microglia/macrophages in inflammatory lesions -tautwopathy distinct from degenerative tauopathies-. Acta Neuropathol. 2005:109:159-164

7. Uchihara T, Shibuya K, Nakamura A, Yagishita S. Silver stains distinguish tau-positive structures in corticobasal degeneration/progressive supranuclear palsy and in Alzheimer's disease--Comparison between Gallyas and Campbell-Switzer methods--. Acta Neuropathol. in press(10.1007/s00401-004-0947-7)
8. Uchihara T, Tsuchiya K, Nakamura A, Akiyama H. Silver staining profiles distinguish Pick bodies from neurofibrillary tangles of Alzheimer type --Comparison between Gallyas and Campbell-Switzer methods--. Acta Neuropathol. in press(10.1007/s00401-005-09488-6)
9. Uchihara T, Sanjo N, Nakamura A et al. Transient abundance of presenilin 1 fragments / nicastrin complex associated with synaptogenesis during development in rat cerebellum. Neurobiol. Aging. in press
10. Orimo S, Kanazawa T, Amino T, Uchihara T. et al. Preserved Cardiac Sympathetic Nerve Accounts for Normal Cardiac Uptake of MIBG in PARK2. Mov. Disord. (in press)
11. Orimo S, Amino T, Ito Y , Uchihara T. et al. Cardiac sympathetic denervation precedes neuronal loss in the sympathetic ganglia in Lewy body disease. Acta Neuropathol (in press)
12.内原俊記　原発性進行性失語　Clin. Neurosci. 2005:23(3):294-6
13.内原俊記 封入体の神経病理-蛋白沈着の疾患特徴的様式と萎縮・変性との関係-神経進歩 2004:48:334-345
14. 内原俊記　脳のかたち、病のかたち ーまだまだわからない脳の不思議ー「こころを育む脳のはたらきー育てることと守ること」（2004世界脳週間講演録）クバプロ(印刷中)

2.学会発表

1.渋谷克彦、内原俊記、中村綾子、山岡恵子、高橋竜哉、柳下三郎，皮質基底核変性症(CBD)と進行性核上性麻痺(PSP)のtau2陽性構造物に対する界面活性剤の影響，第４５回日本神経病理学会総会学術研究会，前橋市（2004, 5.26) [Neuropathology, 24(2004)62]
2.中村綾子、内原俊記、渋谷克彦、柳下三郎，タウ陽性構造物の嗜銀性について免疫染色/銀染色の多重染色による検討，第45回日本神経病理学会総会学術研究会，前橋市 (2004, 5.26) [NEUROPATHOLOGY, 24(2004)63]
3.内原俊記、渋谷克彦、中村綾子、柳下三郎，タウ陽性構造物の嗜銀性の違いーGallyas法とCampbell-Switer法の比較，第45回日本神経病理学会総会学術研究会，前橋市 (2004, 5.26) [NEUROPAHOLOGY, 24(2004)64]
4.馬原孝彦、内原俊記、土谷邦秋、中村綾子、小山俊一、岩本俊彦、高橋　優、池田研二, Pick嗜銀球における14-3-3蛋白およびisoformの局在，第45回日本神経病理学会学術研究会，前橋市（2004, 5.26) [NEUROPATHOLOGY, 24(2004)66]
5.内原俊記、中村綾子、三條伸夫、セントジョージヒスロップピーター，生後ラット小脳におけるニカストリンとプレセニリン1の発現，第45回日本神経病理学会学術研究会，前橋市（2004, 5.26) [NEUROPATHOLOGY, 24(2004)68]
6児矢野繁、黒岩義之、柳下三郎、岩淵　繁、内原俊記, SCA2おける病変の分布及び病理学的特徴(ataxin-2,polyglutamineおよびubiquitin蛋白の分布，第45回日本神経病理学会学術研究会，前橋市（2004, 5.28) [NEUROPATHOLOGY, 24(2004)150]
7.内原俊記、武田嘉恵、小林高義、春日　孟、石川欽也、姜　喜玲、水澤英洋、遠藤太嘉志、広川勝イク　、黒岩俊彦, SCA1遺伝子に軽度の延長を認めた脊髄小脳変性症の一剖検例，第45回日本神経病理学会学術研究会，前橋市（2004, 5.28) [NEUROPATHOLOGY, 24(2004)151]
8.児矢野繁、黒岩義之、柳下三郎、岩淵　潔、内原俊記, SCA2における病変の分布および病理学的特徴(ataxin-2,polyglutamineおよびubiquitin蛋白の分布)，第45回日本神経病理学会総会学術研究会，前橋市 (2004, 5.28) [NEUROPATHOLOGY, 24(2004)150]
9.内原俊記、武田嘉恵、小林高義、春日　孟、石川欽也、姜　喜玲、水澤英洋、遠藤太嘉志、広川勝いく、黒岩俊彦, SCA1遺伝子に軽度の延長を認めた脊髄小脳変性症の一剖検例，第45回日本神経病理学会総会学術研究会，前橋市 (2004, 5.28) [NEUROPAHOLOGY, 24(2004)151]
10.古城徹、内原俊記、高橋敦、網野猛志、織茂智之，パーキンソン病の交感神経障害は心臓に特異的か？，第4回Parkinson's

disease Forum, 2004, 千葉県浦安市（東京ベイホテル東急）（2004, 8.28）

11.織茂智之、金澤俊郎、篠栗弘貴、網野猛志、高橋敦、古城徹、内原俊記、若林孝一、高橋均, パーキンソン病の心臓交感神経変性は病早期から逆行性に進行する。, 第4回Parkinson's disease Forum, 2004, 千葉県浦安市（東京ベイホテル東急）（2004, 8.28）

12.金澤俊郎、網野猛志、水澤英洋、織茂智之、横地正之、古城徹、内原俊記、若林孝一、高橋均、服部信隆, PARK2では心臓交感神経は障害されず心臓のMIBG集積は保たれる。, 第4回Parkinson's disease forum, 2004, 千葉県浦安市（東京ベイホテル東急）（2004, 8.28）

13.内原俊記、池田研二、中村綾子、土谷邦秋, Pick小体の嗜銀性の特徴-Alzheimer病との違い-, 第23回日本痴呆学会, 江戸川総合区民ホール（2004, 9.30）[Dementia Japan, 18(2004)156]

14.町田 明、小林 禅、三明裕知、綿引定清、内原俊記, 随意呼吸障害、運動性失語、軟口蓋挙上障害を伴い進行性の発語失行を呈した一例, 第171回日本神経学会関東地方会, 東京都千代田区平河町（全教連ビル）（2004, 11.27）[, 0]

15.内原俊記、中村綾子、三條伸夫、St.George-Hyslop,P.,Fraser,P., 生後ラット小脳におけるニカストリンの発現, 第45回日本神経学会総会, 東京都品川区（高輪プリンスホテル）（2004, 5.11）[臨床神経, 44(2004)1043]

16.小林高義、秋本秀昭、小松清秀、遠藤太嘉志、内原俊記, ペニシリン耐性肺炎連鎖球菌による重症髄膜農園(PRME)の臨床的病理学的研究, 第45回日本神経学会総会, 東京都品川区（新高輪プリンスホテル）（2004, 5.11）[臨床神経, 44(2004)1061]

17.児矢野繁、黒岩義之、岩淵潔、柳下三郎、内原俊記, 遺伝性脊髄小脳変成症における橋神経細胞の病理学的特徴, 第45回日本神経学会総会, 東京都品川区（新高輪プリンスホテル）（2004, 5.11）[臨床神経, 44(2004)1067]

18.織茂智之、網野猛志、古城徹、内原俊記、高橋敦、若林孝一、高橋均, パーキンソン病（PD）の心臓交感神経病変はごく早期に始まる可能性がある, 第45回日本神経学会総会, 東京都品川区（新高輪プリンスホテル）（2004, 5.14）[臨床神経, 44(2004)1150]

19.古城轍、内原俊記、高橋敦、網野猛志、織茂智之, パーキンソン病（PD）に伴う交感神経障害の各種臓器における免疫組織化学的検討, 第45回日本神経学会総会, 東京都品川区（新高輪プリンスホテル）（2004, 5.14）[臨床神経, 44(2004)1150]

20.馬原孝彦、内原俊記、土谷邦秋、中村綾子、小山俊一、岩本俊彦、高崎優, Pick嗜銀球における14-3-3蛋白の免疫染色性, 第45回日本神経学会総会, 東京都品川区（新高輪プリンスホテル）（2004, 5.14）[臨床神経, 44(2004)1174]

21.金澤俊郎、網野猛志、織茂智之、横地正之、内原俊記、若林孝一、高橋均、服部信隆、水澤英洋, PARK2では心臓交感神経は障害されず心臓のMIBG集積は保たれる, 第45回日本神経学会総会, 東京都品川区（新高輪プリンスホテル）（2004, 5.14）[臨床神経, 44(2004)1185]

22.網野猛志、金澤俊郎、島津誠一郎、内原俊記、織茂智之、水澤英洋, 正常 mouse における心臓交感神経の分布ーパーキンソン病の自律神経障害の解明に向けてー, 第 45 回日本神経学会総会, 東京都品川区（新高輪プリンスホテル）（2004, 5.14）[臨床神経, 44(2004)1185]

H.知的財産権の出願・登録状況
特記すべきことなし

別紙５

研究成果の刊行に関する一覧表

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
横田隆徳	家族性ビタミンＥ欠乏症の発見と発症機序の解明および治療法の確立	ビタミン	78	87-92	2004
横田隆徳	ビタミン欠乏による神経症状	薬の知識	57	In press	2005

別紙４

研究成果の刊行に関する一覧表レイアウト（平成１６年度）

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
M. Shibata, J. Ishii, H. Koizumi, N. Shibata, N. Dohmae, K. Takio, H. Adachi, M. Tsujimoto, and H. Arai	Type F scavenger receptor SREC-I interacts with advillin, a member of the gelsolin/villin family, and induces neurite-like outgrowth in mouse fibroblastic L cells	J. Biol. Chem.	279	40084-40090	2004
Tamura Y, Osuga J, Adachi H, Tozawa R, Takanezawa Y, Ohashi K, Yahagi N, Sekiya M, Okazaki H, Tomita S, Iizuka Y, Koizumi H, Inaba T, Yagyu H, Kamada N, Suzuki H, Shimano H, Kadowaki T, Tsujimoto M, Arai H, Yamada N, Ishibashi S	Scavenger receptor expressed by endothelial cells I (SREC-I) mediates the uptake of acetylated low density lipoproteins by macrophages stimulated with lipopolysaccharide.	J. Biol. Chem.	279,	30938-30944	2004)
F. Bonin, S. D. Ryan, L. Migahed, F. Mo, J. Lallier, D. J. Franks, H. Arai, and S. A. L. Bennett	Anti-apoptotic actions of the platelet activating factor acetylhydrolase Ib catalytic subunit	J. Biol. Chem.,	279,	52425-52436	2004

研究成果の刊行に関する一覧表

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
Uchihara T, Duyckaerts C, Iwabuchi K, Iwata M, et al.	Was Pierre Marie s ataxia Machado-Joseph disease?	Arch Neurol	61	784-790	2004
Nakamura A, Uchihara T	Dual enhancement of tripleimmunofluorescence using two antibodies froma the same species	J Neurosci Methods	135	67-70	2004
Umahara T, Uchihara T, Tsuchiya K, et al	14-3-3 proteins and zeta isoform containing NFTs in patients with Alzheimer s disease	Acta Neuropathol	108	279-286	2004
Umahara T, Uchihara T , Tsuchiya K et al.	Immunolocalization of 14-3-3 isoforms in brains with Pick body disease	Neurosci Lett	371	215-219	2004
Amino T, Orimo S, Itoh Y, Takahashi A, Uchihara T, Mizusawa H.	Profound cardiac sympathetic denervation occurs in Parkinson s disease	Brain Pathol	15	29-34	2005
Uchihara T, Duyckaerts C, Seilehean D et al.	Exclusive induction of tau2 epitope in microglia/macrophages in inflammatory lesions	Acta Neuropathol	109	159-164	2005
Uchihara T, Shibuya K, Nakamura A, Yagishita S.	Silver stains distinguish tau-positive structures in CBD/PSP and in AD.	Acta Neuropathol	10.1007/ s00401-004-0947-7		
Uchihara T, Tsuchiya K, Nakamura A, Akiyama H.	Silver staining profiles distinguish Pick bodies from NFTs of Alzheimer type	Acta Neuropathol	10.1007/ s00401-005-09488-6		
内原俊記	原発性進行性失語	Clin Neurosci	23	294-6	2005
内原俊記	封入体の神経病理	神経進歩	48	334-345	2004

総合論文

家族性特発性ビタミンE欠乏症の発見と発症機序の解明および治療法の確立*

東京医科歯科大学 神経内科**

横田 隆徳

Vitamins (Japan), 78 (2), 87-92 (2004)

Pathophysiology and Therapy for Familial Idiopathic Vitamin E Deficiency

Takanori YOKOTA

Department of Neurology, Tokyo Medical and Dental University

I found the new disease 'familial ataxia with idiopathic vitamin E deficiency (AVED)' without fat malabsorption. I identified the causative gene: α-tocopherol transfer protein (αTTP). By impairing αTTP function in the liver, α-tocopherol cannot be transferred to VLDL, resulting in low concentration of serum vitamin E. I establish the disease entity and made clear the clinical picture of posterior column ataxia and retinitis pigmentosa. Furthermore, by investigating αTTP knockout mouse, I clarify the mechanism of vitamin E deficiency and neuronal degeneration. In this disease, almost symptoms can be prevented to progress by vitamin E supplementation.

Key words: vitamin E, α-tocopherol transfer protein, ataxia, retinitis pigmentosa

(*Received October 6, 2003*)

1. 原因遺伝子発見までの背景

慢性の脂肪吸収障害が運動失調や視覚障害などの進行性の症状をきたすことは知られていた．1981年Burckら[1]，1985年Hardingら[2]により特発性のビタミンE(E)単独欠乏による重篤なFriedreich失調症型の脊髄小脳変性症の小児例の報告がされたのに引き続いて，1987年に我々は[3]初めて中年以降の成人発症の比較的症状の軽度の家系を報告し，さらにE欠乏の機序がEの吸収不全ではなく，血清からのEの消退速度の亢進であること明らかにした．現在ではfamilial isolated vitamin E deficiency(FIVE)，またはataxia with isolated vitamin E deficiency(AVED)の名称で報告されている．

1995年に症状の均一なチュニジアの家系で連鎖解析が進められ，第8染色体長腕に原因遺伝子が連鎖することが明らかとなり，同年にAritaら[4]によってによりクローニングされたヒトα-tocopherol転移タンパク(α-TTP)遺伝子が同じく第8染色体長腕に存在することが判明し，フランスのKoenig，日本のArai，アメリカのKaydenらによる共同研究により北アフリカ，イタリアの例の家系においてα-TTP遺伝子frame-shift変異が発見された[5]．我々もGotoda，Araiらと共同で我々の成人発症例の検索を進め，同年に同遺伝子にmissense変異があり，これがα-TTPの機能障害の原因であることを証明した[6]．

我々はこの成人発症のE欠乏症の症例を集積してい

*本論文は日本ビタミン学会第55回大会(平成15.5.29～30，出雲市)における奨励賞講演をまとめたものである．

**〒113-8519 東京都文京区湯島1-5-45

くうちに，しばしば重度の網膜色素変性症を伴う例が稀でないことに気付いた．これらより，α-TTP が網膜色素変性症の新しい原因遺伝子であり，また網膜色素変性症を伴う Friedreich 型失調症という新しい症候群と考えている[7)8)]．

さらに我々は α-TTP ノックアウトマウスを作製して，神経変性の発生機序とE欠乏との因果関係を明らかとし，この疾患がE投与で治療可能であることを証明し[9)]，神経変性疾患の中で最初の原因治療の確立となった．

2．遺伝子異常と臨床症状[8)10)]

基本的な神経症状は著明な運動失調と深部感覚障害，Romberg 徴候，四肢腱反射消失でほぼ全例に認められる．症状は下肢に強い．多くの例に構音障害を，約半数の例に Babinski 徴候，頭部振戦を認める．一部の例に四肢末端の表在感覚障害，網膜色素変性症，側湾症，凹足，四肢筋力低下，筋萎縮を認める．知能障害，眼振，fasciculation，自律神経症状は認めない．

一般検査所見では，他の脂溶性ビタミンであるビタミンA，Dは正常でEの単独欠損である．劣性遺伝でEの運搬タンパクの一つである β-リポプロテインの先天欠損が原因の無b-リポプロテイン症は類似の症状を示すが，β-リポプロテインが低値であり脂溶性ビタミンが正常であることから鑑別できる．肝臓の α-TTP の機能評価として α-Toc の経口負荷試験で上述のように α-Toc の吸収は正常だが，その消退速度が亢進していることが示される．画像診断では頭部 MRI では小脳，脳幹の萎縮，輝度異常は認めず，SPECT も正常である．脊髄 MRI で脊髄に萎縮を認めることがある．

電気生理学的所見では，正中神経体性感覚誘発電位で N13 と皮質電位の消失を認める一方，Erb の電位は認められ，末梢神経伝導検査では運動神経は正常，感覚神経では伝導速度正常，振幅低下もごく軽度に留まることが特徴的である．その他の詳細な検討は我々の検索した H101Q 変異のみであるが，経頭蓋的磁気刺激による運動誘発電位で中枢運動時間の延長，網膜電図の振幅低値，視覚誘発電位で P100 の延長を認める[8)]．これらの神経症状，検査所見から神経系の運動失調の主な責任病巣は脊髄後索と網膜で，加えて，視覚伝導路，錐体路，末梢感覚神経の障害が考えられた．構音障害がある例で小脳症状の有無については深部感覚障害が著明であるためその評価が困難だが，MRI，SPECT や神経耳科的な検索では小脳障害は明らかではなかった(自検例)．

遺伝子異常は現在まですべての exon にて変異が知られている[10)]．北アフリカ，イタリアからの報告は最も頻度の高い 744delA をはじめ 486delA，530AG→GTAAGT，513insTT はいずれも frame-shift 変異のホモ接合体である．最近西日本で発見された G552A は exon 3 の 3'端の変異で exon 3 の skipping をおこし 57 % の truncation でタンパクの機能はほとんどないと考えられる[11)]．これらの症例の発症は 20 歳以下で 30 歳までに自力歩行不能となるなど症状は重く，血清Eの濃度は著しく低い(＜1.0 mg/L)．744delA はタンパクのC末端の 11 % のみが欠損するのみだが，その例の症状や血清Eの濃度低下は重度であり，この事実はタンパクのC末端に重要な働きが存在することを示唆する[5)12)]．

一方，我々の報告した4家系(H101Q)は missense 変異であり発症も 30～50 歳台遅く，症状も軽くて血清Eの濃度低下も比較的軽度である(＞1.0 mg/L)．事実，剖検肝には正常対象と同じ分子量の α-TTP が発現しており[30)]，変異 cDNA による recombinant タンパクの α-Toc の *in vitro* での転送機能は正常タンパクの 11 % ではあるが残存していた[6)]．513insTT と 574G→A の frame-shift 変異と missense 変異の複合ヘテロ接合体である例[10)]の症状や血清Eの濃度低下は軽度で，劣性遺伝である本症の遺伝形式から肝臓 α-TTP の機能は missense 変異のアリルに依存すると考えるとよく説明できる．ただし，H101Q のヘテロ接合体は無症状だが，血清E濃度は正常範囲内だが平均値は正常より約 25 % 低下しており，肝臓で変異タンパクが正常タンパクの機能を阻害したり，変異タンパクと正常タンパクがダイマーを作る可能性も考えられている[6)]．

GAA repeat の伸長を伴う Friedreich 失調症と α-TTP 遺伝子の non-conservative 変異による本症の基本症状，経過はよく似ているが，神経症状では本症の約半数の例に見られる頭部振戦や一部の例でみられる網膜色素変性症は Friedreich 失調症では認められない点と，検査所見では感覚神経の末梢神経伝導検査で Friedreich 失調症ではほとんど導出不能になるのに対して本症では振幅が正常下限ながら導出される点が異なる[8)]．また，本症では画像診断上小脳脳幹の萎縮が明らかでなく，四肢末梢部に高度の感覚障害を示し腱反射が消失することから，特に我々の報告したような症状の軽い conservative な missense 変異では電気生理学的検査の解釈が不十分であると感覚性多発ニューロパチーと考えてしまうこともあるので注意が必要である[13)]．

3. 神経病理学的所見

剖検例は744delAの例[14]とH101Qの例[15]の報告がある．両者の基本的な病理像は共通しており，主たる病理所見としてはspheriodとcorpora amylaceaを伴う後索変性と，主に神経細胞へのceroid-lipofuscin沈着であった．後索変性は吻側ほど変化が強く，Goll束，Burdach束両者に認められるが，Goll束により高度な変化であった．一方，後根神経節細胞の減少はごく軽度でNageotte結節も稀であった．さらに後根神経節の末梢軸索側である感覚神経線維は軽度の大径有髄線維の減少は認めるものの有髄線維全体の線維密度は正常範囲内でその変化は後索変性に比して極めて軽度であった．これらの病変パターンは後根神経節の中枢軸索のdying-back型変性といえる．Ceroid-lipofuscinの異常沈着は特に後根神経節細胞，脊髄前角細胞に著明だが，744delA例では大脳皮質第3相，脳神経核，小脳歯状核，視床，線状体，外側膝状体などH101Q例に比してより広範に沈着を認めている．

程度は軽度であるが重要な所見として2例ともに軽度ながら小脳Purkinje細胞の脱落を認めている．744delA例では小脳歯状核の神経細胞も軽度低下していた．また，H101Q例では網膜に局所的に光受容体細胞の変性と網膜色素細胞の多量のceroid-lipofuscin沈着を認め視野，視力障害の責任病巣と考えられた．また，H101Q例では外側膝状体の髄鞘は淡明化しており視覚誘発電位のP100潜時延長を説明する病変かもしれない．皮質脊髄路は744delAでは中等度淡明化していた．これらの病理所見はE欠乏動物[16)-18)]やヒトの慢性脂肪吸収不全[19]の所見と非常によく一致しているが，本症では小脳Purkinje細胞の脱落が明らかな点が異なる．

4. α-tocopherol転移タンパク(α-TTP)

α-TTPは肝細胞に豊富に存在する細胞質タンパクとして同定され，その分子量は約3万でクロマトフォーカシング上等電点5.1および5.0のふたつのアイソフォームを有する．α-TTPは1モル当たり1モルのα-Tocと結合し，メチル基の1つ少ないだけのγ-Tocは親和性は極端に低下することから本タンパクはα-Tocに特異的に結合し[20)21)]，肝細胞からα-Tocをゴルジを介さない経路で細胞外に分泌する働きを担っていると考えられる[22]．

ヒトα-TTP遺伝子cDNAは全長4.5kbで5つのエキソンからなる278のアミノ酸をコードするopen reading frameを持ち，C末端側に疎水性アミノ酸が比較的多くあることからこの部分に基質結合部位が存在するすることが推定されている[20]．α-TTPのアミノ酸配列は網膜細胞に局在するcellular retinaldehde binding protein (CRALBP)と高いホモロジーを示し，その機能が網膜色素細胞内において様々なレチノイドの中から11-cis-レチナールを識別して視細胞に供給する働きであることから，これらはファミリーを形成し，脂溶性生理物質の中から生体に必要なもののみを識別して輸送する機能に関わることが推定されている．

5. α-TTPノックアウトマウスの神経症状[9]

ノックアウトマウスの作製はマウスα-TTPゲノムDNAにexon1をネオマイシン耐性遺伝子に置換することによりα-TTP遺伝子をノックアウトした．ノックアウトマウスでは肝臓のα-TTP mRNAやタンパクは欠損し，血清のα-tocopherol濃度は著明に低下した．ヘテロ接合体マウスでは肝臓のα-TTP mRNA，血清のα-tocopherol濃度ともに半減した．作製されたノックアウトマウスではα-tocopherolの欠乏した餌を与えた群，正常の餌(36 mg/kg)を与えた群，過剰の餌(600 mg/kg)を与えた群を作成すると，過剰の餌によってα-TTPノックアウトマウスの血清のα-tocopherol濃度は正常レベル程度まで回復した．α-TTPノックアウトマウスは生後1年までは明らかな神経症状は示さなかったが，1年3か月頃より運動失調，筋力低下，振戦を示すようになった．進行すると歩行時に両下肢をひきずるようになった．振戦は安静時には明らかではないが，歩行時やストレスが加わるとより明らかとなった．歩行障害は加速度式回転車テストにより野生型に比較してノックアウトマウスの運動障害は明らかであった．これらの振戦や運動障害はα-tocopherol欠乏食群ではより障害が顕著で，α-tocopherolの過剰投与により著明に改善し，過剰食の群では野生型と有意差はほとんどなかった．

さらに電気生理学的に検討したところ，体性感覚誘発電位で前肢，後肢いずれの電気刺激でも末梢神経伝導は正常だが，大脳誘発電位はその振幅がノックアウトマウスでは著明に低下し，α-tocopherolの過剰投与により著明に改善した．網膜電図でもノックアウトマウスではMuller細胞や内神経層の機能を反映すると考えられるa波，光受容体細胞の機能を反映すると考えられるb波ともに低下していた．

中枢神経系の病変の主座は脊髄後索で顕著な神経線維の脱落とスフェロイドを，後索核で線維性グリオー

シス認めた．この変化はBurdach束よりGoll束に高度で，腰髄より頚髄，頚髄より延髄後索核でより顕著である一方，後根神経節では変化はわずかで，dying backタイプの後索変性であった．網膜は外顆粒層，視細胞内節，外節の厚さの減少を認めた．また，自己蛍光を発する大量のリポフスチンを後根神経節，脊髄前角細胞，網膜色素上皮に認めた．これらの病理所見は例の744delAとH101Qの剖検所見にほぼ合致したしていたが，剖検例で認められた軽度の小脳Purkinje細胞の脱落がα-TTPノックアウトマウスでは明らかではなかった．

これらの神経変性の所見はα-tocopherol欠乏食群ではより障害が顕著で，α-tocopherolの過剰投与により著明に改善した．

6．ビタミンE欠乏の機序

自然界のEはα，β，γ，δ-tocopherol(Toc)が主な同族体である．一般に食物にはα-Tocよりもγ-Tocのほうが多く含まれているが，生理的に活性をもつのはα-Tocで，実際ほ乳類の血漿中にはα-Toc濃度はγ-Tocの5～20倍も高い．上述のようにα-TTPは肝細胞の細胞質タンパクでα-Tocを選択的に摂取し，リサイクルして血中濃度を維持する働きを果たしていると考えられている．すなわち，α-Tocとγ-Tocは小腸で同程度の効率で吸収され，カイロミクロンの成分として肝臓に取り込まれる．肝細胞においてγ-Tocは胆汁から腸管内に排泄される一方，α-Tocは選択的に肝臓で合成される超低比重リポタンパク(VLDL)に取り込まれて再び血中に戻る．血中でVLDLは低比重リポタンパク(LDL)に変化し，LDL受容体を介して各組織の細胞内に取り込まれる[23)-25)]．

AVED患者のE欠乏の機序は肝臓のα-TTPの機能異常でよく説明できる．正常人にα-Toc経口摂取させると小腸で吸収され血清中のα-Toc濃度は上昇し6～8時間後にピークとなるが，AVED患者ではこのα-Tocの吸収は正常かむしろ亢進している[3)26)27)]．しかし，その後のα-Toc濃度はAVED患者では正常に比して急速に低下する．ラジオラベルしたα-Tocを経口摂取させ，その脂質代謝を詳細に検討すると，カイロミクロン内のα-Tocの動態は正常と同じだが，VLDL，LDL，HDL内ではα-Tocは急速に低下する[25)]．また，ラジオラベルしたα-Toc(d_6-RRR-α-Toc)とγ-Toc(d_6-SRR-α-Toc)を用いて両者の識別能を検討すると，E欠乏が高度で後にα-TTP遺伝子のframe-shift変異を持つことが判明したAVED患者では正常で認められるd_6-RRR-とd_6-SRR-α-Tocの識別能は全く消失している一方，E欠乏の比較的軽度なmissense変異を持ったAVED患者は正常と高度のE欠乏患者との中間的な比較的軽度の識別能の低下を示した[28)]．

7．神経変性とα-Toc欠損の因果関係

α-TTPが少量ながら中枢神経にも発現していることから，α-TTPの中枢神経内での機能が注目されていた．α-TTPの中枢神経内の局在についてはバーグマングリア[29)]，プルキンエ細胞[30)]などの報告があるが決着はついていない．そこでα-TTPノックアウトマウスの中枢神経内のα-tocopherol濃度について検討した．α-TTPノックアウトマウスでは末梢血，中枢神経内にはα-tocopherolは検出感度以下であった．α-tocopherol過剰食の群では血中のα-tocopherolは正常近くまで上昇するが，中枢神経内のα-Toc濃度は正常の5～10％程度までしか上昇しなかった[9)]．すなわち，中枢神経内にα-TTPが欠損すると血中のα-tocopherolを中枢神経内に効率良く輸送できない，あるいは保持できないと解釈できる．しかし，重要な点は血中のα-tocopherol濃度を上昇させてやると少量ではあるが中枢神経内にα-tocopherolが移行する点である．すなわち，α-TTPの中枢神経内への移行はα-TTP以外にも別のルートがマイナーながら存在することを示している．今回作製したα-tocopherol欠乏食の野生型の群では中枢神経内にα-Toc濃度ある程度保たれた．これは，妊娠にα-tocopherolが必要であるために欠乏食にできずに胎生期に中枢神経内に入ったα-tocopherolがなかなか抜けないことによるのかも知れない．このα-Toc欠乏食の野生型マウスは神経症状を発症しなかったが，その中枢神経内のα-tocopherol濃度はα-tocopherol過剰食のα-TTPノックアウトマウスの中枢神経内のα-Toc濃度より低いことに注目したい．このことは，中枢神経内のα-Toc濃度は正常の5～10％程度であれば神経変性を防ぐには十分であることを示唆している．

α-TTP遺伝子異常症例やノックアウトマウスの神経症状や神経病理所見が従来報告されている欠乏食によるα-tocopherol欠乏動物のそれらによく似ていることからα-TTP遺伝子異常による神経変性は血中のα-tocopherol欠乏が考えられていた．しかし，今回の実験からα-TTPは血中の保持のみならず，中枢神経系への移行にも中核的な働きをしており，欠損は中枢神経にさらに高度のα-tocopherol欠乏状態が生じさせていることが判明した．そして組織内のα-tocopherol補充で神経変性

がほぼ完全に防止ができたことは，神経変性の主たる原因が組織内のα-tocopherol欠乏にあり，中枢神経内α-TTPのα-tocopherol輸送以外の未知の機能の障害ではなさそうであることが示された．

8．AVED患者の治療法

上記のα-TTPノックアウトマウスからの知見より，AVED患者にE投与が神経変性進行の防止に意義をもつという理論的裏付けができたと考えている．α-TTPがα-tocopherolの中枢神経内への移行をも行っているという事実から，AVED患者のE投与は血中のα-tocopherol値が正常範囲内になったというだけでは不十分で，正常の2～3倍のレベルに保つことにより神経症状の進行を停止させることに成功している．

9．今後の展望

中枢神経系は脂質の含有量が多く最も酸化の受けやすい組織と言われている．老化やアルツハイマー病や筋萎縮性側索硬化症などの神経変性疾患の病態生理に酸化ストレスが関与しているという知見は多く報告されている．しかし，動物モデルで，酸化ストレスによる神経細胞死をきたすのはいずれも急性のモデルで，α-TTPノックアウトマウスは慢性の酸化ストレスによる初めての神経変性のモデルといえる．現在，このマウスとアルツハイマー病などの神経変性疾患のモデルマウスとの掛け合わせ実験を進行中で，それぞれの神経細胞死の機序における酸化ストレスの関与を検索する有用な方法になると期待している．

謝　辞

本研究は東京大学大学院薬学部衛生化学，新井洋由先生，東京大学大学院第3内科，後藤田貴成先生，中外製薬創薬資源研究所，鈴木宏志先生，東京都神経科学総合研究所神経病理，内原俊記先生などとの共同研究であり各先生に深謝する．

(平成15. 10. 6受付)

文　献

1) Burck U, Goebel HH, Kuhlendahl HD, Meier C, Goebel KM (1981) Neuromyopathy and vitamin E deficiency. *Neuroperiatrics* 12, 267-278

2) Harding AE, Matthews S, Jones S, Ellis CJ, Booth IW, Muller DP (1985) Spinocerebellar degeneration associated with selective defect of vitamin E absorption. *N Engl J Med* 313,32-35

3) Yokota T, Wada Y, Furukawa T, Tsukagoshi H, Uchihara T, Watabiki S (1987) Adult-onset spinocerebellar syndrome with idiopathic vitamin E deficiency. *Ann Neurol* 22, 84-87

4) Arita M, Sato Y, Miyata A, Miyata A. Tanabe T, Takahashi E, Kayden HJ, Arai H, Inoue K (1995) Human α-tocopherol transfer protein: cDNA cloning, expression and chromosomal localization. *Biochem J* 306, 437-443

5) Ouahchi K, Arita M, Kayden HJ, Hentani F, Ben Hamida M, Sokol R, Arai H, Inoue K, Mandel J-L, Koenig M (1995) Ataxia with isolated vitamin E deficiency is caused by mutations in the α-tocopherol transfer protein. *Nat Genet* 9, 141-145

6) Gotoda T, Arita M, Arai H, Inoue K, Yokota T, Fukuo Y, Yazaki Y, Yamada N (1995) Adult-onset spinocerebellar dysfunction caused by a mutation in the gene for the α-tocopherol-transfer protein. *N Engl J Med* 333, 1313-1318

7) Yokota T, Shiojiri T, Gotoda T, Arai H (1996) Retinitis pigmentosa and ataxia caused by mutation in the α-tocopherol transfer protein gene. *N Engl J Med* 335, 1770-1771

8) Yokota T, Shiojiri T, Gotoda T, Arita M, Arai H, Ohga T, Kanda T, Suzuki J, Imai T, Matsumoto H, Harino S, Kiyosawa M, Mizusawa H, Inoue K (1997) Freidreich-like ataxia and retinitis pigmentosa caused by His101Gln mutation of the α-tocopherol-transfer protein gene. *Ann Neurol* 41, 826-832

9) Yokota T, Igarashi K, Uchihara T, Jishage K, Inaba A, Tomita H, YiLi, Arita M, Suzuki H, Mizusawa H, Arai H (2001) Delayed onset ataxia in mice lacking -tocopherol transfer protein: Model for neuronal degeneration caused by chronic oxidative stress. *Proc Natl Acad USA* 98, 15185-15190

10) Cavalier L, Ouahchi K, Keyden HJ, Di Donato S, Reutenauer L, Mandel JL, Koenig M (1998) Ataxia with isolated vitamin E deficiency: heterogeneity of mutations and phenotypic variability in a large number of families. *Am J Hum Genet* 62, 301-310

11) Tamaru Y, Hirano M, Kusaka H, Ito H, Imai T, Ueno S (1997) α-tocopherol-transfer protein gene: exon skipping of all transcripts causes ataxia. *Neurology* 49, 584-588

12) Hentani A, Deng HX, Hung WY, Nayer M, Ahmed MS. He X, Tim R, Stumpf DA, Siddique T (1996) Human α-tocopherol-transfer protein: gene structure and mutations in familial vitamin E deficiency. *Ann Neurol* 39, 295-300

13) 松谷　学，松本博之，千葉　進，柏木　基，笠原理子 (1994) 網膜色素変性症と伴い感覚性多発ニューロパチーを主徴とした特発性ビタミンE欠乏症の1例．脳神経 46, 989-994

14) Lamaout A, Belal S, Zouari M, Fki M, Ben Hamida C, Goebel HH, Ben Hamida M, Hentati F (1997) Friedreich ataxia with isolated vitamin E deficiency: a neuropathological study of a Tunisian patient. *Acta Neuropathol* 93, 633-637

15) Yokota T, Kumagai J, Uchihara T, Shiojiri T, Seko , Pang, Mizusawa H, Inoue K (2000) Autopsy of ataxia with retinitis pigmentosa caused by the His101Gln mutation of the α-tocopherol tranfer protein. *J Neurol Neurosurg Psychiatry* 68, 521-212

16) Nelson JS, Fitche CD, Fische VW, Broun GD, Chou AC (1981) Progressive neuropathologic lesions in vitamin E deficient rhesus monkey. *J Neuropathol Exp Neurol* 40, 166-186

17) Southam E, Thomas PK, King RHM, Goss-Sampson MA, Muller DP (1991) Experimental vitamin E deficiency in rats. *Brain* 114. 915-936

18) Robinson WG, Kuwabara T, Bier JG (1980) Vitamin E deficiency and the retina: photoreceptor and pigment epithelial changes. *Invest Ophthalmol Visual Sci* **18**, 683-690
19) Muller DPR, Lloyd JK, Wolff OH (1983) Vitamin E and neurological function. *Lancet* **1**, 225-227
20) Sato Y, Hagiwara K, Arai H, Inoue K (1991) Purification and characterization of the α-tocopherol transfer protein from rat liver. *FEBS Lett* **288**, 41-45
21) Yoshida H, Yusin M, Ren I, Kuhlenkamp J, Hirano T, Stolz A, Kaplowitz N (1991) Identification, purification and immunochemical characterization of α-tocopherol-binding protein from rat liver cytosol. *J Lipid Res* **33**, 343-350
22) Arita M, Nomura K, Arai H, Inoue K (1997) α-tocopherol-transfer protein stimulates the secretion of α-tocopherol from a cultured liver cell line through a brefeldin A-insensitive pathway. *Proc Natl Acad Sci USA* **94**, 12437-12441
23) Kayden HJ, Traber MG (1993) Absorption, lipoprotein transport, and regulation of plasma concentrations of vitamin E in humans. *J Lipid Res* **34**, 343-358
24) Traber W, Ramakrishnan, Kayden HJ (1994) Human plasma vitamin E kinetics demonstrate rapid recyclin g of plasma RRR-α-tocopherol. *Proc Natl Acad Sci USA* **91**, 10005-10008
25) Traber MG, Sokol RJ, Burton GW, Ingold KU, Papas AM, Huffaker JE, Kayden HJ (1990) Impaired ability of patients with familial isolated vitamin E deficiency to incorporate α-tocopherol into lipoproteins secreted by the liver. *J Clin Invest* **85**, 397-407
26) Stampf DA, Sokol R, Bettis D, Neville H, Ringel S, Angelini C, Bell R (1987) Freidreich disease: V. Variantform with vitamin E deficiency and normal fat absorption. *Neurology* **37**, 68-74
27) Sokol RJ, Kayden HJ, Bettis DB, Traber MG, Neville H, Ringel S, Wilson B, Stampf DA (1988) Isolated vitamin E deficiency in the absence of fat malabsorption—familial and sporadic cases: characterization and investigation of causes. *J Lab Clin Med* **111**, 548-559
28) Traber M, Sokol RJ, Kohlschutter A, Yokota T, Muller DPR, Dufour R, Kayden HJ (1993) Impaired discrimination between stereoisomers of α-tocopherol in patients with familial isolated vitamin E deficiency. *J Lipid Res* **34**, 201-210
29) Hosomi A, Goto K, Kondo H, Iwatsubo T, Yokota T, Ogawa M, Arita M, Aoki J, Arai H, Inoue K (1998) Localization of alpha-tocopherol transfer protein in rat. *Neurosci Lett* **1256**, 159-162
30) Copp RP, Wisniewski T, Hentati F, Larnaout A, Ben Hamida M, Kayden HJ (1999) Localization of alpha-tocopherol transfer protein in the brains of patients with ataxia with vitamin E deficiency and other oxidative stress related neurodegenerative disorders. *Brain Res* **822**, 80-87

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 279, No. 38, Issue of September 17, pp. 40084–40090, 2004
Printed in U.S.A.

Type F Scavenger Receptor SREC-I Interacts with Advillin, a Member of the Gelsolin/Villin Family, and Induces Neurite-like Outgrowth*

Received for publication, April 7, 2004, and in revised form, June 30, 2004
Published, JBC Papers in Press, July 9, 2004, DOI 10.1074/jbc.M403844200

Mami Shibata‡§, Junko Ishii‡§¶, Hiroyuki Koizumi‡, Norihito Shibata‡¶, Naoshi Dohmae||, Koji Takio||, Hideki Adachi¶, Masafumi Tsujimoto¶, and Hiroyuki Arai‡**

From the ‡Department of Health Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan and the ¶Laboratory of Cellular Biochemistry and ||Division of Biomolecular Characterization, RIKEN (The Institute of Physical and Chemical Research), 2-1 Wako-shi, Saitama 351-0198, Japan

The scavenger receptor expressed by endothelial cells (SREC) was isolated from a human endothelial cell line and consists of two isoforms named SREC-I and -II. Both isoforms have no significant homology to other types of scavenger receptors. They contain 10 repeats of epidermal growth factor-like cysteine-rich motifs in the extracellular domains and have unusually long C-terminal cytoplasmic domains with Ser/Pro-rich regions. The extracellular domain of SREC-I binds modified low density lipoprotein and mediates a homophilic SREC-I/SREC-I or heterophilic SREC-I/SREC-II trans-interaction. However, the significance of large Ser/Pro-rich cytoplasmic domains of SRECs is not clear. Here, we found that when SREC-I was overexpressed in murine fibroblastic L cells, neurite-like outgrowth was induced, indicating that the receptor can lead to changes in cell morphology. The SREC-I-mediated morphological change required the cytoplasmic domain of the protein, and we identified advillin, a member of the gelsolin/villin family of actin regulatory proteins, as a protein binding to this domain. Reduction of advillin expression in L cells by RNAi led to the absence of the described SREC-I-induced morphological changes, indicating that advillin is a prerequisite for the change. Finally, we demonstrated that SREC-I and advillin were co-expressed and interacted with each other in dorsal root ganglion neurons during embryonic development and that overexpression of both SREC-I and advillin in cultured Neuro-2a cells induced long process formation. These results suggest that the interaction of SREC-I and advillin are involved in the development of dorsal root ganglion neurons by inducing the described morphological changes.

Scavenger receptors are defined by their ability to bind and metabolize modified low density lipoproteins (LDLs),[1] such as acetylated LDL (AcLDL) and oxidized LDL (OxLDL), and have been regarded as relevant in the pathogenesis of atherosclerosis (1, 2). Mammalian cells have several different classes of scavenger receptors, and their relative contributions to lipid metabolism in pathophysiological conditions, such as atherosclerosis, are the subject of intense investigation (2).

Endothelial cells express several distinct scavenger receptors, such as SR-BI (3–5), LOX-1 (6), and FEEl-1/stabilin-1 (7). We cloned a novel scavenger receptor from a DNA library prepared from human umbilical vein endothelial cells employing expression cloning and termed it SREC (scavenger receptor expressed by endothelial cells)-I (8). Subsequently, we also succeeded in cloning a homologous protein, SREC-II, by a data base search (9). These two receptors are now classified as type F scavenger receptors (2, 9).

Both SREC-I and -II have no significant homology to other types of scavenger receptors. They contain 10 repeats of epidermal growth factor-like cysteine-rich motifs in their extracellular domains and unusually long C-terminal cytoplasmic domains with Ser/Pro-rich regions (8, 9). SREC-I mediates the binding and degradation of AcLDL and OxLDL in endothelial cells, whereas SREC-II has little scavenger receptor activity, making it likely that these type F scavenger receptors have biological functions not linked to scavenger receptor activity. We showed previously (9) that SREC-I and -II display respective homophilic interaction through their extracellular domains between separate cells (trans-interaction) and strong SREC-I/SREC-II heterophilic trans-interaction. The homophilic and heterophilic trans-interactions of SREC-I and -II were effectively suppressed by the presence of scavenger receptor ligands, such as AcLDL and OxLDL.

The cytoplasmic domains of SREC-I and -II consisting of ~400 amino acids contain several potential phosphorylation sites for kinases A, C, and G, suggesting that these domains transduce intracellular signals generated by the receptors. SREC-I and -II may transduce different signals because of low sequence similarity and different potential phosphorylation sites. However the biological role of the cytoplasmic domain of SRECs is so far totally unknown.

In this study, we focused on the function of the SREC-I cytoplasmic domain. We previously employed murine L cells to elucidate the receptor/receptor trans-interaction (9), because this cell type is commonly used in experiments demonstrating trans-interaction of various cell adhesion molecules (10–13). In the experiments, we had noticed that prolonged culture of SREC-I-transfected L cells induced striking morphological cell changes. Based on these preliminary observations, we show here that SREC-I, but not SREC-II, induces neurite-like long processes after overexpression in murine fibroblastic L cells and that the cytoplasmic domain of SREC-I is a prerequisite for

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to the results of this work.
** To whom correspondence should be addressed. Tel.: 81-3-5841-4720; Fax: 81-3-3818-3173; E-mail: harai@mol.f.u-tokyo.ac.jp.

[1] The abbreviations used are: LDL, low density lipoprotein; AcLDL, acetylated LDL; OxLDL, oxidized LDL; DRG, dorsal root ganglion; SREC, scavenger receptor expressed by endothelial cells; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; GFP, green fluorescent protein; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate.

This paper is available on line at http://www.jbc.org

this activity. Moreover, we demonstrated that advillin, a member of the gelsolin/villin family of actin regulatory proteins, binds specifically to the cytoplasmic domain of SREC-I and is required for this morphological cell change. The biological implications of this activity are discussed.

EXPERIMENTAL PROCEDURES

Cell Culture—Murine L cells (CCL-1, American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. Chinese hamster ovary cells were maintained in Ham's F-12 medium supplemented with 50 units/ml penicillin, 50 mg/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. The murine neuroblastoma line Neuro-2a cells (CCL-131, American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine.

Plasmid Construction—The EcoRI-XhoI fragment of the mouse cDNA for SREC-I, SREC-I cytoplasmic domain deletion mutant that lacks amino acid residues 451–820 (SREC-I-ΔC370), SREC-II, SR-A, SR-BI, and advillin were subcloned into the mammalian expression vector pcDNA3 (Invitrogen), and expression plasmids were termed pcDNA3-SREC-I, pcDNA3-SREC-I-ΔC370, pcDNA3-SREC-II, pcDNA3-SR-A, pcDNA3-SR-BI, and pcDNA3-advillin, respectively. We noted that a hemagglutinin tag was added at the C terminus of advillin in pcDNA3-Advillin.

Uptake of DiI-AcLDL—L cells (1×10^5 cells/well) in 24-well plates were mock transfected or transfected with either pcDNA3-SREC-I, pcDNA3-SREC-I-ΔC370, pcDNA3-SREC-II, pcDNA3-SR-A, or pcDNA3-SR-BI using LipofectAMINE reagent (Invitrogen) according to the manufacturer's instructions. The cells were incubated for 72 h, incubated again in the presence of 2 μg/ml DiI-AcLDL (Biomedical Technologies Inc.) for 2 h, washed, and then fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. The presence of fluorescent DiI in the fixed cells was determined by visual inspection using fluorescence microscopy.

GST Fusion Proteins—The EcoRI-SalI fragment encoding the first half (C1, amino acid residues 451–643), the central part (C2, amino acid residues 561–752), or the last half (C3, amino acid residues 643–820) of the cytoplasmic domain of mouse SREC-I was subcloned into a multicloning site downstream of the sequence for GST in pGEX-4T-1 (Pharmacia Corporation). This plasmid was transformed into the JM109 strain of *Escherichia coli* and induced with isopropyl-1-thio-β-D-galactopyranoside to produce GST fusion proteins. The bacteria were suspended in PBS, and vigorous sonication was performed before centrifugation at 10,000 × *g* for 20 min. The resulting supernatants were applied to a glutathione-Sepharose column and then eluted with an elution buffer (50 mM Tris-HCl, pH 9.6, 120 mM NaCl, 10 mM glutathione). Purified GST fusion proteins were dialyzed against PBS containing 2 mM EDTA and 1 mM dithiothreitol.

GST Affinity Chromatography and Peptide Sequence Analysis—L cells (6×10^7 cells) were harvested and homogenized in 1 ml of PBS and then centrifuged at 100,000 × *g* for 1 h at 4 °C. The resultant supernatant was used as the cytosolic extract. Recombinant GST-C1 or -C2 fusion proteins, bound to the glutathione-Sepharose column, were used to affinity-purify C1- or C2-binding protein(s). L cell cytosolic extracts were loaded onto the GST-C1 or -C2 glutathione-Sepharose columns and then eluted with the elution buffer. The eluted fractions of affinity chromatography were collected, precipitated by 10% trichloroacetic acid, and subjected to SDS-PAGE. A Coomassie Brilliant Blue-stained band of 90 kDa was cut out and digested with *Acromobacter* protease I (API; a gift from Dr. Masaki, Ibaraki University) (14). The resulting peptides were separated by reverse phase high pressure liquid chromatography on tandemly connected DEAE-5PW (1 × 20 mm; Tosoh, Tokyo, Japan) and Capcel Pak C_{18} UG120 (1 × 50 mm; Shiseido, Tokyo, Japan) columns with a 0–80% gradient of acetonitrile in 0.1% trifluoroacetic acid. Isolated peptides were analyzed by automated Edman degradation on an Applied Biosystems protein sequencer model 477A (PerkinElmer Life Sciences) connected on line to a PTH Analyzer model 120A (PerkinElmer Life Sciences) using an in-house-generated gas phase program and were also examined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry with a Reflex MALDI-TOF (Bruker-Franzen Analytik, Bremen, Germany) in linear mode, with 2-mercaptobenzothiazole used as a matrix.

Antibodies—The polyclonal antibodies against SREC-I and advillin were prepared as follows. Peptides corresponding to the C-terminal

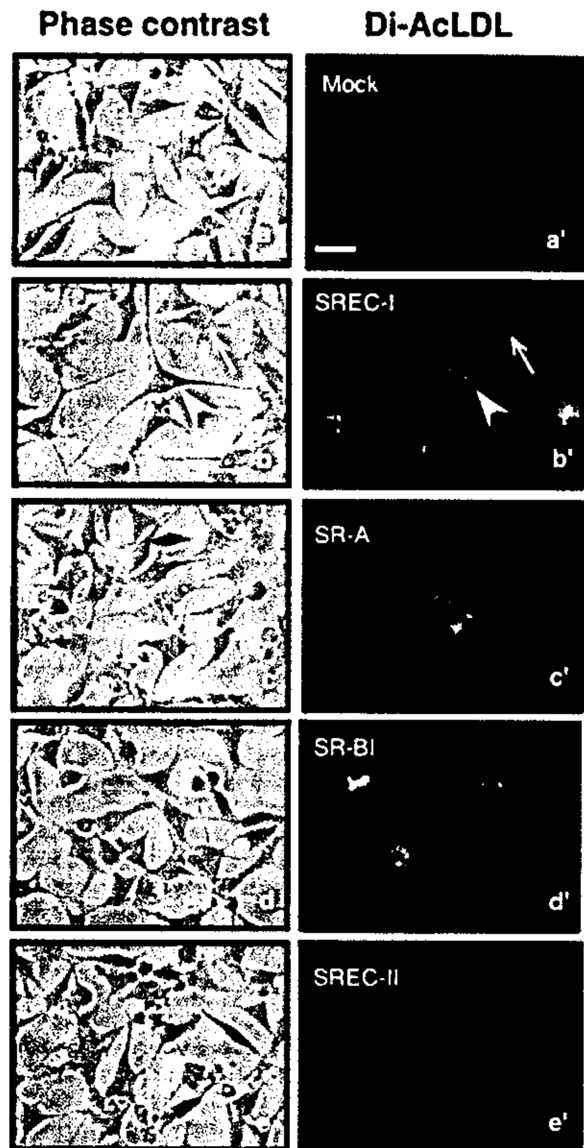

FIG. 1. **SREC-I induces neurite-like long processes in L cells.** Mock-transfected L cells (*a*, *a'*) or L cells transfected with pcDNA3-SREC-I (*b*, *b'*), pcDNA3-SR-A (*c*, *c'*), pcDNA3-SR-BI (*d*, *d'*), or pcDNA3-SREC-II (*e*, *e'*) were incubated with DiI-AcLDL for 2 h. *Left panels*, phase-contrast light micrographs (*a*–*e*). *Right panels*, fluorescence image (*a'*–*e'*) of the same fields as in the *left panels*. Note the long processes with the presence of SREC-I (*arrowhead*) and no morphological change with the absence of SREC-I (*arrow*). *Bar*, 10 μm.

domain of mouse SREC-I (NH_2-KEQEEPLYENVVPMSVPPQH-COOH) and mouse advillin (NH_2-DGEPKYYPVEVLLKGQNQEL-COOH) were synthesized. The synthesized peptides were conjugated with keyhole limpet hemocyanin using an Imject sulfhydryl-reactive antibody production Kit (Pierce). The keyhole limpet hemocyanin peptides were gel-purified and emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI). Female Wistar rats were immunized with the emulsions. These rat sera were collected and purified using an affinity column (Sulfolink Coupling Gel, Pierce) to which the corresponding antigen peptide was coupled.

Small Interference RNA—The mammalian expression vector pSUPER was used for expression of siRNA in the L cells. Three parts of the gene-specific targeting sequence (19-nucleotide sequences: 1, 5'-AGAAGCCATGCCACTGGTA-3'; 2, 5'-CCGCAGCAGAAAGACGTCG-3'; and 3, 5'-CACAAGGATCAAGGATGAC-3') from the target transcript separated by a 9-nucleotide noncomplementary spacer (TTCAAGAGA) from the reverse complement of the same 19-nucleotide sequence were inserted in pSUPER. These vectors were referred to as pSUPER-advillin/RNAi-1, -2, and -3, respectively. L cells were transfected with either pSUPER-advillin/RNAi, control vector (pSUPER), pcDNA3-SREC-I plus pSUPER, pcDNA3-SREC-I plus pSUPER-advillin/RNAi, or

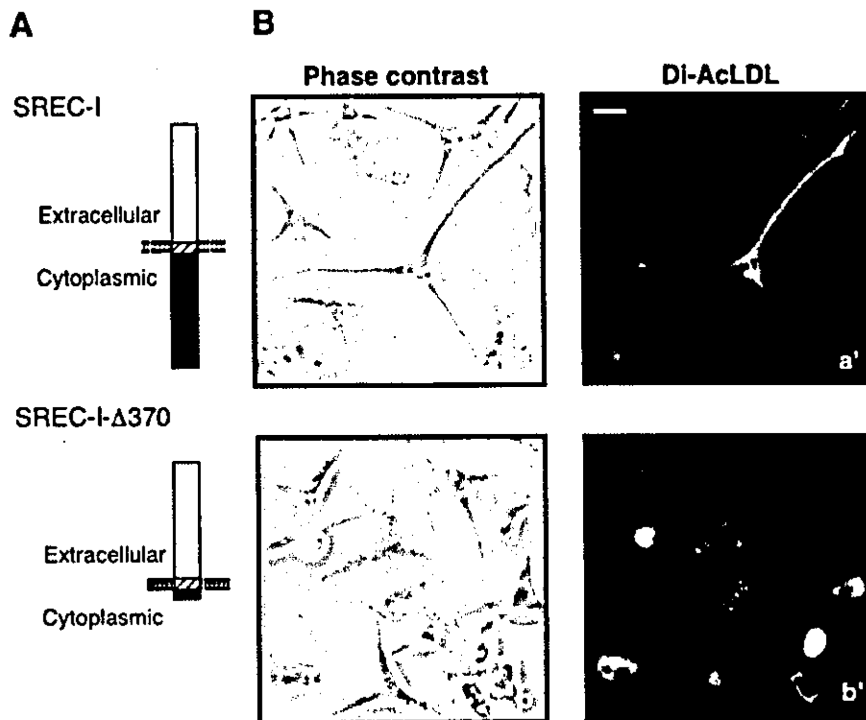

FIG. 2. **SREC-I cytoplasmic domain is required for the induction of morphological change.** *A*, schematic figures of murine SREC-I and SREC-I-ΔC370 showing extracellular, transmembrane, and cytoplasmic sites. *B*, L cells transfected with pcDNA3-SREC-I (*a*, *a'*) or pcDNA3-SREC-I-ΔC370 (*b*, *b'*) were incubated with DiI-AcLDL for 2 h. *Left panels*, phase-contrast light micrographs (*a*, *b*). *Right panels*, fluorescence image (*a'*, *b'*) of the same fields as in the *left panels*. *Bar*, 10 μm.

pcDNA3-GFP plus pSUPER-advillin/RNAi as described above, and the cells were cultured for 72 h. Total cell lysates were prepared in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (w/v) Triton X-100, 0.5% (w/v) Nonidet P-40, 1 mM EDTA, protease inhibitor mixture (Sigma), and 1 mM phenylmethylsulfonyl fluoride). The lysates were cleared by centrifugation at 18,600 × *g* for 20 min at 4 °C, were harvested and homogenized in 1 ml of PBS, and then centrifuged at 100,000 × *g* for 1 h at 4 °C. The resultant supernatants were analyzed by Western blotting with anti-advillin or anti-SREC-I antibodies.

Immunofluorescence Microscopy—L cells were transfected with pcDNA3-SREC-I and pSUPER-advillin/RNAi vector or control vector (pSUPER), incubated for 72 h at 37 °C, fixed with 3.7% formaldehyde in PBS for 20 min at room temperature, permeabilized with 0.1% Triton X-100 for 5 min, and then blocked with 3% bovine serum albumin in PBS for 1 h at room temperature. The cells were then incubated with the anti-SREC-I antibody for 2 h at room temperature, washed 4 times with PBS, incubated again with an Alexa Fluor 594 goat anti-rat IgG(H+L) antibody for 1 h at room temperature, washed thoroughly with PBS, embedded, and then visualized using a fluorescent microscope. Neuro-2a cells were transfected with pcDNA3-SREC-I and pcDNA3-advillin, and incubated for 72 h at 37 °C. The cells were then fixed with 3.7% formaldehyde in PBS for 20 min at room temperature, permeabilized with 0.1% Triton X-100 for 5 min, blocked with 3% bovine serum albumin in PBS for 1 h at room temperature, and incubated with the anti-SREC-I antibody and anti-hemagglutinin antibody for 2 h at room temperature. After that, the cells were washed four times with PBS, incubated again with an Alexa Fluor 594 goat anti-rat IgG(H+L) antibody and Alexa Fluor 488 goat anti-mouse IgG(H+L) antibody for 1 h at room temperature, washed thoroughly with PBS, embedded, and then visualized using a fluorescent microscope.

Western Blot Analysis—C57/BL6 mice (adults or 18-day embryos) were perfused with ice-cold SET buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) containing protease inhibitor mixture and 1 mM phenylmethylsulfonyl fluoride. Thereafter, the brain, spinal cord, and dorsal root ganglion (DRG) were rapidly excised, as described previously (15). The brain and spinal cord were homogenized in 4 volumes (w/v) of SET buffer and then centrifuged at 1,000 × *g* for 10 min at 4 °C. The resultant supernatants were used as the brain and spinal cord total protein lysates. The DRG was homogenized in SET buffer and then centrifuged at 1,000 × *g* for 10 min at 4 °C. The resultant supernatant was concentrated by 10% trichloroacetic acid precipitation. The resultant pellets were suspended in SET buffer and used as the DRG total protein lysate.

The protein concentrations of samples were determined by BCA assay (Pierce). Each total protein lysate (100 μg/lane) was separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% (w/v) skim milk (Wako, Osaka, Japan) in TTBS buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% (w/v) Tween 20) and incubated with anti-advillin or anti-SREC-I antibodies in TTBS. The levels of protein were analyzed with an ECL kit (Amersham Biosciences) according to the manufacturer's instructions.

Immunoprecipitation—DRG extracts were prepared in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (w/v) Triton X-100, 0.5% (w/v) Nonidet P-40, 1 mM EDTA, protease inhibitor mixture (Sigma), 1 mM phenylmethylsulfonyl fluoride). The DRG extracts were precleared for 2 h with protein G-agarose beads (Amersham Biosciences) and then incubated overnight with anti-SREC-I antibody at 4 °C. Immunocomplexes were precipitated with protein G-agarose beads for 45 min, washed three times with lysis buffer, and boiled in SDS sample buffer containing 2-mercaptoethanol. The supernatants were subjected to SDS-PAGE and Western blotting.

RESULTS

SREC-I-induced Morphological Change of L Cells—First, L cells were transfected with vectors for various scavenger receptors, and morphological changes were monitored. As shown in Fig. 1, transfection of the expression vector for SREC-I into the cells caused significant morphological changes with generation of neurite-like long processes. On the other hand, no change in cell morphology was observed when other scavenger receptors, such as SR-A, SR-BI, or even SREC-II were overexpressed in these cells. These results suggested that SREC-I is the specific scavenger receptor that can induce neurite-like outgrowth when overexpressed in L cells. When SREC-I was expressed in Chinese hamster ovary cells, no morphological cell change was observed (data not shown).

SREC-I contains a large cytoplasmic domain consisting of ~400 amino acids. To elucidate the role of this cytoplasmic domain, deletion mutants lacking C-terminal fragments of 370 amino acids were created, and their effects on cell shape were examined. We have shown previously (9) that this truncated receptor was expressed in L-cells to a degree similar to the full-length receptor and showed a comparable level of AcLDL uptake activity. In contrast to these findings, the mutant protein did not induce a change in cell morphology (Fig. 2*B*). These results indicated that the cytoplasmic domain is required for the induction of neurite-like outgrowth, and we hypothesized

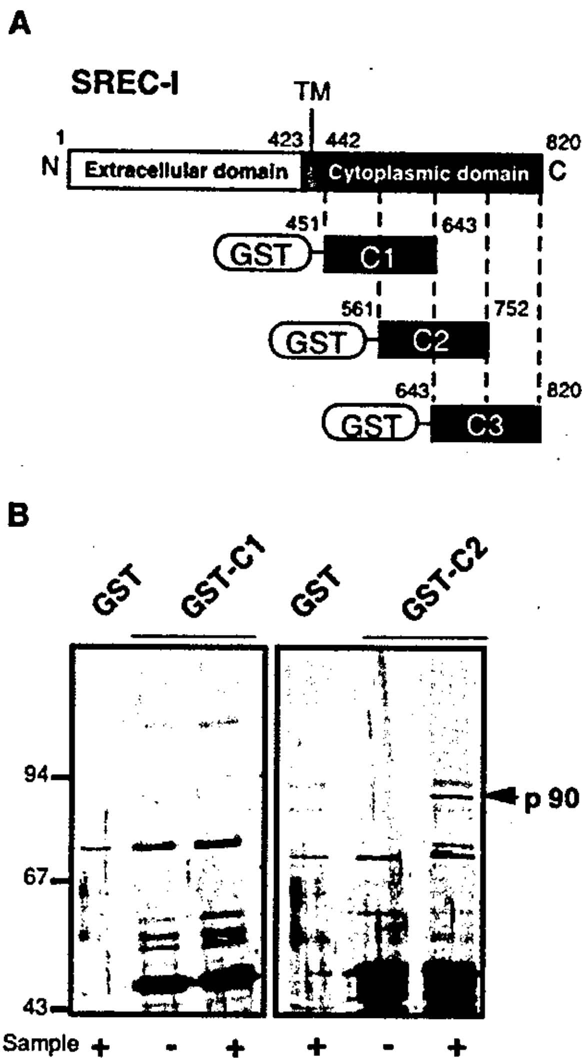

FIG. 3. **Identification of SREC-I cytoplasmic domain-binding proteins.** *A*, the constructs of various deletion mutants of SREC-I fused with GST are shown schematically. *N*, N terminus; *C*, C terminus; *TM*, transmembrane. *Small numbers* refer to amino acid residues. *B*, the L cell cytoplasmic fraction was loaded onto glutathione-Sepharose columns coated with the indicated GST fusion proteins. The bound proteins were eluted by the addition of glutathione. The eluates were subjected to SDS-PAGE followed by silver staining. The *arrow* denotes the position of p90.

that proteins interacting with this domain are required to mediate the effects of this receptor.

Identification of Binding Proteins to the Cytoplasmic Domain of SREC-I—To identify the proteins that bind to the cytoplasmic domain of SREC-I, we prepared several expression vectors for GST-fused C-terminal fragments of the SREC-I protein (Fig. 3*A*, *C1–C3*). Among others, we could successfully express GST-C1 and -C2 fragments in *E. coli* and analyze their binding activities. The cytoplasmic fraction prepared from L cells was loaded onto either a GST-C1 or -C2 affinity column, and the proteins bound to the respective column were co-eluted with GST-fused peptide by the addition of glutathione. As shown in Fig. 3*B*, we could not detect any proteins specifically binding to the C1 fragment. On the other hand, a protein with a molecular mass of ~90 kDa was specifically detected in the glutathione eluate from a GST-C2 column onto which the cytoplasmic fraction of L cells was loaded, although the column loaded with control solution yielded no such protein. These results indicated that the identified 90-kDa protein could bind to the amino acid sequence between residues 643 and 752 of the SREC-I cytoplasmic domain.

The 90-kDa protein was then subjected to amino acid sequencing. Seven peptides derived from the protein were determined, and all were the partial sequences of advillin, an actin regulatory protein belonging to the gelsolin/villin family (16).

Advillin Is Required for SREC-I-mediated Morphological Cell Change—To examine whether advillin is involved in SREC-I-mediated morphological cell change, the RNAi technique was applied (17–20). L cells were treated with several constructs (RNAi-1 to -3; see "Experimental Procedures") of advillin siRNA. First, we confirmed by Western blotting that murine fibroblastic L cells intrinsically expressed advillin (Fig. 4*A*, *lanes 1* of *(a)* and *(b)*, *Advillin*). Moreover, we found that the RNAi-1 vector was most efficient in decreasing the expression of advillin (Fig. 4*A*). Thereafter, L cells were co-transfected with SREC-I and advillin siRNA (RNAi-1) vectors, and morphological cell change was monitored. The expression vector for SREC-I was transfected into the cells, and the expressed protein was analyzed by Western blotting. Two bands with M_r of 141,000 and 147,000 were observed. These two bands were not detected when the green fluorescent protein expression vector was transfected, indicating the heterogeneity of the expressed SREC-I, most probably because of the varying glycosylation of the protein. Co-transfection of RNAi-1 caused a significant decrease in the expression of endogenous advillin without affecting the expression pattern of SREC-I (Fig. 4*B*). As shown in Fig. 4*C*, transfection of the SREC-I vector alone induced neurite-like outgrowth, whereas this phenomenon was impaired significantly by the co-transfection of the RNAi-1 vector. When counting the cells showing long processes, it was apparent that co-transfection of the RNAi-1 vector together with that of SREC-I caused a dramatic decrease in cell number. These results indicate that advillin is required for the SREC-I-mediated induction of neurite-like outgrowth in L cells. It should be noted that unlike in the L cells, advillin was undetectable in Chinese hamster ovary cells in which SREC-I had no apparent ability to induce the described morphological cell change (data not shown).

SREC-I Is Expressed in Peripheral Nerve Neurons—Because it was reported that advillin is expressed in the peripheral nervous system in areas such as the DRG and the superior cervical ganglion and plays a role in the neurite outgrowth of neuronal cells (21), we examined whether SREC-I is also expressed in peripheral nerve neurons. We focused on the embryonic expression of the protein, because it was reported that advillin is expressed in peripheral nerve neurons, especially during embryonic development (16). SREC-I protein was barely detectable in the brain, spinal cord, or DRG of the adult mouse but was clearly detectable in each of these tissues in 18-day embryonic mice (Fig. 5*A*). These results suggested that SREC-I is expressed transiently in the nervous system during fetal development. On the other hand, both in adult and embryonic mice, advillin was detectable in DRG but not in brain or spinal cord.

To determine whether SREC-I interacts with advillin in the DRG of 18-day embryonic mice, we performed immunoprecipitation studies with the anti-SREC-I antibody. Immunoblot analysis revealed that the immunoprecipitates contained advillin in addition to SREC-I (Fig. 5*B*). No anti-SREC-I or anti-advillin signals were detected in the immunoprecipitates treated with normal rabbit serum (data not shown). These results demonstrated that SREC-I interacts with advillin in the DRG of 18-day embryonic mice.

Next, we examined whether the interaction of SREC-I and advillin induces neurite outgrowth in neuronal cells. Because most of the neuronal cell lines intrinsically expressed neither SREC-I nor advillin, we used a murine neuroblastoma cell line,

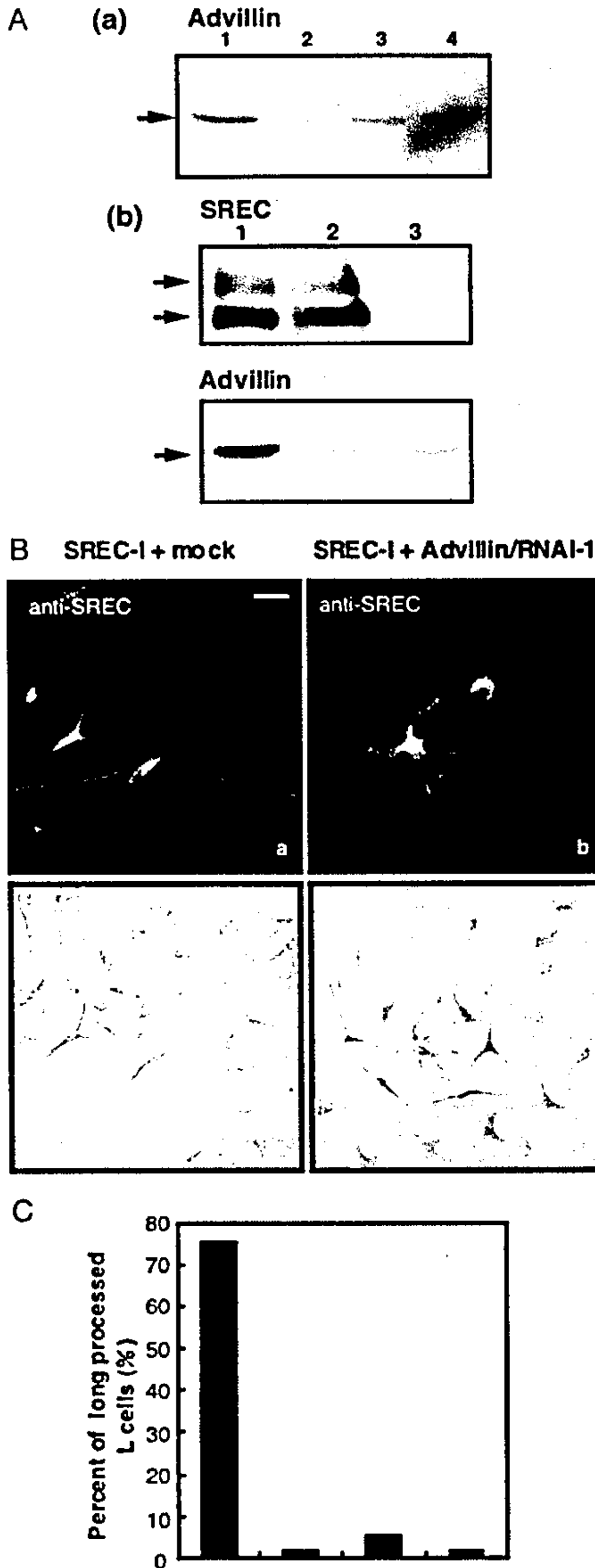

FIG. 4. **Advillin is required for the SREC-I-induced morphological change.** *A*, suppression of advillin expression by pSUPER-advillin/RNAi. (*a*), pSUPER (*lane 1*) or pSUPER-advillin/RNAi-1 to -3 (*lanes 2–4*) were transfected into L cells as described under "Experimental Procedures." Total cell lysates were subjected to SDS-PAGE and immunoblotted to detect advillin proteins. (*b*), pcDNA3-SREC-I and pSUPER (*lane 1*) or pcDNA3-SREC-I and pSUPER-advillin/RNAi-1 (*lane 2*) or pcDNA3-GFP and pSUPER-advillin/RNAi-1 (*lane 3*) were transfected into L cells as described under "Experimental Procedures." Total cell lysates were subjected to SDS-PAGE and immunoblotted to detect SREC-I (*upper panel*) and advillin (*lower panel*) proteins. *B*, L cells transfected with pcDNA3-SREC-I and pSUPER (*left panels*) or pcDNA3-SREC-I and pSUPER-advillin/RNAi-1 (*right panels*) were immunostained with polyclonal antibody against SREC-I. *Upper panels*, fluorescence images. *Lower panels*, phase-contrast light micrographs of the same fields as in the *upper panels*. *Bar*, 10 μm. C, percent of >40-μm long processed L cells.

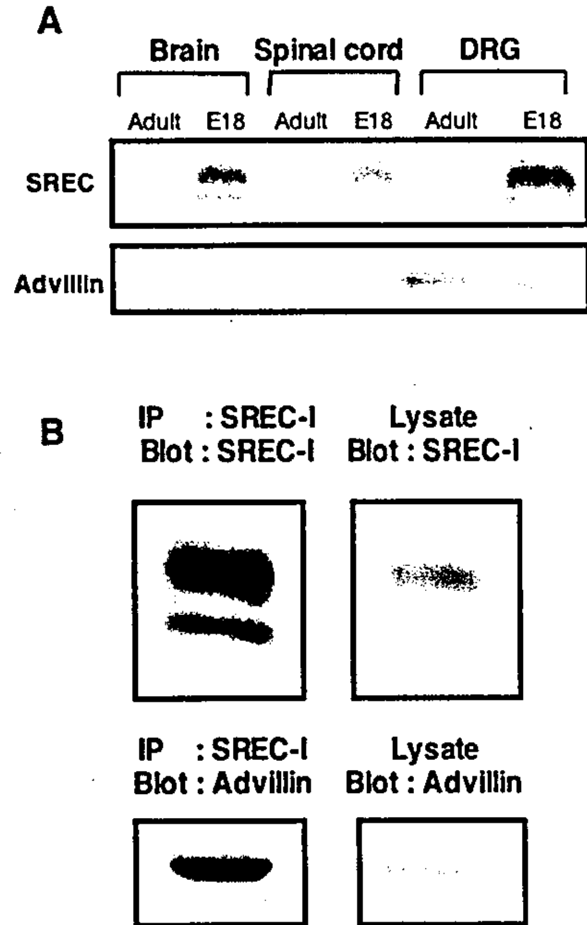

FIG. 5. **Western blot analysis of SREC-I and advillin in murine brain and DRG.** *A*, immunoblot analysis of lysates from mouse brain, spinal cord, and DRG with polyclonal antibody against SREC-I (*upper panel*) and advillin (*lower panel*). Each total protein lysate (100 μg/lane) was separated by SDS-PAGE and subjected to Western blotting. *B*, mouse DRG lysates were immunoprecipitated with polyclonal antibody against SREC-I. Immunoblot analysis of the immunoprecipitates and the lysates with polyclonal antibody against SREC-I (*upper panels*) and advillin (*lower panels*).

Neuro-2a, to perform transfection experiments. The cells were transfected with the SREC-I and/or advillin vectors, and morphological cell change was monitored. As shown in Fig. 6, the cells co-expressing SREC-I and advillin showed neurite outgrowth, whereas the cells expressing either SREC-I or advillin did not show this phenomenon, indicating that the interaction of SREC-I and advillin induces neurite formation in cultured neuronal cells.

DISCUSSION

The present results demonstrate that SREC-I is capable of interacting with advillin (16) through its large Ser/Pro-rich cytoplasmic domain and thereby is capable of inducing neurite-like outgrowth. Members of this actin regulatory protein family are capable of capping and severing actin filaments (16).

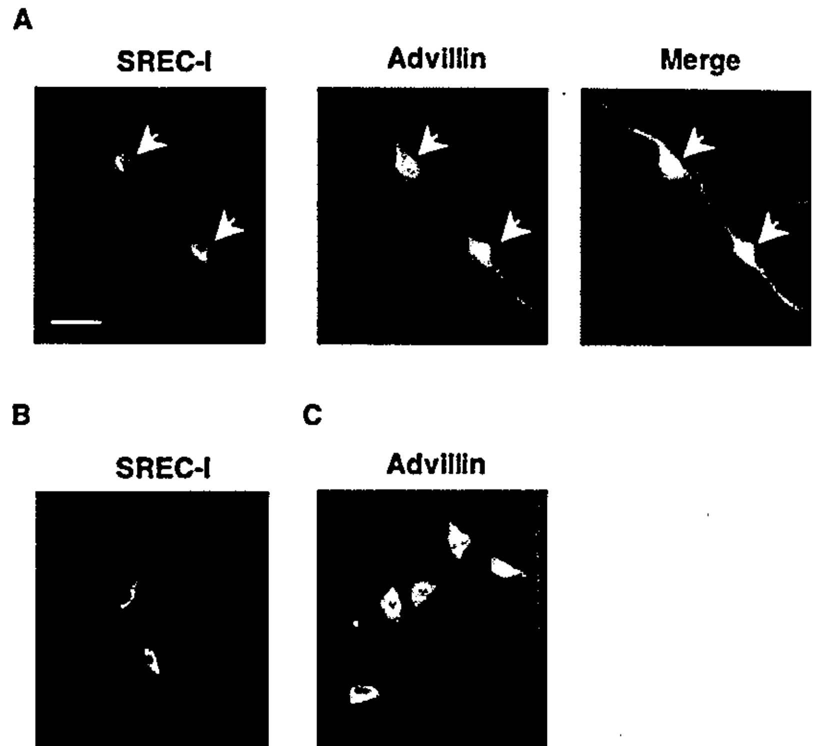

FIG. 6. **Co-expression of SREC-I and advillin induces neurite outgrowth in Neuro-2a cells.** Neuro-2a cells were transfected with pcDNA3-SREC-I and pcDNA3-advillin (*A*), pcDNA3-SREC-I (*B*), or pcDNA3-advillin (*C*). The cells were immunostained with rat polyclonal antibody against SREC-I (*red*) and mouse monoclonal antibody against hemagglutinin-tagged (*green*). Note the long processes with the presence of SREC-I and advillin (*arrowheads*). *Bar*, 20 μm.

Among these proteins, advillin is most closely related to villin in its domain structure, including the C-terminal F-actin-binding headpiece domain (16). Villin is expressed mainly in differentiated epithelial tissues possessing a brush border, such as intestinal villi or proximal renal tubules (22, 23), and most likely plays an important role in the morphogenesis of microvilli (24–26). Advillin, however, is highly expressed in the dorsal root and trigeminal ganglia during embryonic development and only at low levels in adult uterine and intestinal epithelial cells (16). Transfection of the advillin expression vector to primary cultures of rat DRG sensory neurons resulted in increased neurite outgrowth (21), indicating that the protein plays a significant role in the morphogenesis of peripheral neurons through an actin-bundling domain. It is therefore reasonable that the cytoplasmic domain of SREC-I binds to advillin and regulates the intracellular cytoskeletal organization, resulting in the generation of neurite-like long processes.

Unexpectedly, murine fibroblastic L cells were found to intrinsically express advillin (Fig. 4*A*). The parent L strain was derived from normal subcutaneous areolar and adipose tissue of male C3H/An mice according to the CCL-1 catalogue. Intrinsic expression of advillin made it possible to elucidate the function of SREC-I in advillin-mediated morphological changes in L cells. Depletion of endogenous advillin protein in L cells by RNAi inhibited the generation of long processes, indicating that advillin is indispensable for this change. Chinese hamster ovary cells, in which advillin was undetectable, displayed no morphological changes upon SREC-I overexpression. This provides further evidence for the necessity of advillin.

Our results are the first to show that SREC-I is expressed not only in endothelial cells but also in neuronal cells. Furthermore, SREC-I and advillin are co-expressed and interact with each other in DRG neurons, especially during embryonic development. We also demonstrated that overexpression of both SREC-I and advillin in cultured Neuro-2a cells induces the formation of long processes. These results suggest that the interaction of SREC-I and advillin are involved in the development of DRG neurons by inducing the described morphological changes.

SREC-I is characterized by its extremely large cytoplasmic domain. The present data indicate that this domain is a prerequisite for the receptor-mediated morphological change of L cells, which shows that the cytoplasmic domain of SREC-I plays a role in transducing intracellular signals of SREC-I. We showed that advillin binds to the C2 peptide (amino acid residues 562–752) but not to the C1 peptide (amino acid residues 452–643) of the cytoplasmic domain. This suggests that the region within 643–752 is responsible for advillin binding. This region is also rich in Ser and Pro, but it is not similar to the known domains that transduce signals into the cell interior. Interestingly, although ~20% homology was observed in the entire cytoplasmic domains of SREC-I and -II, this region is less homologous (<10%), which can explain why SREC-II exerted no morphogenetic activity on L cells.

It remains unclear how the SREC-I signal is transduced into the cell interior. We have demonstrated previously that SREC-I shows a homophilic trans-interaction between separate cells through its extracellular domain (9). This homophilic trans-interaction of SREC-I may serve as a signal for the induction of neurite-like long processes. However, this may not be the case, because the L cells that display long processes did not necessarily show contact with neighboring SREC-I-expressing L cells (Figs. 1, 2, and 4*B*). Moreover, although homophilic trans-interaction of SREC-I is effectively disrupted by the addition of AcLDL or OxLDL (9), these ligands had little effect on the formation of long processes (data not shown), which supports the idea that trans-interaction of SREC-I between cells is not obligatory for the transduction of the signal into the cells. In our preliminary study, we observed that SREC-I forms an oligomer in the membrane (homophilic *cis*-interaction), possibly a dimer when overexpressed in L cells. The extracellular domain may be indispensable for the oligomerization, because upon co-transfection of native full-length SREC-I and the ΔC370 deletion mutant, which lacks most of the cytoplasmic domain into L cells, both receptors could be co-immunoprecipitated. Interestingly, in most of these cells, long process formation was greatly diminished, indicating that the ΔC370 deletion mutant may function as a dominant

negative effector for SREC-I activity. These observations suggest that close association of the SREC-I cytoplasmic domain themselves may send a signal to L cells that induces the formation of long processes. In *in vivo* situations, some ligands might stimulate SREC-I oligomerization producing a signal in the cells like other growth hormone receptors. SREC-II, which shows a strong heterophilic trans-interaction with SREC-I, is a possible natural ligand for SREC-I. However, in our preliminary experiments, the expression levels of SREC-II in the mouse brain and DRG were very low compared with SREC-I, suggesting that other factor(s) serve as ligands for SREC-I extracellular domains. Further studies are needed to identify the natural ligand in these tissues and to elucidate the mechanism of advillin activation through the SREC-I cytoplasmic domain.

SREC-I was originally identified from a human endothelial cell line (9). Endothelial cells play important roles in vasculogenesis, angiogenesis, and the repair of injuries along the endothelium (27). Under these situations, endothelial cells actively migrate along the substratum in a coordinated and polarized fashion. This process involves extension of filopodia and lamellipodia, both of which have specific actin-based architectures. SREC-I might be involved in the formation and disruption of actin bundles through the gelsolin/villin family in endothelial cells.

REFERENCES

1. Goldstein, J. L., Ho, Y. K., Basu, S. K., and Brown, M. S. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 333–337
2. Greaves, D. R., Gough, P. J., and Gordon, S. (1998) *Curr. Opin. Lipidol.* **9,** 425–432
3. Acton, S. L., Scherer, P. E., Lodish, H. F., and Krieger, M. (1994) *J. Biol. Chem.* **269,** 21003–21009
4. Yuhanna, I. S., Zhu, Y., Cox, B. E., Hahner, L. D., Osborne-Lawrence, S., Lu, P., Marcel, Y. L., Anderson, R. G., Mendelsohn, M. E., Hobbs, H. H., and Shaul, P. W. (2001) *Nat. Med.* **7,** 853–857
5. Li, X.-A., Titlow, W. B., Jackson, B. A., Giltiay, N. M., Nikolova-Karakashian, M., Uittenbogaard, A., and Smart, E. J. (2002) *J. Biol. Chem.* **277,** 11058–11063
6. Sawamura, T., Kume, N., Aoyama, T., Moriwaki, H., Hoshikawa, H., Aiba, Y., Tanaka, T., Miwa, S., Katsura, Y., Kita, T., and Masaki, T. (1997) *Nature* **386,** 73–77
7. Adachi, H., and Tsujimoto, M. (2002) *J. Biol. Chem.* **277,** 34264–34270
8. Adachi, H., Tsujimoto, M., Arai, H., and Inoue, K. (1997) *J. Biol. Chem.* **272,** 31217–31220
9. Ishii, J., Adachi, H., Aoki, J., Koizumi, H., Tomita, S., Suzuki, T., Tsujimoto, M., Inoue, K., and Arai, H. (2002) *J. Biol. Chem.* **277,** 39696–39702
10. Nagafuchi, A., Shirayoshi, Y., Okazaki, K., Yasuda, K., and Takeichi, M. (1987) *Nature* **329,** 341–343
11. Hatta, K., Nose, A., Nagafuchi, A., and Takeichi, M. (1988) *J. Cell Biol.* **106,** 873–881
12. Aoki, J., Koike, S., Asou, H., Ise, I., Suwa, H., Tanaka, T., Miyasaka, M., and Nomoto, A. (1997) *Exp. Cell Res.* **235,** 374–384
13. Satoh-Horikawa, K., Nakanishi, H., Takahashi, K., Miyahara, M., Nishimura, M., Tachibana, K., Mizoguchi, A., and Takai, Y. (2000) *J. Biol. Chem.* **275,** 10291–10299
14. Masaki, T., Tanabe, M., Nakamura, K., and Soejima, M. (1981) *Biochim. Biophys. Acta* **660,** 44–50
15. Akopian, A. N., and Wood, J. N. (1995) *J. Biol. Chem.* **270,** 21264–21270
16. Marks, P. W., Arai, M., Bandura, J. L., and Kwiatkowski, D. J. (1998) *J. Cell Sci.* **111,** 2129–2136
17. Mise-Omata, S., Montagne, B., Deckert, M., Wienands, J., and Acuto, O. (2003) *Biochem. Biophys. Res. Commun.* **301,** 704–710
18. Hannon, G. J. (2002) *Nature* **418,** 244–251
19. Scherr, M., Morgan, M. A., and Eder, M. (2003) *Curr. Med. Chem.* **10,** 245–256
20. Doi, N., Zenno, S., Ueda, R., Ohki-Hamazaki, H., Ui-Tei, K., and Saigo, K. (2003) *Curr. Biol.* **8,** 41–46
21. Ravenall, S. J., Gavazzi, I., Wood, J. N., and Akopian, A. N. (2002) *Eur. J. Neurosci.* **15,** 281–290
22. Ezell, R., Chafel, M. M., and Matsudaira, P. (1989) *Development (Camb.)* **106,** 407–419
23. Gröne, H., Weber, K., Helmchen, U., and Osborn, M. (1986) *Am. J. Pathol.* **124,** 298–302
24. Franck, Z., Footer, M., and Bretscher, A. (1990) *J. Cell Biol.* **111,** 2475–2485
25. Friederich, E., Huet, C., Arpin, M., and Louvard, D. (1989) *Cell* **59,** 461–475
26. Friederich, E., Vancompernolle, K., Huet, C., Goethals, M., Finidori, J., Vandekerckhove, J., and Louvard, D. (1992) *Cell* **70,** 81–92
27. Kiosses, W. B., Daniels, R. H., Otey, C., Bokoch, G. M., and Schwartz, M. A. (1999) *J. Cell Biol.* **147,** 831–843

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 30, Issue of July 23, pp. 30938–30944, 2004
Printed in U.S.A.
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

Scavenger Receptor Expressed by Endothelial Cells I (SREC-I) Mediates the Uptake of Acetylated Low Density Lipoproteins by Macrophages Stimulated with Lipopolysaccharide*

Received for publication, December 1, 2003, and in revised form, May 11, 2004
Published, JBC Papers in Press, May 15, 2004, DOI 10.1074/jbc.M313088200

Yoshiaki Tamura‡, Jun-ichi Osuga‡, Hideki Adachi§, Ryu-ichi Tozawa‡, Yasukazu Takanezawa¶, Ken Ohashi‡, Naoya Yahagi‡, Motohiro Sekiya‡, Hiroaki Okazaki‡, Sachiko Tomita‡, Yoko Iizuka‡, Hiroyuki Koizumi¶, Toshihiro Inaba‖, Hiroaki Yagyu‖, Nobuo Kamada, Hiroshi Suzuki‡‡, Hitoshi Shimano§§, Takashi Kadowaki‡, Masafumi Tsujimoto§, Hiroyuki Arai¶, Nobuhiro Yamada§§, and Shun Ishibashi‡‖¶¶**

*From the ‡Department of Metabolic Diseases, Faculty of Medicine, University of Tokyo 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan, the §Laboratory of Cellular Biochemistry, Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351-0198, Japan, the ¶Department of Health Chemistry, Graduate School of Pharmaceutical Science, University of Tokyo 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, the ‖Department of Internal Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi 329-0498, Japan, the **Chugai Research Institute for Medical Science Incorporated, 1-135 Komakado, Gotenba, Shizuoka 412-8513, Japan, the ‡‡Unit for Functional Genomics, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Nishi-2, Inada, Obihiro, Hokkaido 080-8555, Japan, and §§Metabolism, Endocrinology, and Atherosclerosis, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*

Scavenger receptor expressed by endothelial cells I (SREC-I) is a novel endocytic receptor for acetylated low density lipoprotein (LDL). Here we show that SREC-I is expressed in a wide variety of tissues, including macrophages and aortas. Lipopolysaccharide (LPS) robustly stimulated the expression of SREC-I in macrophages. In an initial attempt to clarify the role of SREC-I in the uptake of modified lipoproteins as well as in the development of atherosclerosis, we generated mice with a targeted disruption of the *SREC-I* gene by homologous recombination in embryonic stem cells. To exclude the overwhelming effect of the type A scavenger receptor (SR-A) on the uptake of Ac-LDL, we further generated mice lacking both SR-A and SREC-I (*SR-A$^{-/-}$;SREC-I$^{-/-}$*) by cross-breeding and compared the uptake and degradation of Ac-LDL in the isolated macrophages. The contribution of SR-A and SREC-I to the overall degradation of Ac-LDL was 85 and 5%, respectively, in a non-stimulated condition. LPS increased the uptake and degradation of Ac-LDL by 1.8-fold. In this condition, the contribution of SR-A and SREC-I to the overall degradation of Ac-LDL was 90 and 6%, respectively. LPS increased the absolute contribution of SR-A and SREC-I by 1.9- and 2.3-fold, respectively. On the other hand, LPS decreased the absolute contribution of other pathways by 31%. Consistently, LPS did not increase the expression of other members of the scavenger receptor family such as CD36. In conclusion, SREC-I serves as a major endocytic receptor for Ac-LDL in LPS-stimulated macrophages lacking SR-A, suggesting that it has a key role in the development of atherosclerosis in concert with SR-A.

* This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture and by the Takeda Medical Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶¶ To whom correspondence should be addressed: Division of Endocrinology and Metabolism, Department of Internal Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi 329-0498, Japan. Tel.: 81-285-58-7355; Fax: 81-285-40-6035; E-mail: ishibash@jichi.ac.jp.

Scavenger receptors mediate the endocytosis of chemically modified lipoproteins such as acetylated low density lipoprotein (LDL),[1] thereby contributing to the development of atherosclerosis (1). The scavenger receptor gene family comprises a series of unlinked genes encoding membrane proteins with diverse ligand binding activity (2). The class A type I/type II scavenger receptor (SR-A) is the prototype receptor belonging to this family (3) and accounts for ~80% of the uptake of Ac-LDL in macrophages (4, 5).

Recently, we identified scavenger receptor expressed by endothelial cells I (SREC-I), which encodes a protein of 830 amino acids and binds fluorescent DiI-labeled Ac-LDL when expressed in Chinese hamster ovary cells (6), and its paralogous gene, *SREC-II* (7). The SREC-I protein is composed of an N-terminal extracellular ligand binding domain with seven epidermal growth factor receptor-like cysteine pattern signatures, a membrane-spanning domain, and an unusually long C-terminal cytoplasmic domain that includes a Ser/Pro-rich region followed by a Gly-rich region. SREC-II encodes an 834-amino acid protein with 35% homology to SREC-I. Although SREC-II has little activity to internalize modified LDL, SREC-I-expressing fibroblasts are intensely aggregated with SREC-II-expressing fibroblasts, indicating the association of SREC-I and SREC-II (7). However, the precise functions of these two proteins are currently unknown.

In atherosclerotic lesions, macrophages are laden with lipids and immunologically activated (8). In line with this, the development of atherosclerosis is accelerated by LPS (9), a major component of Gram-negative bacteria that stimulates the production of various cytokines *in vivo*, thereby contributing to the pathogenesis of endotoxin shock (10). Conversely, the absence of toll-like receptor 4, a receptor for LPS, inhibits its progression (11). These considerations have prompted us to examine the effects of LPS on the expression of SREC-I. In the present study, we show that LPS robustly stimulated the expression of

[1] The abbreviations used are: LDL, low density lipoprotein; SR-A, class A type I/type II scavenger receptor; SR-BI, class B type I scavenger receptor; SREC, scavenger receptor expressed by endothelial cells.

This paper is available on line at http://www.jbc.org

SREC-I in macrophages. In an initial attempt to clarify the role of SREC-I in the uptake of modified lipoproteins as well as in the development of atherosclerosis, we generated mice with targeted disruption of the *SREC-I* gene by homologous recombination in embryonic stem cells. To exclude the overwhelming effect of SR-A on the uptake of Ac-LDL, we further generated mice lacking both SR-A and SREC-I ($SR\text{-}A^{-/-};SREC\text{-}I^{-/-}$). By comparing the uptake and degradation of Ac-LDL in peritoneal macrophages isolated from these mice, we found that SREC-I plays a significant role in the uptake of Ac-LDL in the setting of SR-A deficiency, especially when stimulated with LPS. From these results, we propose that SREC-I contributes to the development of atherosclerosis in concert with SR-A.

EXPERIMENTAL PROCEDURES

General Methods—Standard molecular biology techniques were used (12). The current experiments were performed in accordance with institutional guidelines for animal experiments at the University of Tokyo and the Jichi Medical School.

SREC-I Antibody Preparation—Two milligrams of the carboxyl-end peptide of mouse SREC-I (amino acid residues 801–820, KEQEEPLY-ENVVPMSVPPQH) was conjugated with keyhole limpet hemocyanin using the Imject sulfhydryl-reactive antibody production kit (Pierce). The keyhole limpet hemocyanin-peptide was gel-purified and emulsified with an equal volume of complete Freund's adjuvant (Calbiochem). A female Wister rat was immunized with the emulsions. One week after the boost injection, blood was collected, and the antiserum was purified and eluted through an affinity column (Sulfolink coupling gel; Pierce) to which the antigen peptide was coupled.

Mice—SR-A knock-out mice were generated previously (4). ApoE knock-out mice were purchased from the Jackson Laboratory (Bar Harbor, ME) (13). Both mice had been back-crossed to C57BL/6J genetic background and fed a normal chow diet (MF diet from Oriental Yeast Co., Tokyo, Japan) that contained 5.6% (w/w) fat with 0.09% (w/w) cholesterol, and the mice were allowed access to water and food *ad libitum*.

Cells—Thioglycolate-elicited peritoneal macrophages (14) and mouse embryonic fibroblasts (15) were prepared as described previously. Cells were treated with varying concentrations of LPS (*Escherichia coli* O127:B8; Sigma) for 12 h before the experiments.

Northern Blot Analysis—For the SREC-I cDNA probe, two probes were prepared, namely Probe A, a 5′ 2.0-kb fragment spanning the extracellular and intracellular domains, and Probe B, a 0.1-kb fragment consisting of only the transmembrane domain. Poly $(A)^+$ RNA was purified using Oligotex-dT30™, an oligo(dT) latex (Roche Applied Science) from 100–150 μg of total RNA that was extracted by TRIzol reagent (Invitrogen) from either cultured cells or tissues. One to three milligrams of poly$(A)^+$ RNA was subjected to 1% agarose gel electrophoresis in the presence of formalin, transferred to Hybond N (Amersham Biosciences), and hybridized to the ^{32}P-labeled probes for SREC-I and other scavenger receptors as described previously (16).

Western Blot Analysis—Cells were lysed with 0.1% SDS. After centrifugation, 50 μg of the supernatant was subjected to SDS-PAGE and transferred to Hybond ECL™, a nitrocellulose membrane (Amersham Biosciences). After incubation with the anti-SREC-I antibody (1:400 dilution), the membrane was incubated with a goat anti-rat IgG conjugated with horseradish peroxidase (1:2000 dilution; Amersham Biosciences). The secondary antibody was visualized by an enhanced chemiluminescence kit (Amersham Biosciences).

Generation of the SREC-I Knock-out Mice—The *SREC-I* gene was cloned from the 129/Sv mouse genomic library (Clontech) using the mouse cDNA as a probe. A replacement-type targeting vector was constructed so that a 35-bp segment in exon 8, which encodes 3′ two-thirds of the transmembrane domain, was replaced with a polIIneo cassette (Fig. 5*A*). Long arm consists of a 10-kb NotI/KpnI fragment spanning the 5′ untranslated region and exon 8; short arm consists of a 0.9-kb SacI/XbaI fragment within intron 9. These were inserted together into the vector pPolIIshort-neobpA-HSVTK, as described previously (17). After digestion with SalI, the vector was electroporated into JH-1 embryonic stem cells (a generous gift from Dr. Herz at University of Texas Southwestern Medical Center at Dallas, TX). Targeted clones, which had been selected in the presence of G418 and 1-(2-deoxy, 2-fluoro-β-D-arabinofuranosyl)-5 iodouracil, were identified by PCR using the primers 5′-GATTGGGAAGACAATAGCAGGCATGC-3′ and 5′-CAGAGAGTGTCACCACAACAAGAGGA-3′ (Fig. 5*A*). Homologous recombination was verified by Southern blot analysis after digestion with EcoRI using a 0.5-kb SpeI/SmaI fragment, which was downstream of the short arm, as a probe (Fig. 5*A*). Targeted embryonic stem clones were injected into C57BL/6J blastocysts, yielding one line of chimeric mice that transmitted the disrupted allele through the germline.

Generation of the SR-A/SREC-I Double Knock-out Mice—The $SR\text{-}A^{-/-}$ mice were crossed with the $SREC\text{-}I^{-/-}$ mice, which were a C57BL/6J × 129/Sv hybrid, to obtain $SR\text{-}A^{+/-};SREC\text{-}I^{+/-}$ mice, which were interbred to obtain four types of mice, namely wild-type, $SR\text{-}A^{+/+};SREC\text{-}I^{-/-}$, $SR\text{-}A^{-/-};SREC\text{-}I^{+/+}$, and $SR\text{-}A^{-/-};SREC\text{-}I^{-/-}$ mice. Thus, the genetic background of these mice was 75% C57BL/6J and 25% 129/Sv. Littermates were used for the experiments.

Biochemical Analyses—Blood was collected from the retro-orbital venous plexus after a 12-h fast. Plasma glucose (ANTSENSE II, Bayer Medical, Tokyo, Japan), cholesterol (Determiner TC, Kyowa Medex, Tokyo), and triglycerides (TGLH; Wako Chemicals, Tokyo, Japan) were measured.

Histology—Mice were sacrificed by decapitation. Tissues were excised, fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin-eosin.

Preparation of Lipoproteins—LDL (*d* 1.019–1.063 g/ml) and lipoprotein-deficient serum (*d* >1.21 g/ml) were prepared by stepwise ultracentrifugation from plasma obtained from healthy volunteers. The lipoproteins and lipoprotein-deficient serum were dialyzed against 10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.01%(w/v) EDTA, and 0.01% (w/v) NaN_3. LDL was acetylated with acetic anhydrate and radioiodinated by the iodine monochloride method as described (18). Protein concentrations were determined by the BCA protein assay reagent kit (Pierce).

Cellular Uptake and Degradation of ^{125}I-Ac-LDL—Peritoneal macrophages were plated in 12-well plates at a density of 1×10^6/well and treated with or without 100 ng/ml of LPS for 12 h. After stringent washing with PBS, the cells were incubated with a medium containing varying concentrations of ^{125}I-Ac-LDL and 5 mg/ml lipoprotein-deficient serum, with or without a 50-fold excess of unlabeled Ac-LDL, for 5 h at 37 °C. The amounts of ^{125}I-Ac-LDL either degraded by or associated with the cells were measured according to a modified method (19) of Goldstein *et al.* (18).

Statistics—The differences of the means were compared by Student's *t* test.

RESULTS

Tissue Distribution of mRNA Expression of SREC-I and -II—We performed Northern blot analyses to examine the expression of SREC-I and II in various organs of a mouse (Fig. 1). SREC-I was expressed in a wide variety of organs, most predominantly in liver, lung, kidney, and heart. On the other hand, the expression of SREC-II was restricted to lung and kidney.

LPS Stimulates the Expression of SREC-I in Peritoneal Macrophages—LPS robustly increased the mRNA expression of both SREC-I and SR-A in macrophages (Fig. 2). The peak of the stimulation was reached by the 12-h time point of the stimulation (Fig. 2*A*), and the maximal responses were obtained at the concentration of 10 ng/ml (Fig. 2*B*). The relative increase in the expression of SREC-I was 4-fold, which was more prominent than that of SR-A (1.8-fold) (Fig. 3). It is of note that the treatment with LPS did not significantly change the expression of MARCO (macrophage receptor with collagenous structure) (20) and SR-BI (21) and that it even decreased the expression of CD36 (22) and FEEL-1 (fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1) (23).

Expression of SREC-I in Aortas—We compared the mRNA expression levels of SREC-I in the atherosclerotic aortas, which were taken from 12-month-old apoE knock-out mice, with normal aortas, mouse embryonic fibroblasts, peritoneal macrophages, kidney, or lung from wild-type mice (Fig. 4). Normal and atherosclerotic aortas expressed 1.7- and 2.1-fold higher levels of SREC-I mRNA than the non-stimulated macrophages, respectively. The expression levels were comparable with those in the kidney, but much lower than those of the LPS-treated macrophages.