L3 は野生型マウスの網膜では生後3週齢以降、主に視細胞内節に局在していた。UCH-L3 遺伝子欠損マウスの網膜は発達期の生後10日齢までは明らかな組織学的変化は認められなかったが、3週齢で視細胞内節から萎縮が始まり12週齢では視細胞層はほぼ消失した。TUNEL 陽性細胞は3週齢以降では UCH-L3 遺伝子欠損マウスの網膜外顆粒層に有意に増加していた。電子顕微鏡において UCH-L3 遺伝子欠損マウスの視細胞内節に空胞変性およびミトコンドリアの膨潤が観察され、クリステの面積比は有意に減少していた。また、UCH-L3 遺伝子欠損マウスでは視細胞内節に酸化ストレスを示す COX、Mn-SOD、AIF の発現が高かった。カスパーゼ依存性アポトーシスのマーカーである caspase-1と活性型 caspase-3 の発現および cytochrome-C の細胞質への移行は認められなかったが、カスパーゼ非依存性アポトーシスの指標となる Endo G の外顆粒層への核移行像が観察された。これらの結果より、UCH-L3 遺伝子欠損マウスの視細胞死は、ミトコンドリアの変化と酸化ストレスマーカーの上昇を伴うカスパーゼ非依存性アポトーシスであることが示唆された。

D. 考察

研究代表者は以前神経軸索ジストロフィーを主病変に持つ gracile axonal dystrophy (gad)マウスの原因遺伝子が UCH-L1 であることをみいだした。神経軸索ジストロフィーは脊椎動物神経系で認められるもっとも普遍的な加齢所見を考慮すれば、脱ユビキチン化酵素が老化と密接に関わっていることを示す貴重な発見であった。UCH-L1 を始めとする脱ユビキチン化酵素の生物学的意義に着目した研究を展開したところ、UCH-L1 が多機能蛋白質として機能し、神経細胞体においては神経細胞死との関連性において抗アポトーシス蛋白質や prosurvival 蛋白質と機能的リンクを形成し神経細胞の生存に密接に関わることが明らかになった。今年度は UCH-L1 が神経発生・新生制御にも関わっていることを見いだし、また

UCH-L3 が神経細胞死の重要な規定因子であることを示すなど脱ユビキチン化酵素の神経細胞における生物学的意義が明らかになった。これらの成果は脱ユビキチン化酵素、UCH-L1とUCH-L3を機軸にした神経細胞老化の分子メカニズムの解明と脱ユビキチン化酵素の機能モニタリングによる神経系老化の評価系の構築をめざすうえで基盤形成を果たすものである。ユビキチンシステムを機軸にした神経系老化の遺伝子・蛋白質ネットワークを新たに描出し、老化がもたらす病態の克服に必要な標的分子を明らかにすることが展望出来るようになったと言えよう。

他方、水溶液中における UCH-L1 の微細構造に関しサブナノレベルで非破壊的に解析を行い決定することが出来たが、この成果はこれまでに見出した酸化ストレスによる UCH-L1 の機能低下の発見と合わせ細胞傷害センサー、細胞老化センサーとしての UCH-L1、UCH-L3 の構造生物学的証明を行う上で今後貢献する成果であった。今後両分子の機能変化が醸し出す細胞老化の分子機序をゲノム、プロテオームの面から明らかにし老化プロセスの解明に新たなメスを入れるとともに、治療戦略上必要不可欠な遺伝子・蛋白質素子を抽出することを行うが、今年度の成果は目標達成に向けて研究が着実かつ独創性高く展開されていることを示すものである。高齢者社会を迎えた我が国においては老化がもたらす様々な病態の克服は医療行政だけでなく、健全な国家財政の形成のためにも必要不可欠な社会的急務である。本研究の継続発展はこれら社会的要請に対して革新的治療法の提供という回答を出すだけでなく、UCH-L1、UCH-L3 に結合しその機能状態を検出できるプローブの開拓を行い、老化初期変化を画像的に捉える技術開発、すなわち老化の初期過程の検出という予防診断法を提供すると期待される。

E. 結論

UCH-L1、UCH-L3 がそれぞれ神経発生、神経細胞死の重要な調節因子である可能性を見いだし、また中性子小角散乱法を活用し UCH-L1 の水溶液中における蛋白構造を決定した。

F. 健康危険情報

特になし

G. 研究発表

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H. 知的所有権の出願・登録状況(予定を含む)

1. 特許取得

(出願中)

特許出願番号:2005-170412「神経細胞分化誘導剤または神経新生作用剤のスクリーニング方法」、発明人:和田圭司他2名、出願人:国立精神・神経センター、他 1 名、出願年月日:平成17年6月10日

特許出願番号:2005-170413「神経分化誘導剤のスクリーニング方法」、発明人:和田圭司他 5 名、出願人:国立精神・神経センター、他 1 名、出願年月日:平成17年6月10日

2. 実用新案登録

なし

3. その他

なし

別紙５

研究成果の刊行に関する一覧表レイアウト

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kwon, J., Mochida, K., Wang, Y.L., Sekiguchi, S., Sankai, T., Aoki, S., Ogura A., Yoshikawa, Y. and Wada, K.	Ubiquitin C-terminal hydrolase L1 is essential for the early apoptotic wave of germinal cells and for sperm quality control during spermatogenesis.	**Biol. Reprod.**	73	29-35	2005
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Kwon, J., Sekiguchi, S., Wang, Y.L., Setsuie, R. Yoshikawa, Y. and Wada, K.	The region-specific functions of two ubiquitin C-terminal hydrolase isozymes along the epididymis.	**Exp. Anim.**	55(1),	35-43	2006

研究成果の刊行物・印刷

Exp. Anim. 55(1), 35–43, 2006

The Region-Specific Functions of Two Ubiquitin C-Terminal Hydrolase Isozymes along the Epididymis

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Abstract: *We previously showed that* gad *mice, which are deficient for ubiquitin C-terminal hydrolase L1 (UCH-L1), have a significantly increased number of defective spermatozoa, suggesting that UCH-L1 functions in sperm quality control during epididymal maturation. The epididymis is the site of spermatozoa maturation, transport and storage. Region-specific functions along the epididymis are essential for establishing the environment required for sperm maturation. We analyzed the region-specific expression of UCH-L1 and UCH-L3 along the epididymis, and also assessed the levels of ubiquitin, which has specificity for UCH-L1. In wild-type mice, western blot analysis demonstrated a high level of UCH-L1 expression in the caput epididymis, consistent with ubiquitin expression, whereas UCH-L3 expression was high in the cauda epididymis. We also investigated the function of UCH-L1 and UCH-L3 in epididymal apoptosis induced by efferent duct ligation. The caput epididymides of* gad *mice were resistant to apoptotic stress induced by efferent duct ligation, whereas* Uchl3 *knockout mice showed a marked increase in apoptotic cells following ligation. In conclusion, the response of* gad *and* Uchl3 *knockout mice to androgen withdrawal suggests a reciprocal function of the two UCH enzymes in the caput epididymis.*
Key words: *apoptosis, epididymis, ubiquitin, UCH*

Introduction

The mammalian epididymis is a highly convoluted tubule that connects the efferent ducts of the testis to the vas deferens [2, 8]. The epididymis is composed of three distinct compartments, caput (head), corpus (body) and cauda (tail), each having a specific role in sperm maturation, sustenance, transport, and storage [2, 6]. However, the molecular basis for the maturation process remains largely unknown.

It has been suggested that the epididymis acts as a quality control organ to eliminate defective spermato-

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zoa before ejaculation [37]. The epididymis is an organ with voluminous protein traffic between the epithelium and lumen. Numerous proteins, secreted in an apocrine manner by the epididymal epithelium, are implicated in spermatozoa maturation [18]. Two major components of the ubiquitin-dependent proteolytic pathway, ubiquitin and UCH-L1 (PGP9.5), are expressed in epididymal tissue [10, 35]. Ubiquitin is present in human seminal plasma [26], and defective spermatozoa become ubiquitinated during epididymal passage [23, 37]. Our previous work showed that UCH-L1 associates with monoubiquitin and stabilizes its expression [31]. In addition, it has been suggested that UCH-L1 functions as a regulator of apoptosis via the ubiquitin pathway [13, 23, 25]. We found that testes of gracile axonal dystrophy (*gad*) mice, which lack UCH-L1, have reduced ubiquitin levels and are resistant to cryptorchid injury–mediated germ cell apoptosis [25]. Furthermore, our recent work demonstrated that the percentage of morphologically abnormal spermatozoa is significantly higher in *gad* mice, compared with wild-type mice [23].

Two mouse UCH isozymes, UCH-L1 and UCH-L3, are strongly but reciprocally expressed in the testis during spermatogenesis [25], suggesting that these proteins have distinct functions in the testis [23], even though they have high amino acid sequence identity and share significant structural similarity [21]. The functional regionalization of the epididymis is delineated at the molecular level by regional differences in gene expression [16–19]. Regional differences along the epididymis might be essential characteristics of the environment required for sperm quality control. Although it has been shown that UCH-L1 and UCH-L3 have reciprocal functions with respect to cryptorchid injury, their molecular functions in regulating sperm quality during epididymal passage are not fully understood. Thus, we examined the epididymal expression of UCH-L1 and UCH-L3 with regard to their involvement in the regulation of apoptosis. In addition, we assessed the reciprocal functions of these two proteins in the epididymis.

Materials and Methods

Animals

We used *gad* (CBA/RFM) [34] and *Uchl3* knockout (C57BL/6J) [21] male mice at 10 weeks of age. The *gad* mouse is an autosomal recessive mutant that was obtained by crossing CBA and RFM mice. The *gad* line has been maintained by intercrossing for more than 20 generations [34]. *Uchl3* knockout mice were generated by the standard method [21] using homologously recombinant ES cells, and the knockout line has been back-crossed several times to C57BL/6J mice. Both strains are maintained at our institute. Animal care and handling were in accordance with our institutional regulations for animal care and were approved by the Animal Investigation Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry.

Unilateral efferent duct ligation

Animals were either left intact to serve as controls or were unilaterally ligated at the efferent duct [9, 38]. Four mice in each group were anesthetized with pentobarbital (Abbott Laboratories, North Chicago, IL), and the testis and epididymis on the right side were exposed through a scrotal incision. The thin avascular attachment joining the initial segment of the epididymis to the tunica albuginea was cut to permit exposure of the efferent ducts coursing above and parallel to the vascular supply. A silk suture was passed by needle through the thin sheet of connective tissue between the ductules and the blood vessels, and the efferent ducts were occluded by ligation. Mice were sacrificed 2 or 4 days after ligation. Both epididymides were immersed in 4% paraformaldehyde for at least 24 hr before they were dehydrated and embedded in paraffin [22].

Histological and immunohistochemical assessment of the epididymis

The caput, corpus and cauda epididymides along the epididymal region embedded in paraffin were cut into 4-μm sections and stained with hematoxylin and eosin. Light microscopy was used for routine observations. For immunohistochemical staining, the sections were incubated with 10% goat serum for 1 h at room temperature followed by incubation overnight at 4°C with a rabbit polyclonal antibody raised against peptides within UCH-L1 or UCH-L3 (1:1,000 dilution; peptide antibodies [24]) and ubiquitin (1:500; DakoCytomation, Glostrup, Denmark) in PBS containing 1% BSA. Sections were then incubated for 1 h with biotin-conjugated anti-rabbit IgG diluted 1:200 in PBS, followed by

Vectorstain ABC-PO (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Sections were developed using 3,3'-diaminobenzidine and counterstained with hematoxylin.

In situ apoptosis was detected by TUNEL (TdT-mediated nick end-labeling) staining with the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI) according to the manufacturer's instructions, to identify apoptotic cells in situ via specific labeling of nuclear DNA fragmentation. Quantification was performed using four mice on each of postoperative days 0, 2 and 4. The total number of apoptotic cells was determined by counting the positively stained nuclei in each caput epididymis section [9]. Four sections from each mouse and 100 total circular tubules per group were processed.

Western blotting

Western blots were performed as previously reported [24]. Total protein (10 μg/lane) from each epididymal region including spermatozoa was subjected to SDS-polyacrylamide gel electrophoresis using 15% gels (Perfect NT Gel, DRC, Japan). Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) and blocked with 5% non-fat milk in TBS-T (50 mM Tris base, pH 7.5, 150 mM NaCl, 0.1% (w/v) Tween-20). The membranes were incubated individually with primary antibodies to monoubiquitin (1:1,000; u5379, Sigma-Aldrich, St. Louis, MO), UCH-L1 and UCH-L3 (1:1,000 dilution; anti-peptide antibodies [24]), p53, Bax, and Bcl-xL (1:1,000 dilution; all from Cell Signaling Technology, Beverly, MA), and Bcl-2 (1:500; Transduction Laboratories, Franklin Lakes, NJ). Blots were further incubated with peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:5,000 dilution; Pierce, Rockford, IL) for 1 h at room temperature. Immunoreactions were visualized using SuperSignal West Dura Extended Duration Substrate (Pierce) and analyzed using a ChemiImager (Alpha Innotech, San Leandro, CA). Each protein level was normalized to α-tubulin following analysis with a ChemiImager using AlphaEase software.

Statistical analysis

The mean and standard deviation were calculated for all data (presented as mean ± SD). Student's *t*-test was used for statistical analysis.

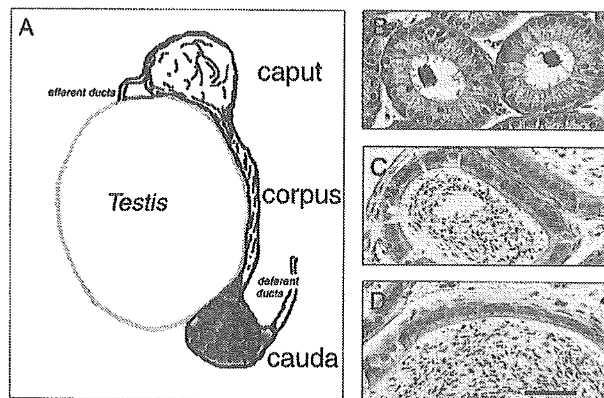

Fig. 1. A: Diagram of the epididymis. B–D: Morphology of the caput (B), corpus (C) and cauda (D) epididymidis from a wild-type mouse. Magnification, 200×. *Scale bar*, 40 μm.

Results

Levels of UCH-L1 and UCH-L3 in individual epididymal regions

The epididymis is a single long, coiled tubule situated on the surface of the testis (Fig. 1A). The epididymal epithelium is composed of four major cell types, principal cells, basal cells, clear cells and narrow cells [7], and can be divided anatomically and functionally into three regions, the caput, corpus and cauda epididymis (Fig. 1B, C, D). We used western blotting to characterize UCH-L1 and UCH-L3 levels along the epididymis (Fig. 2). In wild-type mice, the level of UCH-L1 was highest in the caput epididymis and that of UCH-L3 was highest in the cauda epididymis. UCH-L1 and UCH-L3 were not observed in *gad* and *Uchl3* knockout mice, respectively (Fig. 2; comparison of UCH-L1 and UCH-L3 levels with those in wild-type control mice).

Immunohistochemistry of UCH-L1, UCH-L3 and ubiquitin in the epididymis

Under light microscopy, granular and diffuse UCH-L1 and UCH-L3 immunoreactivity was detected in many epithelial cells of the caput, corpus and cauda epididymis in wild-type mice (Fig. 3A, C). Granular immunoreactivity to ubiquitin was seen in the epithelial cells of the epididymis (Fig. 3B). The distribution of ubiquitin in the corpus and cauda epididymal epithelial cells was similar to that of the caput epididymis,

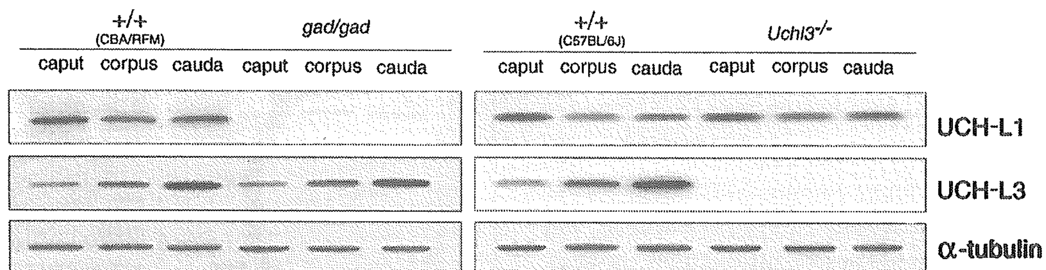

Fig. 2. Comparison of UCH-L1 and UCH-L3 expression by western blotting of caput, corpus and cauda epididymis lysates from two wild-type (CBA/RFM and C57BL/6J), *gad* and *Uchl3* knockout mice. Blots were reprobed for α-tubulin, which was used to normalize the protein load. Images are representative of four independent experiments.

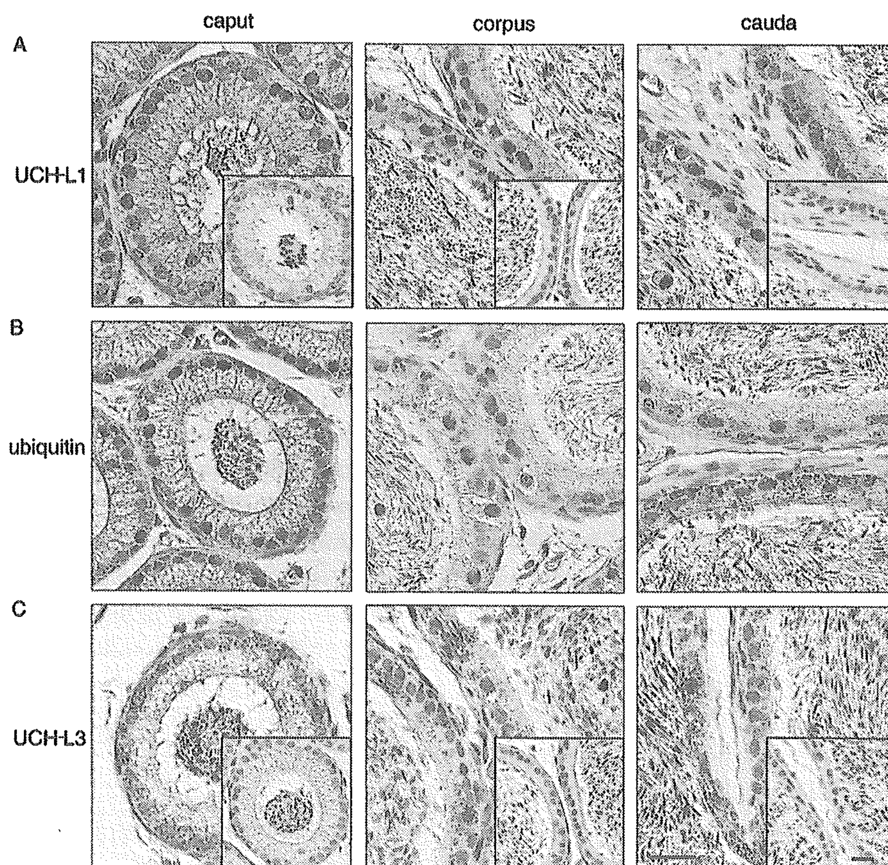

Fig. 3. Immunohistochemistry of UCH-L1, UCH-L3, and ubiquitin in the individual epididymal regions of wild-type mice. Each of the protein-positive cells in the caput, corpus and cauda epididymis is stained by DAB. The insets show that no cells are positive for UCH-L1 and UCH-L3 in the individual epididymal compartments from *gad* (A) and *Uchl3* knockout (C) mice, respectively. A: UCH-L1-positive cells. B: Ubiquitin-positive cells. C: UCH-L3-positive cells. Magnification, 400×. *Scale bar*, 20 μm.

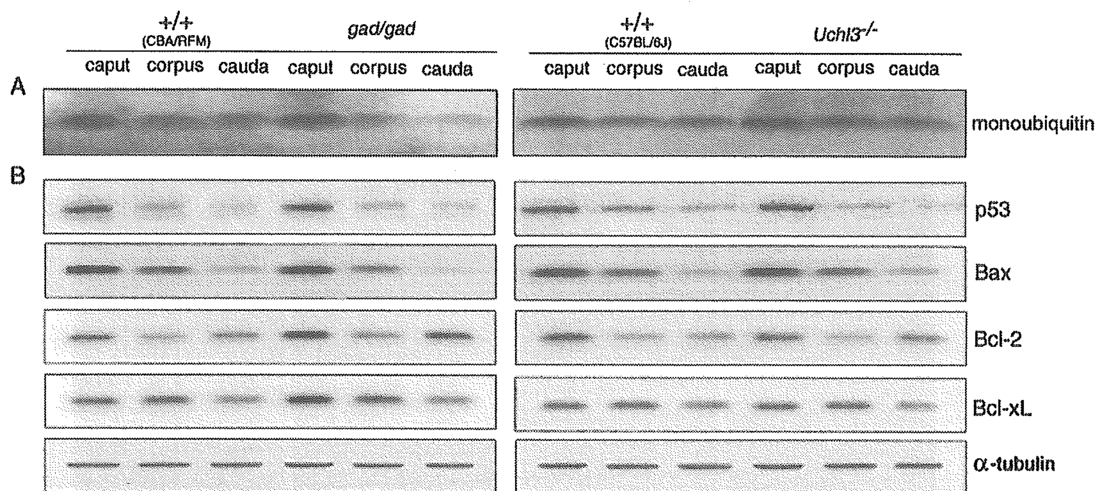

Fig. 4. Western blot analyses showing monoubiquitin and apoptotic proteins in the individual epididymal regions. Total protein (10 μg per lane) was prepared from the caput, corpus and cauda epididymidis from two wild-type (CBA/RFM and C57BL/6J), *gad* and *Uchl3* knockout mice. The blots show the expression levels of monoubiquitin (A) and apoptotic proteins (p53 and Bax) and antiapoptotic proteins (Bcl-2 and Bcl-xL) (B). Blots were reprobed for a-tubulin, which was used to normalize the protein load. Images are representative of four independent experiments.

the ubiquitin staining in these epididymal regions was less intense (Fig. 3B). Immunoreactivity to both UCH-L1 and ubiquitin was intense in the caput epididymal epithelial cells, which was consistent with the expression level (Fig. 2 and Fig. 4A). Diffuse cytoplasmic immunoreactivity in the epididymal epithelial cells to UCH-L3 was intense in the cauda epididymis (Fig. 3C). As shown previously [24], no UCH-L1 or UCH-L3 immunoreactivity was found in the epididymal epithelial cells of *gad* and *Uchl3* knockout mice, respectively (Fig. 3A, C. inset).

Region-specific localization of ubiquitin and apoptotic proteins in the caput epididymis

We previously reported that UCH-L1 binds ubiquitin, and that the level of ubiquitin is decreased in *gad* mice [25, 31]. To determine whether UCH-L1 is associated with the ubiquitin level in the epididymis, we performed western blot analysis of the individual epididymal regions. The monoubiquitin level was markedly higher in the caput epididymis than in the corpus and cauda epididymis, and the low level of monoubiquitin in *gad* mice is consistent with our previous report [25] (Fig. 4A). The epididymis of *Uchl3* knockout mice did not show a difference in ubiquitin level compared with the corresponding wild-type controls.

To explore whether apoptotic phenomenon of spermatozoa in the caput epididymis is in accord with the high expression of apoptotic proteins, we used western blot analysis to verify the expression levels of p53 and Bcl-2 family proteins, which are associated with cell death [12, 28, 29]. The levels of p53 and Bax protein, considered to be proapoptotic, were strikingly high in the caput epididymis, consistent with the pattern of the monoubiquitin level (Fig. 4B). In the *gad* mouse, the levels of the antiapoptotic proteins, Bcl-2 and Bcl-xL, were markedly elevated compared in wild-type mice in the caput epididymis [23] as well as a possible increase in the corpus and cauda epididymis, whereas the levels of apoptotic proteins, p53 and Bax, were unchanged (Fig. 4B). However, we did not detect a difference in the analyzed protein levels between the epididymis of *Uchl3* knockout and wild-type mice.

Region-specific apoptosis in the epididymis following unilateral efferent duct ligation

Androgen deprivation by efferent duct ligation induces glandular epithelial cell death via an apoptotic mechanism [9, 38]. We previously showed that germ cell apoptosis differs between *gad* and *Uchl3* knockout mice following cryptorchid injury [25]. To detect apoptosis in the epididymis following efferent duct li-

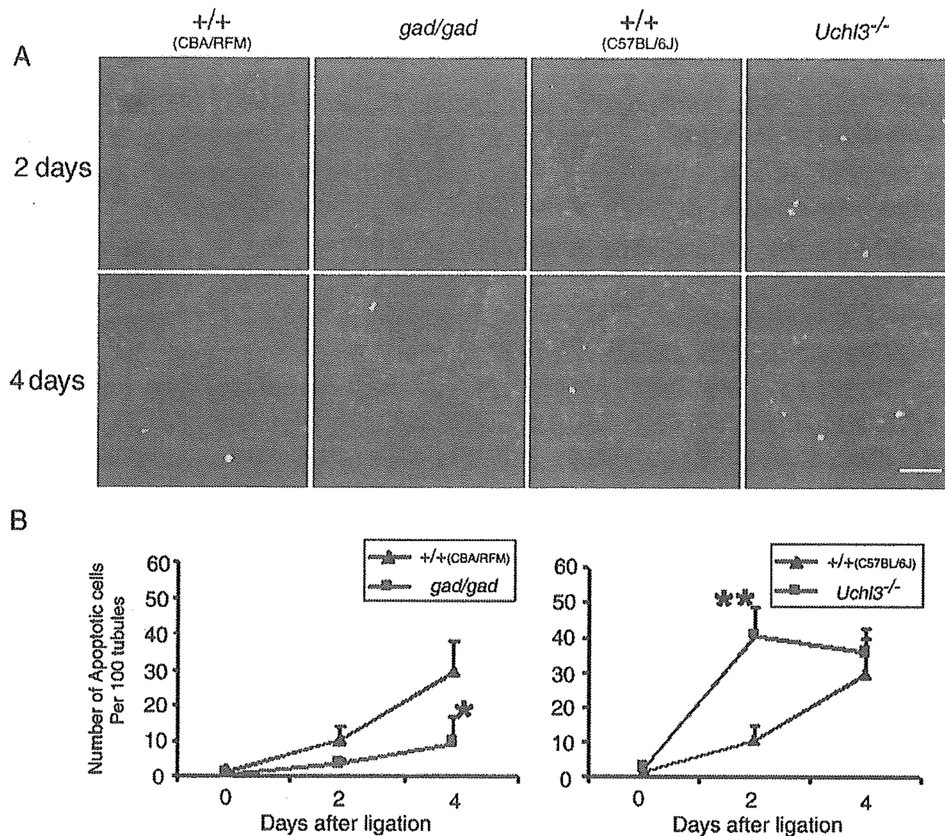

Fig. 5. TUNEL staining of apoptotic cells in the caput epididymis following unilateral efferent duct ligation. A: TUNEL staining in the caput epididymis cross-sections on days 2 and 4 after ligation. Green fluorescence, TUNEL-positive cells; red fluorescence, nuclei stained with propidium iodide. Magnification, 200×. *Scale bar*, 30 μm. B: Quantitation of epithelial cell apoptosis in the caput epididymis following efferent duct ligation. The number of apoptotic epithelial cells in *gad* and wild-type mice is shown on the left. Each value represents the mean ± SD; **P*<0.05. The number of apoptotic epithelial cells in *Uchl3* knockout and wild-type mice is shown on the right. Each value represents the mean ± SD; ***P*<0.01.

gation, we used an *in situ* TUNEL assay to examine apoptosis in *gad* and *Uchl3* knockout mice on postoperative days 2 and 4 (Fig. 5). After efferent duct ligation, epithelial cell apoptosis was observed only in the caput epididymis (mostly the initial segment). The caput epididymis showed a time-dependent increase in epithelial cell apoptosis after efferent duct ligation and epithelial cell apoptosis was found mainly in the principal cells (Fig. 5A). Compared with wild-type mice, the caput epididymis of *Uchl3* knockout mice showed a marked increase in apoptotic epithelial cells on postoperative day 2, whereas *gad* mice resisted efferent duct-ligated epithelial cell apoptosis (Fig. 5A). By postoperative day 2, the number of apoptotic cells per 100 tubules increased with statistical significance (***P*<0.01, *n*=4) in the caput epididymis of *Uchl3* knockout mice as compared with wild-type mice (Fig. 5B). However, *gad* mice showed resistance to ligation-induced apoptosis in the caput epididymis relative to wild-type mice by postoperative day 4 (**P*<0.05, *n*=4) (Fig. 5B).

Discussion

After leaving the testis via the testicular rete, spermatozoa collect in the epididymis, where they undergo final maturation and storage [2, 36, 37]. During epididymal passage, ubiquitination may trigger apoptotic mechanisms that recognize and eliminate abnormal sper-

matozoa, and ubiquitination is believed to play an important role in controlling sperm quality to ensure the production of intact, functional spermatozoa [10, 27, 37]. Ubiquitination of abnormal spermatozoa predominantly occurs in the caput epididymis [37].

Previous studies have shown that two closely-related UCH isozymes, UCH-L1 and UCH-L3 have distinct expression patterns during spermatogenesis [24] and reciprocal functions following cryptorchid injury [25]. We have proposed that UCH-L1 might function as a regulator of apoptosis. Indeed, UCH-L1-deficient *gad* mice are resistant to apoptotic stress [13, 23, 25], and this apoptotic resistance leads to alterations in sperm motility and morphology as well as an increased number of defective spermatozoa in the epididymis of *gad* mice [23]. Our present study demonstrated that UCH-L1 and UCH-L3 have distinct expression patterns along the epididymis in wild-type mice (Fig. 2). We detected a high level of UCH-L1 in the caput epididymis, the main maturation organ, whereas the UCH-L3 level was high in the cauda epididymis, the main storage organ [10]. These region-specific variations in UCH-L1 and UCH-L3 level suggest that they have different functions in the epididymis. The regional differentiation of the epididymis, as suggested by region-specific gene expression, reflects different luminal environments between the regions [16, 19].

We also determined the expression pattern/level of the major component of the proteolytic pathway, ubiquitin, which has specificity for UCH-L1. UCH-L1 associates with monoubiquitin [31], and the monoubiquitin level is reduced in *gad* mice relative to wild-type mice [25, 31]. Predictably, monoubiquitin expression pattern showed similar patterns to UCH-L1 and the monoubiquitin level was reduced in the epididymis of *gad* mice, which had its highest level in the caput epididymis relative to the corpus or cauda epididymis in wild-type mice (Figs. 3 and 4A). Ubiquitin induction is important for regulating programmed cell death, which is a fundamental component of spermatogenesis [1, 23, 32]. Under specific circumstances, the caput epididymis contains a high level of ubiquitin, which may serve to maintain apoptotic mechanisms that eliminate abnormal spermatozoa [37]. This is consistent with the high levels of apoptotic p53 and Bax observed in the caput epididymis compared with the corpus and cauda epididymis (Fig. 4B). Protein p53 and Bax are classically thought to be involved in regulating apoptotic processes, and are targets for ubiquitination [5, 7, 29, 30]. The role of p53 in mediating apoptosis in the male genital tract has been demonstrated in several mice lines [28, 29, 42]. However, p53-independent apoptosis is suggested in the prostate and seminal vesicles by androgen withdrawal or in the rat epididymis by deprivation of luminal factors [3, 11, 14, 38]. Previous studies indicated that Bcl-2 family proteins are involved in the induction or prevention of apoptosis [12, 33, 39, 40]. In *gad* mice, in the present study, the levels of the antiapoptotic proteins, Bcl-2 and Bcl-xL, were markedly increased in the caput epididymis (Fig. 4B), although there was no difference in the levels of the apoptotic proteins, p53 and Bax, relative to wild-type mice. The high levels of Bcl-2 and Bcl-xL in the caput epididymis of *gad* mice is consistent with a previous report that the percentage of morphologically abnormal spermatozoa is significantly higher in *gad* mice [23]. Therefore, the variations of in the levels of Bax, and Bcl-2 and Bcl-xL combined in the caput epididymis probably maintain the regulation of apoptosis [4].

Our previous work focused on the reciprocal functions that UCH-L1 and UCH-L3 exhibit, a distinct feature in testicular germ cells following cryptorchid-induced apoptosis [25]. To characterize the distinct functions of UCH-L1 and UCH-L3 in the epididymis, *gad* and *Uchl3* knockout mice were examined after efferent duct ligation. The epididymal epithelium of the two mutant mice showed differences in apoptotic induction following efferent duct ligation (Fig. 5), after which the circulating androgen level decreases rapidly as a result of apoptotic cell death [9, 20, 38]. After duct ligation, the number of apoptotic cells increased in the caput epididymis of *Uchl3* knockout mice compared with wild-type mice, whereas *gad* mice showed relative resistance in this regard (Fig. 5B). In *gad* mice, the resistance to apoptotic stress can be explained by the high levels of Bcl-2 and Bcl-xL combined in the caput epididymis (Fig. 4B). The tissue androgen level is higher in the caput epididymis than in the corpus or cauda epididymis [15, 38]; thus, apoptotic cells showed in the caput epididymis rather than in the corpus and cauda epididymis following efferent duct ligation. These results may suggest that UCH-L1 and UCH-L3 have reciprocal functions in the caput epididymis fol-

lowing apoptotic stress induced by androgen withdrawal, as was shown with cryptorchid stress [25].

We cannot explain the profound apoptotic phenomenon observed in the present study in the caput epididymis of *Uchl3* knockout mice after efferent duct ligation by the balance of the Bcl-2 family proteins alone. Although our previous report showed that the Nedd8 expression level increased in the testis of *Uchl3* knockout mice [25], we found no difference in the present study (data not shown). The mechanism with regard to the antiapoptotic function of UCH-L3 requires further study. Our present study demonstrated that UCH-L1 and UCH-L3 have distinct expression levels along the epididymis as well as reciprocal functions in response to apoptotic stress induced by androgen withdrawal.

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