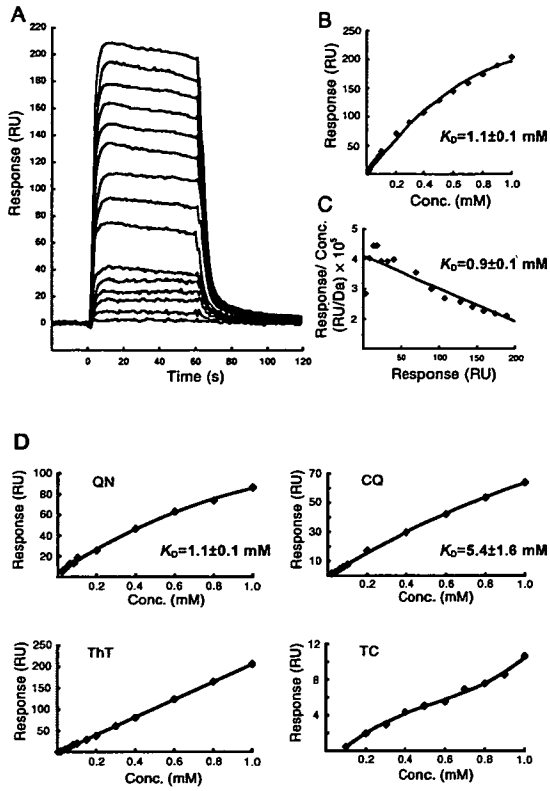

Fig. 3. Kinetic Analyses of Low Affinity Compounds

(A) Sensorgrams, (B) dose response curve and (C) scatchard plot of QC. (D) Dose response curves of QN, CQ, ThT, and TC.

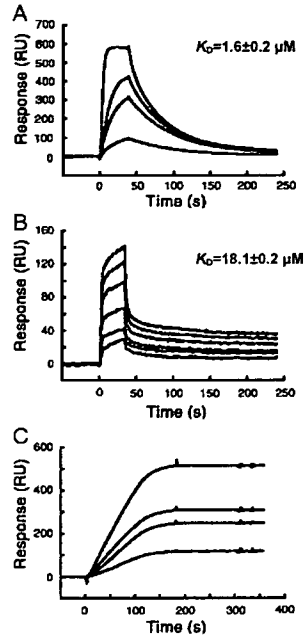

Fig. 4. Kinetic Analyses of High Affinity Compounds

(A) Sensorgrams of CR at concentrations of 1, 2, 3.3 and 5 μM, and its K_D value. (B) Sensorgrams of PcTS at concentrations of 1, 5, 10, 50, 75 and 100 μM, and its K_D value. (C) Sensorgrams of ThS at concentrations of 1, 2, 3 and 5 μg/ml, and its K_D value could not be calculated because of its undetermined structure and molecular weight.

CQ, respectively showed a similar monophasic pattern in dose response curves, yielding K_D of 1.1 mM and 5.4 mM (Fig. 3D). These K_D values, however, were of rough estimation and might be a little underscored due to lack of the data at concentrations of more than 1 mM. Unstable solubility of the compounds at such high concentrations hindered further analyses.

On the other hand, ThT gave a linear dose–response curve within a concentration of up to 1 mM and TC showed a biphasic pattern (Fig. 3D). Therefore, the saturation levels and K_D values of these compounds could not be determined, indicating that these compounds have a very low or no affinity with PrP121—231. Of them, TC is known to revert abnormal physicochemical properties of PrPres *in vitro*,[18] and interaction between TC and human PrP 106—126 peptides is revealed by NMR analysis.[26] Their data appear to be inconsistent with the data in this study. However, this discrepancy might be attributable to the lack of a TC binding site in the PrP121—231 used in our study.

Each sensorgram of high affinity compounds showed a very slow dissociation phase and was individually characteristic (Fig. 4). The structural and stoichiometric binding details of the compounds with PrP121—231 have not yet been established, but CR or PcTS is a symmetrical molecule and either half of the molecule has anti-prion activity (Doh-ura K, unpublished data). Consequently, the K_D value for the compound was deduced after the data were fit to a binding model assuming a bivalent analyte. The K_D of CR was calculated to be 1.6 μM from the sensorgrams of 1, 2, 3.3 and 5 μM (χ^2=20.9±2.1) (Fig. 4A). The K_D of PcTS was calculated as 18.1 μM from the sensorgrams of 1, 5, 10, 50, 75 and 100 μM (χ^2=28.1±2.9) (Fig. 4B). The K_D of ThS was incalculable to an exact degree because it is presumed to be a mixture of compounds formed by methylation and sulfonation of primulin; their structures and molecular weights have not been determined.

Comparison between PrP Affinity and Anti-prion Activity The IC_{50} value for the inhibition of PrPres formation in ScNB cells, either previously reported or examined in this study, was used as an anti-prion activity in this study. It was compared with the K_D or with the binding response. The latter, an index for estimating the interaction, was obtained from the R_{eq} value or the maximum response value at a concentration of 1 mM divided by the molecular weight (Table 1).

From data of all compounds except ThT, TC and ThS, statistical analyses demonstrated a significant linear correlation between the reciprocal of binding response and the IC_{50} (r=0.985, p=0.0005) (Fig. 5). This relation appeared to be also observed in TC, but not in ThT showing the next highest binding response to QC but no inhibition of PrPres formation within a non-toxic dose range. However, ThT demonstrated cell-toxicity at such a low dose as 0.05 μM.

For ThS, assuming that its minimum molecular weight deduced from presumable structures was 520 Da, its binding response was estimated to be 5.03 RU/Da; the IC_{50} was estimated to be about 2 μM, corresponding to about 1 μg/ml. However, these values seem to be underestimates because some constituents of ThS might interact with PrP121—231 or have inhibitory activity for PrPres formation. Therefore, active constituents of ThS might be expected to inhibit PrPres formation in ScNB cells at a submicromolar dose, similar to the other high-affinity compounds.

Screening by SPR Findings suggested that a compound

Table 1. Binding Response, Dissociation Constant (K_D) and 50% PrPres Inhibition Dose in ScNB Cells (IC_{50})

Compound	Binding response[a] (RU/Da)	K_D[b] (mM)	IC_{50}[c] (μM)
Low-affinity			
Quinacrine (QC)	0.25±0.00	1.1±0.1 (0.9±0.1)	0.3 (7)
Quinine (QN)	0.05±0.00	1.1±0.1 (1.4±0.1)	6.0 (11)
Chloroquine (CQ)	0.07±0.01	5.4±1.6 (3.5±0.8)	4.0 (7)
Thioflavin T (ThT)	0.16±0.01	n.d.[d] (n.d.[d])	No effect[f]
Tetracycline (TC)	0.01±0.00	n.d.[d] (n.d.[d])	No effect[g]
High-affinity			
Congo red (CR)	8.74±0.64	$1.6\pm0.2\times10^{-3}$	1.5×10^{-2} (4)
Phthalocyanine tetrasulfonate (PcTS)	1.82±0.06	$18.1\pm0.2\times10^{-3}$	0.5 (17)
Thioflavin S (ThS)	n.d.[e]	n.d.[e]	*ca.* 1 μg/ml

a) Binding response value was calculated from the R_{eq} value divided by the molecular weight for QC, QN, CQ and CR, or from the response value at a concentration of 1 mM divided by the molecular weight for ThT, TC and PcTS. *b*) K_D values were determined by steady state analysis for the low-affinity compounds or by bivalent analyte model analysis for CR and PcTS. K_D values from Scatchard plot analyses are shown in parentheses. *c*) IC_{50} values reported in the literature (reference shown in parentheses) or examined in this study. *d*) n.d.: not determined because a saturation level could not be estimated. *e*) n.d.: not determined because its structure and molecular weight were undetermined. *f*) Inhibition of PrPres formation was not observed up to a minimal toxic dose of 0.05 μM. *g*) Inhibition of PrPres formation was not observed up to a minimal toxic dose of 5.0 μM.

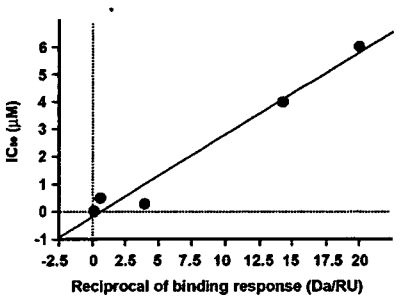

Fig. 5. Correlation between the Reciprocal of Binding Response and the IC_{50}

The data were from five compounds in which both binding response and IC_{50} were determined. Correlation showed a slope of 0.298, an intercept of −0.156 and a correlation coefficient of 0.971 (p=0.002) by simple linear regression analysis.

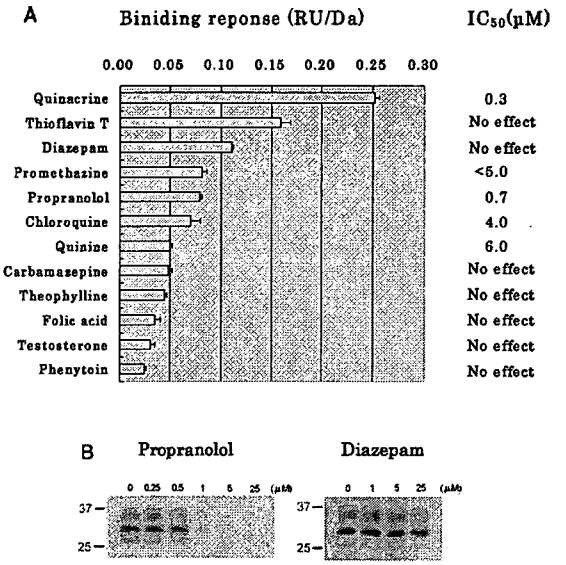

Fig. 6. Screening of Anti-prion Candidates Using the SPR Assay

(A) Binding response of each sample at 100 μM, and its IC_{50} of PrPres formation inhibition in ScNB cells within a non-toxic dose range. (B) Inhibition analyses of PrPres formation in ScNB cells grown in the presence with propranolol or diazepam. Molecular sizes in kDa are shown at the left of each panel.

capable of interacting with PrP121—231 might have a potency of inhibiting PrPres formation in ScNB cells. To verify this inference, several drugs were examined for either their binding response using the SPR method or their IC_{50} in ScNB cells. Eight clinically utilized drugs—carbamazepine, diazepam, folic acid, phenytoin, promethazine, propranolol, testosterone, and theophylline—all of which are low molecular weight compounds capable of crossing the blood brain barrier and share a partial structure similarity with the anti-prion compounds already reported, were examined and compared with the four anti-prion compounds (QC, QN, CQ, and ThT) (Fig. 6A).

Diazepam, promethazine and propranolol showed a higher binding response value than QN, which was the lowest binding response compound among the effective anti-prion compounds examined in this study. Among these, promethazine or propranolol inhibited PrPres formation in ScNB cells (propranolol: IC_{50}=0.7 μM; promethazine: IC_{50}<5.0 μM). Promethazine has already been reported to have anti-prion activity in ScNB cells,[8] whereas propranolol is a novel compound that inhibits PrPres formation in ScNB cells. Diazepam apparently did not inhibit PrPres formation within a non-toxic dose range up to 25 μM (Fig. 6B). Inhibitory activities against PrPres formation in ScNB cells were not observed for other drugs that had lower binding response values than QN.

DISCUSSION

We demonstrated that most anti-prion compounds examined in this study interacted with PrP121—231. The binding response of the compounds correlated with the IC_{50} of PrPres formation inhibition in ScNB cells. In addition, based on this finding, we proved that this interaction analysis using the SPR method was useful for screening to identify new candidates of anti-prion compounds. Three different *in vitro*

screening assays have been reported recently. One is yeast based,[27] one uses ScN2a cells,[10] and the other is based on fluorescence correlation spectroscopy.[28] These assays are suitable for high-throughput screening of large compound libraries to identify novel lead molecules. The SPR method reported here, which easily assayed interactions between compounds and PrP molecules within less than 3 min per compound, is applicable to high-throughput *in vitro* assay for screening of large compound libraries if more highly performing SPR machines are used. The usefulness of this method in screening for PrP binding ligands is also reported very recently by other researchers.[29]

Two chemicals, ThT and diazepam, showed high binding response but did not inhibit PrPres formation within a non-toxic dose range. Of them, ThT exhibited very low or no affinity with PrP121—231 but the next highest binding response to QC. This suggests that ThT might interact with PrP121—231 non-specifically. For diazepam, similar non-specific interaction with PrP121—231 might be occurred, or the interaction might be specific but unrelated to conversion to PrPres. These inferences, however, remain unsupported by other experimental results obtained here.

On the other hand, such high-affinity compounds as CR and PcTS showed large amounts of binding to PrP121—231. One possible interpretation for this is that the compounds might have two or more binding sites per molecule. In fact, structure–activity relationship analysis for these symmetrical compounds indicates that either half of the molecule has anti-prion activity (Doh-ura K, unpublished data), and their sensorgrams looked very similar to those of anti-PrP antibodies (data not shown). The other is that the compounds might self-assemble to interact with the PrP molecule. It has long been known that CR and many other bis-azo dyes self-assemble in water solutions, and this property is proposed to associate with binding capability.[30]

Instead of the full length of mouse PrP, a carboxy-terminal domain of mouse PrP (PrP121—231) was used in the study because of instability of the full length PrP during the experiment. This carboxy-terminal domain is the only autonomous folding unit of PrP with a defined three-dimensional structure[19,23,24] and contains epitopes recognized by a majority of antibodies bearing anti-prion activity.[31—37] Taken together with our findings suggesting that most of anti-prion compounds might exert their effects by interacting with this domain, targeting the carboxy-terminal domain should not necessarily be either inefficient or inappropriate for looking for new anti-prion compounds.

In conclusion, our study indicated that most anti-prion compounds tested here interacted with and had an affinity for recombinant PrP121—231. The SPR binding response to the PrP121—231 correlated with the anti-prion activity in ScNB cells. These observations will allow further discovery of new classes of anti-prion compounds using the SPR assay.

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αヘルペスウイルスの分子生物学

VZVの遺伝子とその産物

The varicella-zoster virus genome and the genes

奥野壽臣

Key words：水痘・帯状疱疹ウイルス，遺伝子，潜伏感染，分子疫学，水痘ワクチン

はじめに

水痘・帯状疱疹ウイルス(varicella-zoster virus: VZV)は，単純疱疹ウイルス(herpes simplex virus: HSV)とともにαヘルペスウイルス亜科に属す．どちらのウイルスも宿主域は広く，増殖速度は比較的速く，知覚神経節に潜伏する．VZVのDNAは1986年，全塩基配列が決定されたが[1]，HSVのDNAとは根本的な相違が見いだされなかった．*in vitro*, *in vivo*における両ウイルスの性質の違いを考えると，遺伝子のわずかな差が生物学的性質に大きな差を生じることに驚きを禁じ得ない．

VZVには日本製の世界で唯一の弱毒生ワクチンがあり各国で使用され，数多くの幼い命を救ってきたが，帯状疱疹の発症予防にも効果のあることが最近示された[2]．このようなワクチンの絶大なる効果を考えるとき，その弱毒化の機構を分子レベルで理解したいと思うのが人情であり，その方面の研究が盛んに行われている．それらも含め，これまでに判明している遺伝子とその産物の機能について概説した．

1. VZVゲノムの構造とその特徴

VZVゲノムはヒトヘルペスウイルスの中で最も小さく，サイトメガロウイルスの約半分の125,000塩基対の線状二本鎖DNAである．遺伝子の基本構造を図1に示す．両端を繰り返し配列のterminal repeat long(TRL)とinternal repeat long(IRL)に囲まれたunique long(UL)(TRL，IRL，ULを合わせてL領域)，terminal repeat short(TRS)とinternal repeat short(IRS)に囲まれたunique short(US)(TRS，IRS，USを合わせてS領域)からなる．TRLとIRL，TRSとIRSは同じ配列が逆向きになっている．TRLとIRLはそれぞれ88.5 bpの長さをもち，HSVのそれ(9.2 kbp)の1%以下である．また，HSVのULとUSの両端に存在し，DNAのカプシドへのパッケージングに必要なa配列がVZVには欠けている．TRSとIRSはHSVと同様に長く(7.3 kbp)，USには複製開始点が存在する．感染細胞内ではL領域とS領域がそれぞれ順方向と逆方向を向いて結合したウイルスゲノムが作られるが，ULが一方向を向きUSが両方向を向いた2種類のDNAがそれぞれ47%ずつ存在し，ULが反対方向を向いたものは6%しか存在しない．

2. 遺伝子とその産物

遺伝子をコードするORF(open reading frame)はゲノムの左端より71番目まで通し番号が付けられ(図2)，ORF9A，ORF33.5，ORFS/Lを加えると74種類となるが，42と45はイントロ

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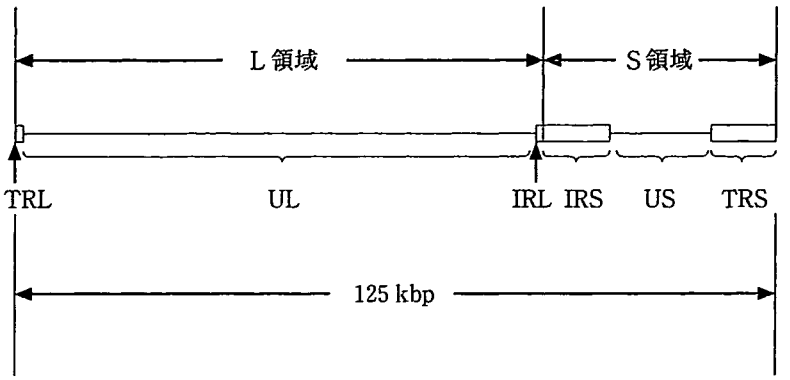

図1 VZVゲノムの基本構造

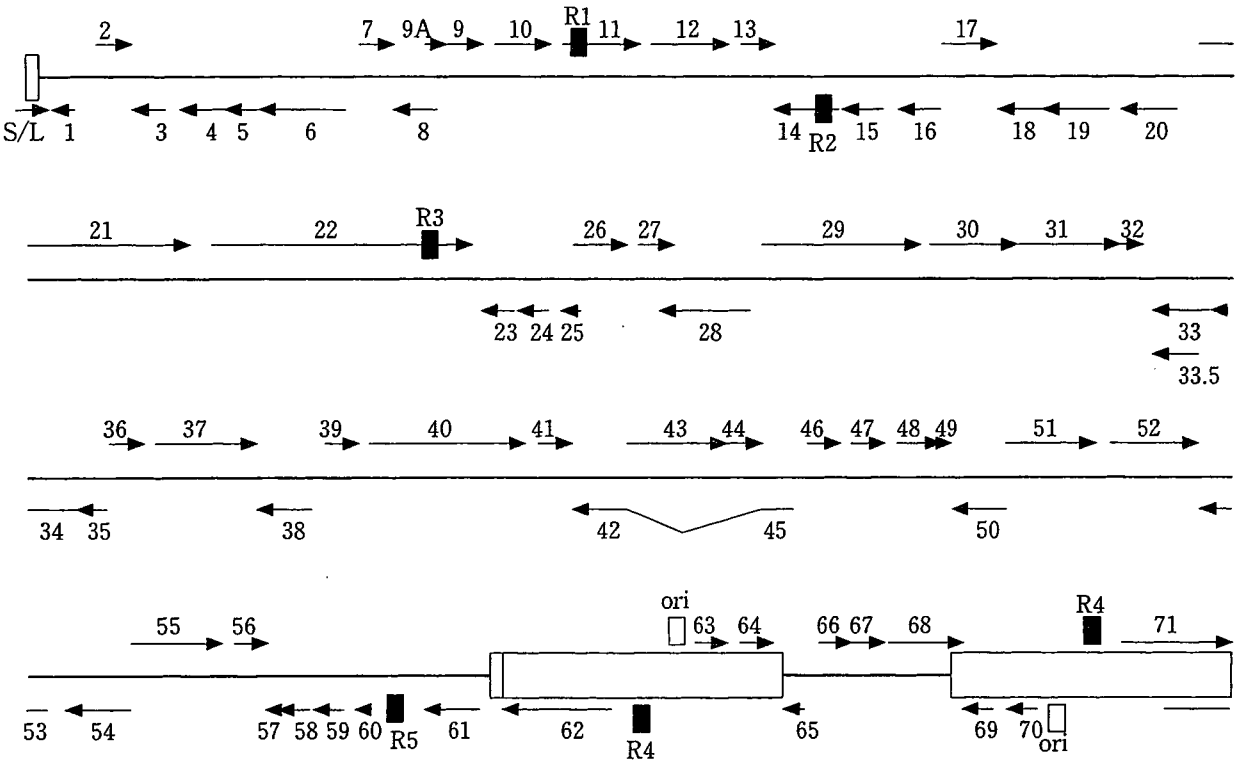

図2 VZVの遺伝子

R1-5: direct sequence repeat, ori: DNA複製開始点.

ンを間に含む1つの遺伝子，ORF62，63，64はそれぞれORF71，70，69と同じ遺伝子であるので，結局ORFは70種類あることになる．そのうち64種類がHSV-1の相同遺伝子で，VZVに固有な6種の遺伝子のうち5種はすべてULに存在する．ORFS/Lは環状になったDNAの結合部分を越えてS領域の右端からL領域の左端に及ぶORFである．ULにはR1，R2，R3という短い繰り返し配列(direct sequence repeat)がそれぞれORF11，14，22の中に存在している．R4はIRSとTRSのORF62，ORF63の間に，R5はORF60，ORF61の間に存在している．これらのdirect sequence repeatは株によってその繰り返し数が異なるので，株間の異同の判定に用いられている．VZVの遺伝子の中で他の7種のヒトヘルペスウイルスにも共通に保存されているものは少なくとも40種以上あり，それらはすべてULに存在する．

表1に各遺伝子の情報をまとめた．これからもわかるように，VZVの各遺伝子の機能の解明

表1 VZV各遺伝子のまとめ

VZV遺伝子	産物の存在場所	遺伝子の発現時期	ウイルス増殖での必要性	潜伏感染時の発現	HSV-1の相同遺伝子	産物の機能
1	感染細胞膜		な し		な し	潜伏感染に必要ない
2	感染細胞膜 粒子にはなし		な し		な し	リン酸化蛋白，潜伏感染に必要ない
3					UL55	
4	テグメント はじめ核，のち細胞質(溶解感染) 細胞質(潜伏感染) 核(再活性時)	IE	必 要	あ り	UL54(ICP27)	転写活性化，リン酸化蛋白 潜伏感染に必要
5	ウイルス粒子 細胞質		必 要		UL53	gK
6					UL52 (helicase-primase complex)	
7					UL51	
8		E	な し		UL50	deoxyuridine triphosphatase
9	テグメント	L			UL49	リン酸化蛋白
9A	ウイルス粒子		な し		UL49A	合胞体形成
10	テグメント	L	な し		UL48(VP16)	転写活性化，潜伏感染に必要ない 皮膚での感染に関与
11					UL47	
12					UL46	
13			な し		な し	thymidylate synthetase 潜伏感染に必要ない
14	粒子にはなし	L	な し		UL44	gC，潜伏感染に必要ない 皮膚での感染に関与
15					UL43	
16					UL42	
17	粒子にはなし	L			UL41	細胞の蛋白合成阻害，37℃の増殖に必要 潜伏感染に必要ない
18	粒子にはなし	E			UL40	ribonucleotide reductase (small subunit)
19	粒子にはなし	E	な し		UL39	ribonucleotide reductase (large subunit)
20	ウイルス粒子	L			UL38	
21	カプシド 核，細胞質	L	必 要	あ り	UL37	潜伏感染に必要ない
22					UL36	
23					UL35	
24					UL34	
25					UL33	
26					UL32	
27					UL31	
28	粒子にはなし	E			UL30	DNA polymerase

（表1つづき）

VZV遺伝子	産物の存在場所	遺伝子の発現時期	ウイルス増殖での必要性	潜伏感染時の発現	HSV-1の相同遺伝子	産物の機能
29	核（溶解感染） 細胞質（潜伏感染） 核（再活性時）			あり	UL29（ICP8）	一本鎖DNA結合蛋白
30					UL28	
31	ウイルス粒子	L			UL27	gB，heparan sulfateとの結合 エンベロープと膜との融合 （gEと共同）
32			な し		な し	リン酸化蛋白 潜伏感染に必要ない
33	カプシド	L			UL26	蛋白分解酵素
33.5					UL26.5	アッセンブリー蛋白
34	ウイルス粒子	L			UL25	
35	核				UL24	皮膚感染に必要
36	粒子にはなし	E	な し		UL23	チミジンキナーゼ
37	ウイルス粒子	L			UL22	gH，エンベロープと膜との融合
38					UL21	
39					UL20	
40	カプシド	L			UL19	主要カプシド蛋白
41					UL18	
42/45					UL15	
43					UL17	
44					UL16	
46					UL14	
47	テグメント	E	な し		UL13	プロテインキナーゼ，リン酸化蛋白 皮膚，T細胞での増殖に必要 潜伏感染に必要ない
48	粒子にはなし				UL12（deoxyribonuclease）	
49					UL11	
50					UL10（gM）	
51	粒子にはなし	E			UL9	複製開始点結合蛋白
52	粒子にはなし	E			UL8（helicase-primase complex）	
53					UL7	
54	ウイルス粒子	L			UL6	
55	粒子にはなし				UL5（helicase-primase complex）	
56					UL4	
57	細胞質		な し		な し	潜伏感染に必要ない
58					UL3	
59			な し		UL2	ウラシルDNAグリコシラーゼ
60					UL1	gL（gHのシャペロン）
61	粒子にはなし		な し		ICP0	転写活性化と抑制化，リン酸化蛋白 潜伏感染に必要ない

(表1つづき)

VZV遺伝子	産物の存在場所	遺伝子の発現時期	ウイルス増殖での必要性	潜伏感染時の発現	HSV-1の相同遺伝子	産物の機能
62, 71	テグメント 核(ORF4蛋白と) 細胞質(ORF66蛋白と)	IE	必要	あり	ICP4	転写活性化，皮膚での増殖に必要 リン酸化蛋白
63, 70	テグメント 核(溶解感染) 細胞質(潜伏感染) 核と細胞質(再活性時)	IE	必要	あり	US1(ICP22)	リン酸化蛋白，潜伏感染に必要 皮膚，T細胞での増殖に必要 転写活性化，抑制化
64, 69	ウイルス粒子	L	なし		US10	皮膚，T細胞での増殖に必要ない
65	ウイルス粒子	L	なし		US9	皮膚，T細胞での増殖に必要ない
66	ウイルス粒子		なし	あり	US3	プロテインキナーゼ，リン酸化蛋白 潜伏感染に必要ない 皮膚での増殖に必要ない T細胞での増殖に必要
67	ウイルス粒子	L	なし		US7	gI，リン酸化蛋白 皮膚，T細胞での増殖に必要 gEとheterodimerでFcレセプター gEの成熟に必要 潜伏感染に必要ない
68	ウイルス粒子	L	必要		US8	gE，リン酸化蛋白 エンベロープと膜との融合(gBと共同) gIとheterodimerでFcレセプター
S/L	細胞質		なし		なし	

はHSVに比べて遅れていたが，これはVZVが細胞依存性が強く高力価のウイルスを得るのが困難だったことと動物モデルが存在しなかったことに起因していた．しかしここ数年来の分子生物学的解析の進歩や，それを利用して生み出された各遺伝子の欠損ウイルス[3,4]および新たなモデル動物[5]を用い，VZVの感染病理，潜伏感染や再活性化機構，ワクチンの弱毒化機構の解明が急速に進んでいる．

3. 潜伏感染機構

VZVは脊髄後根神経節に潜伏感染しているが，ウイルスが潜伏感染を成立，維持，更には再活性化する機構はわかっていない．潜伏時にはORF4，21，29，62，63，66の蛋白が発現しているが，欠損ウイルスを用いた実験から，ORF4と63は潜伏に必要でORF21と66は必要ではないことが判明している．ORF4，63そしてORF29のすべての蛋白は溶解感染のときには核内に存在するが，潜伏時には細胞質内に局在し，再活性化に伴って再び核内に局在するようになるので，これらの蛋白の潜伏感染機構における役割の解明が待たれる．

4. 分子疫学

VZVにはヒトヘルペスウイルスの中で唯一弱毒生ワクチンが開発され世界での使用が広がっているが，ワクチン接種後に水痘，帯状疱疹を発症することがあり，それがワクチン株によるのか野生株によるのかを判別することがますます重要になってくる．そのため，株間のDNAの塩基配列の差を検出する分子疫学が様々試みられている．direct sequence repeatの繰り返し数を判定に用いようとしたが，野生株にもワク

チン株と同数のものがあり，使用には不十分であった．ORF38やORF54をPCR増幅の後それぞれPst1とBgl1で切断する方法があり，ワクチン株と欧米の野生株の判別は可能であった．しかし，ワクチン株と日本の野生株とを分けることはできなかった．その両方を組み合わせた方法[6]や，R2のSSCP(single-strand conformational polymorphism)を加えた方法[7]などが行われて判別の精度が向上した．2002年，五味らは，ワクチン株とその親株のDNAの全塩基配列を解読し比較した[8]．その結果，42カ所の塩基置換があり，そのうち15カ所がORF62に集中していることが判明した．そしてORF6の1カ所とORF62の6カ所の制限酵素切断パターンから，ワクチン株と親株も含めたその他の株とを完全に判別できることを示した．また，制限酵素切断部位のSNP(single nucleotide polymorphism)を解析して，ワクチン株と野生株の判別に有用な制限酵素を決定したことが報告されている[9]．

5. 水痘生ワクチンの弱毒化機構

ワクチンの弱毒を論じるとき，ウイルスのいかなる生物学的変化が毒力の低下をもたらしているのかを同定することは非常に困難である．現在使用されているワクチンには複数の株が混在していることがわかっており[8,10]，話を更に複雑にしている．ORF62は前初期，初期，後期の遺伝子の転写を強く活性化するが，ワクチン株では親株からの変異がこの部分に集中し転写活性化が弱くなっているので，弱毒化に重要な役割を果たしているという指摘がある[8]．ヒトの皮膚の*in vivo*モデル動物を用いた解析では，ORF47，gE，gIプロモーターそれぞれの単独の変異と弱毒との関連が示唆された[11-13]．しかし，弱毒化における複数遺伝子の関与の可能性が同じ研究グループから最近報告されている[14]．

おわりに

第30回国際ヘルペスウイルスワークショップ(2005年)でVZVのレセプター発見の発表がなされ，gEと結合してウイルスの中和や細胞間の感染の広がりを抑制する分子としてinsulin degrading enzymeという蛋白が同定された[15]．HSV同様，VZVにおいてもこれから分子レベルの解析がより一層進んでいくものと思われる．

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Evidence of Immunostimulating Lipoprotein Existing in the Natural Lipoteichoic Acid Fraction▽

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Lipoteichoic acid (LTA) is a cell surface glycoconjugate of gram-positive bacteria and is reported to activate the innate immune system. We previously reported that purified LTA obtained from *Enterococcus hirae* has no immunostimulating activity, but a subfraction (Eh-AF) in an LTA fraction possesses activity. In this study, we established a mouse monoclonal antibody neutralizing the activity of Eh-AF and investigated its inhibitory effects. Monoclonal antibody (MAbEh1) was established by the immunization of BALB/c mice with Eh-AF, followed by hybridoma screening based on its inhibitory effect for the production of interleukin-6 (IL-6) induced by Eh-AF. MAbEh1 neutralized the production of IL-6 by LTA fraction from not only *E. hirae* but also *Staphylococcus aureus*, while it failed to block that of lipopolysaccharide, suggesting that the antibody recognized a common active structure(s) in LTA fractions. Synthetic glycolipids in these LTAs did not induce cytokine production, at least in our system. Interestingly, the antibody was found to inhibit the activity of immunostimulating synthetic lipopeptides, Pam_3CSK_4 and FSL-1. These results suggest that MAbEh1 neutralizes the activity of lipoprotein-like compounds which is responsible for the activity of the LTA fraction of *E. hirae* and *S. aureus*.

Lipoteichoic acid (LTA) is a macroamphiphile distributing on the cell surfaces of gram-positive bacteria and is reported to exhibit immunostimulatory and inflammatory activities. LTA has been shown to have an antitumor effect (34, 36) and to induce inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and IL-6 (3, 31, 33). Recent research showed that such immunostimulatory activities of bacterial compounds were mediated by Toll-like receptor (TLR), a type I transmembrane receptor for innate immune activation (32). To date, more than 10 members of the TLR family have been discovered and most of their ligands were identified: TLR4 in combination with the adapter molecule MD-2 for lipopolysaccharide (LPS)/lipid A, an outer membrane component of gram-negative bacteria (21, 24); TLR9 for unmethylated CpG DNA (15); TLR3 and TLR7/8 for double- and single-stranded RNA (1, 14); TLR5 for bacterial flagellin (13); and TLR2 subfamily (TLR1, −2, and −6) for bacterial lipoprotein/lipopeptide (29, 30). LTA was also reported to be a ligand of TLR2 (22).

The structures of LTAs have been well studied and proposed as a glycoconjugates generally composed of a glycolipid anchor part, such as β-kojibiosyldiacylglycerol for *Enterococcus hirae* and *Streptococcus pyogenes* and β-gentiobiosyldiacylglycerol for *Staphylococcus aureus*, and a 1,3-linked poly(glycerophosphate) substituted by sugars and D-alanine at position 2 of the glycerol (4). Previously, we attempted to determine a structure of the LTA responsible for these activities. Fukase et al. prepared chemically synthetic glycoconjugates having fundamental structures of LTA from *E. hirae* and *S. pyogenes* and their glycolipid anchor parts (5, 6). However, these synthetic compounds exhibited no immunostimulating activities (28), suggesting that the proposed structures are not responsible for the activities. Thus, we reinvestigated the activity of LTA and found that an LTA fraction extracted from *E. hirae* by using a hot phenol (PhOH)-water method was able to be separated into two subfractions, a small amount of cytokine-inducing active fraction and an inactive major compound (27). Further, we determined that the structure of the inactive compound was identical to that of LTA (8). Those results suggested that the contaminating minor components in LTA fraction were responsible for the immunostimulation.

Recently, a structure-function relationship of LTA from *Staphylococcus aureus* has been reported. Morath et al. prepared a purified LTA by using a butanol (BuOH)-water extraction, followed by hydrophobic interaction chromatography, and showed that the LTA itself induces cytokine production (19). Further, those researchers synthesized an LTA counter-

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part and its glycolipid part and found that the counterpart displayed activity similar to that of natural LTA and even glycolipid possesses weak but distinct activity (20). From their observations, the researchers concluded that LTA itself was a potent immunostimulatory component in *S. aureus*. However, their conclusion for *S. aureus* disagreed with our data for *E. hirae*. One explanation for the contradictory results is an effect of the differences in the LTA structure, e.g., kojibiosyl is the backbone for glycolipid anchor in *E. hirae* LTA, whereas gentiobiose is the backbone in *S. aureus*, and partially alanylated oligoglucosyl is the substituent on glycerol at position 2 for *E. hirae*, but D-alanyl and glucosaminyl substitutions are made in *S. aureus*. This explanation may be supported by another report which showed that LTA exhibited from *Streptococcus pneumoniae* is 100-fold less potent than staphylococcal LTA (7). Pneumococcal LTA has been reported to be composed of a phosphocholine (PC)-linked tetraglycosylribitolphosphate polymer and a triglycosyldiacylglycerol anchor (2). Differences in extraction methods may be another possibility. Morath et al. also mentioned the critical role of D-alanine content in an LTA molecule from *S. aureus* (19), reporting that alkaline hydrolysis of the active LTA resulted in a loss of alanine substituent in LTA and reduced its activity. PhOH extraction of bacterial cells also decreased alanine, but BuOH extraction prevented alanine cleavage.

These interpretations might explain the inactivity of *E. hirae* LTA but do not clarify our minor active components. Therefore, we intended to reevaluate a principal compound responsible for the activity in *E. hirae*. In the present study, we established a mouse monoclonal antibody that neutralizes the activity of an LTA fraction from *E. hirae* and investigated its inhibitory effects for various bacterial stimuli.

MATERIALS AND METHODS

Bacterial compounds. *Enterococcus hirae* ATCC 9790 and *Staphylococcus aureus* DSM 20231 organisms were grown as previously described (27). The extraction of crude LTA fractions was performed using the BuOH-water method (19). The crude fractions were treated with DNase and RNase to digest contaminating nucleic acids and then subjected to hydrophobic interaction chromatography on octyl-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden), with a batchwise elution using stepwise 1-propanol concentrations (15, 40, and 60%) as described previously (8). Since LTA was eluted mainly with the 40% 1-propanol fractions, these fractions were used as LTA fractions and designated Eh-Bu (for *E. hirae*) and Sa-Bu (for *S. aureus*). The immunostimulatory active fractions HGL-A, HGL-B1, and HGL-B2, previously prepared from the *E. hirae* LTA fraction (9), were combined, and the resulting fraction (designated Eh-AF) was used for immunization. Glycolipid anchors of LTA, β-kosibiosyldipalmytoylglycerol for *E. hirae* (5) and β-gentiobiosyldipalmytoylglycerol for *S. aureus* (data not shown), were synthesized. LPS from *Escherichia coli* O:111 was obtained from Sigma-Aldrich (St. Louis, MO) and subjected to phenol reextraction by using sodium deoxycholate (16). PC, phosphatidylethanolamine, and phosphatidylinositol were also obtained from Sigma-Aldrich. Synthetic lipopeptides, Pam_3CSK_4, O,O′-diacyl-type Pam_2CSK_4, FSL-1, *N*-monoacyl-type $PamCSK_4$, and deacyl-type $dhCSK_4$ were purchased from EMC Microcollections (Tübingen, Germany). Monoclonal antibody for LTA was purchased from Biogenesis (Oxford, United Kingdom).

Establishment of MAbEh1. A monoclonal antibody, MAbEh1, was established according to standard methods. Briefly, BALB/c mice were immunized with Eh-AF (0.25 mg/mouse) with Freund's complete adjuvant (Becton Dickinson, Franklin Lakes, NJ) on days 0 and 21 and spleen cells obtained on day 24 were fused with SP2/0-Ag14 myeloma cells. The hybridoma cells were cultured in hypoxanthine-aminopterin-thymidine medium and subcloned by limiting dilution. Hybridoma-secreting antibody neutralizing Eh-AF activity was screened on the basis of the inhibitory effect against the production of IL-6 in THP-1 cells stimulated with 300 ng/ml Eh-AF, and an antibody was designated MAbEh1. The hybridoma was cultured in CD hybridoma medium (Invitrogen, Carlsbad, CA), and the culture supernatant was used for the antibody stock solution. The stock solution was then subjected to gel filtration chromatography on Bio-Gel A5m (Bio-Rad, Hercules, CA) to give purified antibody. Isotyping of the antibody was performed with a mouse monoclonal isotyping kit (Serotec, Oxford, United Kingdom). Isotype control antibody was purchased from e-Bioscience (San Diego, CA).

Cytokine assays. Human monocytic leukemia cell line THP-1 was obtained from the Health Science Research Resources Bank (Osaka, Japan) and cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (FBS; MBL, Nagoya, Japan), 50 μg/ml penicillin, and 50 μg/ml streptomycin. THP-1 was differentiated with 10^{-7} M 1,25-dihydroxyvitamin D_3 for 3 days before use. Human peripheral blood mononuclear cells (PBMCs) were obtained from heparinized human peripheral blood collected from a healthy volunteer by density gradient centrifugation using Histopaque-1077 (Sigma).

The cells were plated onto 96-well microplates at 1×10^5 cells in 100 μl of RPMI 1640 with or without 10% FBS and stimulated with the indicated dose of the test specimens in the presence or absence of MAbEh1 for 24 h. Culture supernatants were collected and analyzed by using an enzyme-linked immunosorbent assay (ELISA) kit for secreted IL-6 (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction. The concentration of secreted IL-6 from cells was determined using a standard curve of recombinant IL-6 prepared in each assay and presented as the means ± standard deviations (SD). Inhibitory effects of FBS and MAbEh1 were statistically evaluated by using Welch's *t* test.

Luciferase assays. Ba/F3 cells stably expressing p55IgκLuc, an NF-κB/DNA binding activity-dependent luciferase reporter construct (Ba/κB), murine TLR2 and the p55IgκLuc reporter construct (Ba/mTLR2), and murine TLR4/MD-2 and the p55IgκLuc reporter construct (Ba/mTLR4/mMD-2) were kindly provided by K. Miyake (Institute of Medical Science, University of Tokyo, Tokyo, Japan). NF-κB-dependent luciferase activity in these cells was determined as follows. Cells were inoculated onto each well of a 96-well, flat-bottomed plate at 1×10^5 cells in 80 μl of RPMI 1640 supplemented with 10% FBS and stimulated with the indicated concentrations of the test specimens. After 4 h of incubation at 37°C in humidified air containing 5% CO_2, 80 μl of Bright-Glo luciferase assay reagent (Promega, Madison WI) was added to each well and luminescence was quantified with a luminometer ARVO SX multilabel counter (Perkin Elmer, Wellesley, MA). Results are shown as relative luciferase activity, which was the ratio of stimulated activity to nonstimulated activity in each cell line.

Immune blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was performed by the Tris-glycine method using a mini-PAGE chamber AE-6530 and an AE-8450 power supply (ATTO, Tokyo, Japan) with a 15% gel. Materials in the gels were transferred to a nitrocellulose membrane (Bio-Rad) by using a semidry blotter AE-6677 (ATTO). For dot blot analysis, stimulus solution was placed on a nitrocellulose membrane and dried in the air. The membranes were incubated with blocking solution (3% nonfat milk in Tris-buffered saline containing 0.05% Tween 20) for 12 h at 4°C and then with 1/100 of MAbEh1 diluted in the diluent (1% nonfat milk in Tris-buffered saline containing 0.05% Tween 20) for 2 h at room temperature. The antibody was detected by incubation in peroxidase-labeled second antibody (KPL, Gaithersburg, MD; 1/2,000 in the diluent) for 2 h, followed by development using ECL (Amersham Bioscience). Luminescence was recorded with a LAS-1000 luminescence analyzer (Fuji Film, Kanagawa, Japan).

RESULTS

Preparation of LTA fractions. We previously separated small amounts of immunobiologically active fractions from *E. hirae* LTA fractions prepared by hot PhOH-water extraction (27). However, the structural elucidation of an essential compound(s) responsible for the activity was incomplete because of the difficulty of further purification based on its small amount. Recently, Morath et al. reported that an LTA fraction obtained from *S. aureus* by using BuOH-water extraction, followed by hydrophobic interaction chromatography, exhibited higher activity than that obtained by the PhOH method (19). Thus, we prepared LTA fractions by the BuOH method. *E. hirae* and *S. aureus* bacteria were subjected to BuOH extraction to give crude LTA fractions in yields of 1.5 to 2.1% and 1.6 to 2.6%, respectively. The crude extracts were digested with nu-

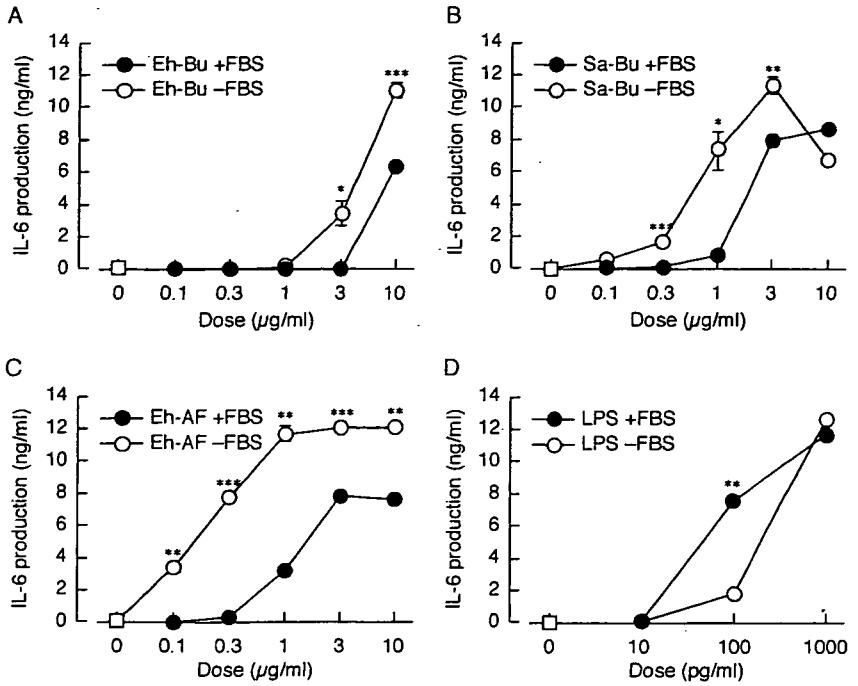

FIG. 1. IL-6 production in human peripheral blood mononuclear cells induced by (A) Eh-Bu, (B) Sa-Bu, (C) Eh-AF, or (D) LPS in the presence or absence of 10% FBS. Cells were stimulated with the indicated doses of stimuli for 24 h, and IL-6 production was determined by ELISA. The results represent the mean values (± SD [error bars]) obtained from three independent experiments. *P* values against stimuli without FBS are indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

clease and then subjected to hydrophobic interaction chromatography to obtain LTA fractions Eh-Bu (15 to 28%, yield from crude LTA fraction) and Sa-Bu (12 to 27%). Both fractions induced IL-6 production in PBMCs (Fig. 1). We previously demonstrated that IL-6 production in THP-1 cells stimulated with the active fraction was suppressed in the presence of FBS (9). Thus, the effect of serum was investigated, and the activities of Eh-Bu and Sa-Bu were found to decrease in the presence of FBS in a manner similar to that with Eh-AF, an active fraction previously prepared from *E. hirae* LTA fraction obtained by the PhOH method (Fig. 1). All of the fractions activated Ba/mTLR2 cells, but Ba/mTLR4/mMD-2 and negative control Ba/κB were not activated significantly (Fig. 2), indicating no endotoxin contamination.

Establishment of MAbEh1. We constructed an antibody that neutralized the activity of the fractions to evaluate a principal compound responsible for the activity. Mouse hybridoma cells were established by the immunization of BALB/c mice with Eh-AF. A hybridoma-secreting monoclonal antibody was screened for a neutralizing effect against the IL-6-inducing activity of Eh-AF in THP-1 cells. One hybridoma was found to secrete a neutralizing antibody named MAbEh1. The culture supernatant of the hybridoma cells in serum-free medium was subjected to gel filtration chromatography, and a fraction containing antibody was used for the solution of MAbEh1 (1.06 mg protein/ml). The isotype of the antibody was immunoglobulin M (IgM). MAbEh1 suppressed the activity of up to 1 μg/ml of Eh-AF dose dependently (Fig. 3). The antibody, in contrast, showed no inhibitory effect on the activity of LPS (Fig. 3). These results showed that MAbEh1 specifically suppresses the activity of the components in Eh-AF.

Neutralizing effects of MAbEh1. We next investigated the neutralizing effect of MAbEh1. The antibody also inhibited the activity of Eh-Bu and Sa-Bu (Fig. 4). These results suggest that structures of active components in Eh-Bu and Sa-Bu are common ones in LTA fractions and are similar to those in Eh-AF. Morath et al. reported that the glycolipid anchor in *S. aureus* LTA induced the production of TNF-α in human whole blood (19). Thus, we investigated the inhibitory effect on synthetic glycolipid anchors of *E. hirae* and *S. aureus*. However, in our assay system, neither glycolipid stimulated IL-6 production in human PBMCs (Fig. 5A and B). We recently showed that lipoproteins are predominant TLR2-activating ligands in *S. aureus* cell wall components (11). Thus, the inhibitory effects of MAbEh1 on the synthetic lipopeptides Pam_3CSK_4 and FSL-1 were studied. The activities of both synthetic counterparts were suppressed by the addition of the antibody dose dependently (Fig. 6A). The activities were also decreased in the presence of serum in a manner similar to those of Eh-AF, Eh-Bu, and Sa-Bu (Fig. 6B and C). Further, MAbEh1 bound lipopeptides Pam_3CSK_4, Pam_2CSK_4, $PamCSK_4$, and FSL-1 in dot blot analysis, but not other lipids (Fig. 7A). The antibody for LTA also failed to recognize these lipopeptides (Fig. 7B). These results suggested that MAbEh1 inhibits the activity of a compound containing lipopeptide structure.

DISCUSSION

LPS is a potent immunostimulatory compound in gram-negative bacteria. Although LPS is known to activate cells through TLR4, LPSs from some bacterial species have been reported to exhibit activity via TLR2 in addition to TLR4 (17,

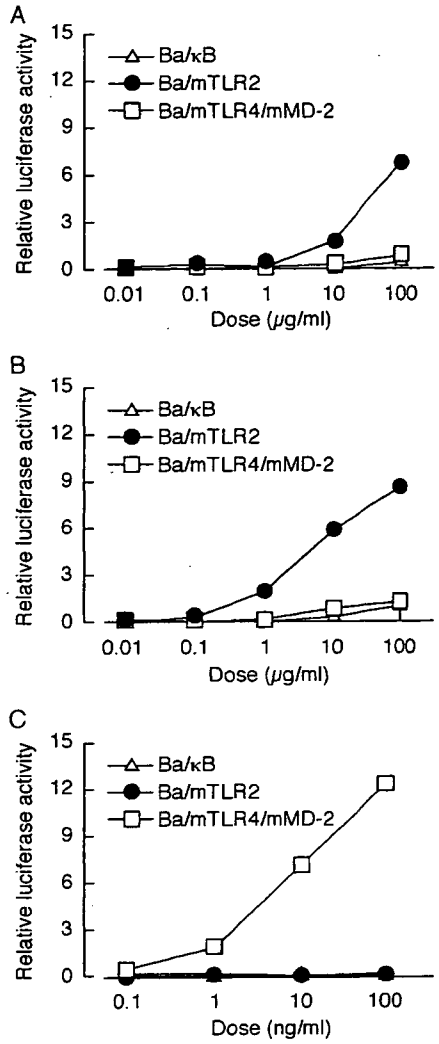

FIG. 2. NF-κB activation in Ba/κB, Ba/mTLR2, or Ba/mTLR4/mMD-2 cells induced by Eh-Bu (A), Sa-Bu (B), and LPS (C). Cells were incubated with the indicated doses of stimuli for 4 h. NF-κB activation was measured with a luciferase assay. Results are shown as relative luciferase activity, which was determined as the ratio of stimulated to nonstimulated activity.

25, 35). Recent research proved that some of the TLR2-activating components were contaminated with small amounts of lipoproteins (10, 18). Lipoproteins are usually extracted from bacterial cells by surfactants such as Triton X-114 (23). LPS, which consists of a long hydrophilic polysaccharide and a hydrophobic lipid A anchor, may act as a surfactant. Since LTA is macroamphiphile, LTA may also work as a surfactant to extract lipoproteins from bacterial cells. In fact, we previously demonstrated that the activity of an LTA fraction, a BuOH extract, of *S. aureus* was not abrogated by hydrofluoric acid (HF) hydrolysis but by the following treatment with lipoprotein lipase, which cleaved acyl groups essential for the activity of lipoprotein and reduced the activity, indicating a possibility of the existence of lipoprotein in the LTA fraction (11). This indicated that lipoprotein but not the LTA molecule is responsible for the activity of LTA fractions. In the present study, we further confirmed the evidence of lipoprotein contamination in

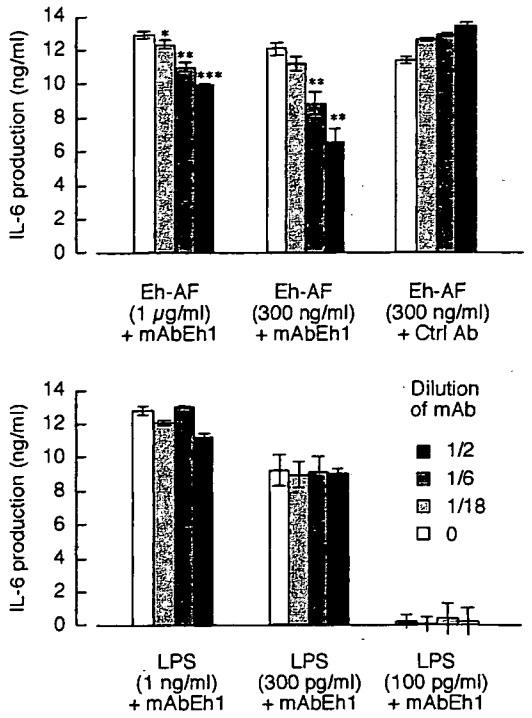

FIG. 3. Inhibitory effects of MAbEh1 or an isotype control antibody on IL-6 production in human peripheral blood mononuclear cells stimulated with Eh-AF or LPS. Cells were stimulated with the indicated doses of stimuli and antibody for 24 h in the absence of FBS, and IL-6 production was determined by ELISA. The results represent the mean values (± SD [error bars]) obtained from three independent experiments. *P* values against stimuli without antibody are indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

LTA fractions of *E. hirae* in addition to those of *S. aureus*. MAbEh1, which inhibited the immunostimulating activity of lipopeptides Pam_3CSK_4 and FSL-1, decreased the activity of the LTA fractions. This showed that lipoproteins were responsible for the activity of minor compounds previously separated from the LTA fraction of *E. hirae* (27).

We also characterized the binding affinity of MAbEh1. Dot blot analysis showed that the antibody binds to the lipopeptides triacylated Pam_3CSK_4, diacylated Pam_2CSK_4 and FSL-1, and

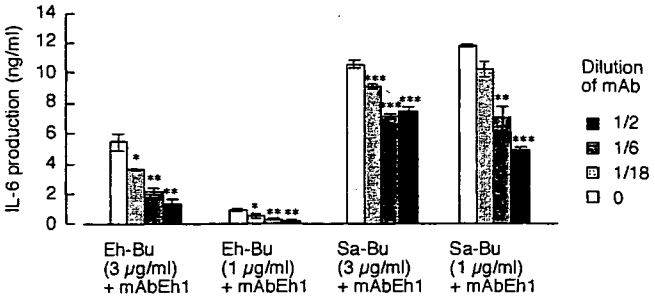

FIG. 4. Inhibitory effects of MAbEh1 on IL-6 production in human peripheral blood mononuclear cells stimulated with Eh-Bu or Sa-Bu. Cells were stimulated with the indicated doses of stimuli and antibody for 24 h in the absence of FBS, and IL-6 production was determined by ELISA. The results represent the mean values (± SD [error bars]) obtained from three independent experiments. *P* values against stimuli without antibody are indicated. *, $P < 0.05$; **, $P < 0.01$; or ***, $P < 0.001$.

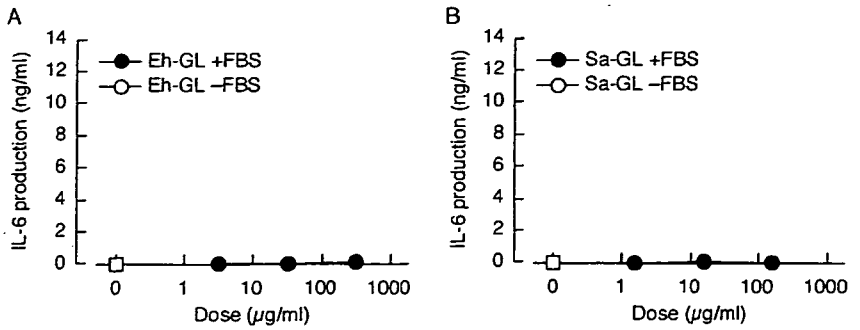

FIG. 5. IL-6 production in human peripheral blood mononuclear cells stimulated with (A) Eh-GL or (B) Sa-GL in the presence or absence of 10% FBS. Cells were stimulated with the indicated doses of stimuli for 24 h, and IL-6 production was determined by ELISA. The results are presented as the means ± SD.

monoacylated $PamCSK_4$ but not to deacylated $dhCSK_4$ (Fig. 7A). The antibody did not bind to LPS or diacylglyceryl lipids PC, phosphatidylethanolamine, and phosphatidylinositol (Fig. 7A). The lipopeptides were not visualized by an LTA antibody which bound to Eh-Bu (Fig. 7B). These results indicated that MAbEh1 recognized the N-terminal lipid moiety of lipopeptide. Unfortunately, both dot blot and Western blotting analysis of the lipoteichoic acid fraction using MAbEh1 failed to visualize any compound (data not shown), although the contamination of lipoprotein in the fraction was expected by the inhibition assay. This might be caused by its low concentration in the fraction as suggested in our previous work (11, 27) and/or low affinity of IgM antibody. In contrast to the specific binding, the inhibitory effect of MAbEh1 against not only lipoteichoic fraction but also synthetic lipopeptides was only partial (Fig. 3, 4, and 6). One interpretation for the partial effect may be the low affinity of IgM. We also assumed another possibility, which was that the inaccessibility of antibody to the N-terminal recognition center of lipoprotein was due to the incorporation into LTA micelles. Our previous observation, that lipoprotein lipase digestion of lipoproteins existing in the *S. aureus* LTA fraction (11) or the *Porphyromonas gingivalis* LPS fraction (10; our unpublished data) is unsuccessful, supported our second assumption.

Previously, we determined that LTA from *E. hirae* was inactive for the innate immune system (8). We also investigated the effect of HF degradation of the LTA fraction derived from *S. aureus* (11). Since HF cleaves the phosphodiester bonds in

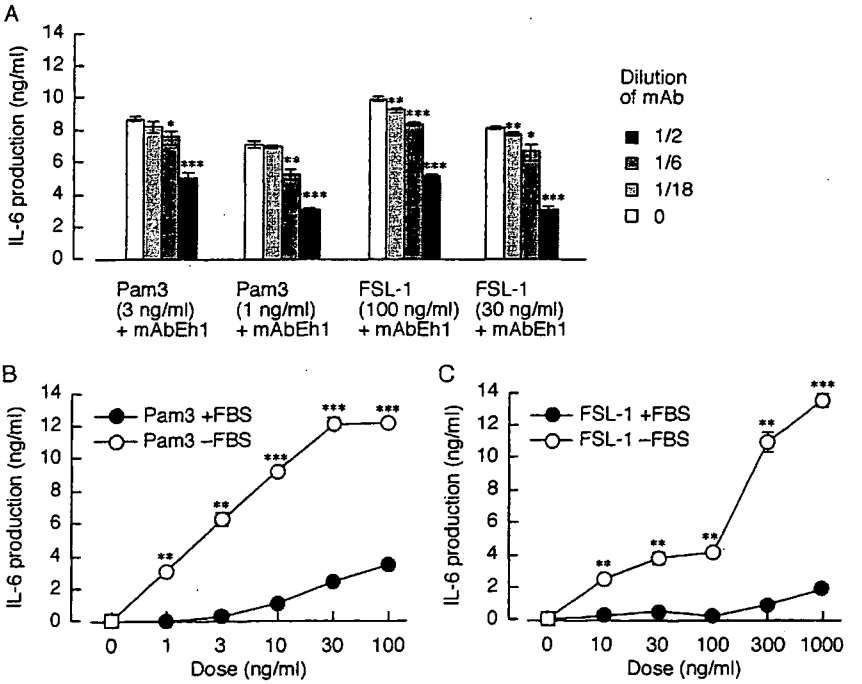

FIG. 6. (A) Inhibitory effects of MAbEh1 on IL-6 production in human peripheral blood mononuclear cells stimulated with synthetic lipopeptides Pam_3CSK_4 (Pam3) or FSL-1. Cells were stimulated with the indicated doses of stimuli and antibody for 24 h in the absence of FBS. (B and C) IL-6 production in human peripheral blood mononuclear cells stimulated with (B) Pam_3CSK_4 or (C) FSL-1 in the presence or absence of 10% FBS. IL-6 production was determined by ELISA. The results represent the mean values (± SD [error bars]) obtained from three independent experiments. *P* values against stimuli without antibody or FBS are indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

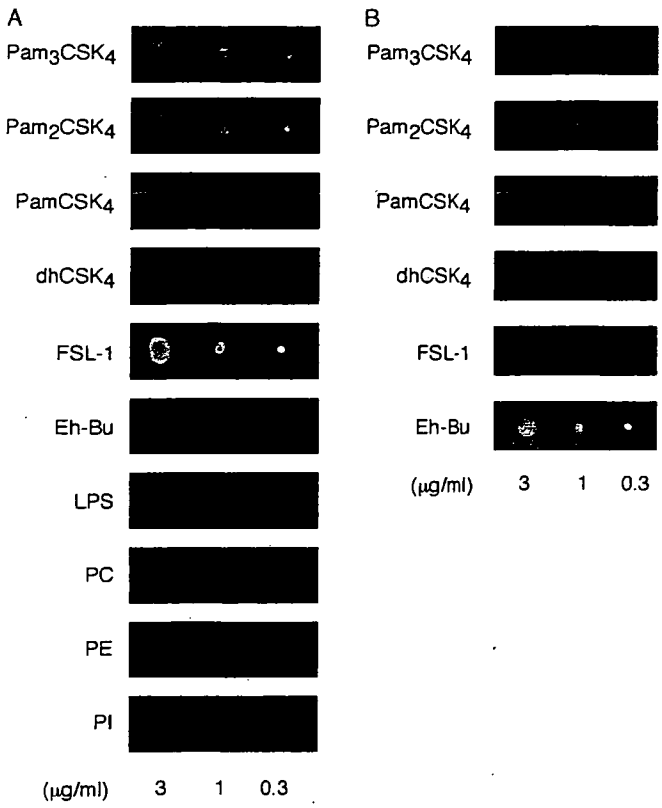

FIG. 7. Dot blot analysis against lipopeptide derivatives and other lipids with (A) MAbEh1 or (B) antibody for LTA. The indicated doses of stimuli were blotted onto a nitrocellulose membrane. The membranes were blocked with nonfat milk and incubated with MAbEh1. The bound antibody was detected with peroxidase-labeled second antibody using ECL reagents.

polyglycerophosphate, a hydrophilic part of LTA, most of the molecular mass of LTA is decomposed into small components, such as phosphate, glycerol, and phosphoglycerol (4). After HF degradation, no Alcian blue-stained band was found in the sodium dodecyl sulfate-PAGE gel, showing the complete decomposition of LTA. The treatment, however, did not abrogate the activity of the LTA fraction. Further, we showed that glycolipid parts of LTA for *E. hirae* and *S. aureus* were both inactive (Fig. 5). These results suggest that LTA itself was not an active molecule. However, we have not confirmed that natural LTA from *S. aureus* was immunobiologically inactive since a selective deletion of lipoprotein was not achieved. It was reported that *S. aureus* LTA was not separated into active and inactive fractions by the hydrophobic interaction and anion-exchange chromatographies which were used for the separation of *E. hirae* LTA (20). Direct lipoprotein lipase digestion of the LTA fraction was not successful (11), probably because contaminated lipoproteins may be incorporated into LTA micelles and the enzyme was not able to approach them. The reextraction of the natural LTA from *S. aureus* with PhOH containing deoxycholate, which was used for the extraction of contaminated lipoprotein from LPS (16), was also unsuccessful (data not shown). Since the reextraction method was also ineffective in some cases, such as for the extraction of lipoprotein from *Porphyromonas gingivalis* LPS (10, 16), it may be considered that the micellation of lipoprotein with LTA is very tight. Recently, we demonstrated that LTA from a lipoprotein diacylglycerol transferase deletion mutant of *S. aureus*, which contains no detectable lipoproteins (26), is 100-fold less active than that from the wild type (12). This result indicated that most of the activity of LTA fraction appears to be caused by lipoproteins. The identification of active lipoprotein species and the determination of chemical structure of compounds responsible for the residual activity in mutant LTA fraction are required for further understanding of biological activity of LTA molecule.

In conclusion, we established a monoclonal antibody that neutralizes the activity of natural LTA and demonstrated that the monoclonal antibody also blocked the activity of lipopeptides. These results strongly suggest that MAbEh1 neutralizes the activity of lipoprotein-like compounds existing in the natural LTA fraction from *E. hirae* and *S. aureus*.

ACKNOWLEDGMENTS

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Signals from intra-abdominal fat modulate insulin and leptin sensitivity through different mechanisms: Neuronal involvement in food-intake regulation

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Summary

Intra-abdominal fat accumulation is involved in development of the metabolic syndrome, which is associated with insulin and leptin resistance. We show here that ectopic expression of very low levels of uncoupling protein 1 (UCP1) in epididymal fat (Epi) reverses both insulin and leptin resistance. UCP1 expression in Epi improved glucose tolerance and decreased food intake in both diet-induced and genetically obese mouse models. In contrast, UCP1 expression in Epi of leptin-receptor mutant mice did not alter food intake, though it significantly decreased blood glucose and insulin levels. Thus, hypophagia induction requires a leptin signal, while the improved insulin sensitivity appears to be leptin independent. In wild-type mice, local-nerve dissection in the epididymis or pharmacological afferent blockade blunted the decrease in food intake, suggesting that afferent-nerve signals from intra-abdominal fat tissue regulate food intake by modulating hypothalamic leptin sensitivity. These novel signals are potential therapeutic targets for the metabolic syndrome.

Introduction

The explosive increase in obesity has become a major public health concern in most industrialized countries (Flier, 2004; Friedman, 2003). Insulin resistance is a fundamental contributor to the metabolic syndrome associated with type 2 diabetes, hypertension, hyperlipidemia, and atherosclerosis. Major advancements in this field include the discoveries of adipocyte-derived humoral factors, such as leptin (Friedman and Halaas, 1998). Leptin conveys energy-storage information from adipose tissue to the central nervous system, leading to food-intake suppression. However, in patients with ordinary obesity, serum leptin levels are increased in proportion to body fat (Considine et al., 1996), but the responses to leptin are impaired (Heymsfield et al., 1999), which defines a state of leptin resistance. Leptin resistance also contributes to the development of obesity and obesity-related metabolic disorders.

Fat accumulation in intra-abdominal fat tissue is involved in development of the metabolic syndrome (Bjorntorp, 1992; Matsuzawa et al., 1995) associated with insulin and leptin resistance (Friedman, 2003). Therefore, in this study, to examine whether the metabolic changes in intra-abdominal fat tissue affect insulin and leptin resistance as well as systemic glucose metabolism, we attempted to express uncoupling protein 1 (UCP1), which functions to dissipate energy as heat (Klingenberg and Huang, 1999), in epididymal fat tissue (Epi) in mice with obesity and diabetes.

Results and discussion

C57BL/6 mice were subjected to direct injection of the UCP1 adenovirus vector into Epi (UCP1 mice) after the development of diabetes associated with obesity in response to high-fat chow preloading for 4 weeks. Mice given the LacZ adenovirus were used as controls (LacZ mice). Immunoblotting detected adenovirus-mediated UCP1 expression in Epi (see Figure S1A in the Supplemental Data available with this article online), and this expression was restricted to Epi (Fig. S1A). UCP1 expression in Epi was detectable on the first day after adenoviral injection and was increased on day 3 but had fallen to very low levels by day 7 (Figure S1B). However, expression levels were far below those of endogenous protein in BAT: on day 3, approximately 5% per unit weight protein (Figure S1B). UCP1 expression was restricted to very limited portions of the tissue (left panel of Figure 1B). Judging from the intensity of immunostaining, UCP1 expression levels in UCP1-expressing white adipocytes did not reach those in brown adipocytes (right panel of Figure 1B). UCP1-expressing adipocytes were significantly smaller than UCP1-nonexpressing adipocytes in the same tissue (Figure 1C), suggesting enhanced metabolism in the former.

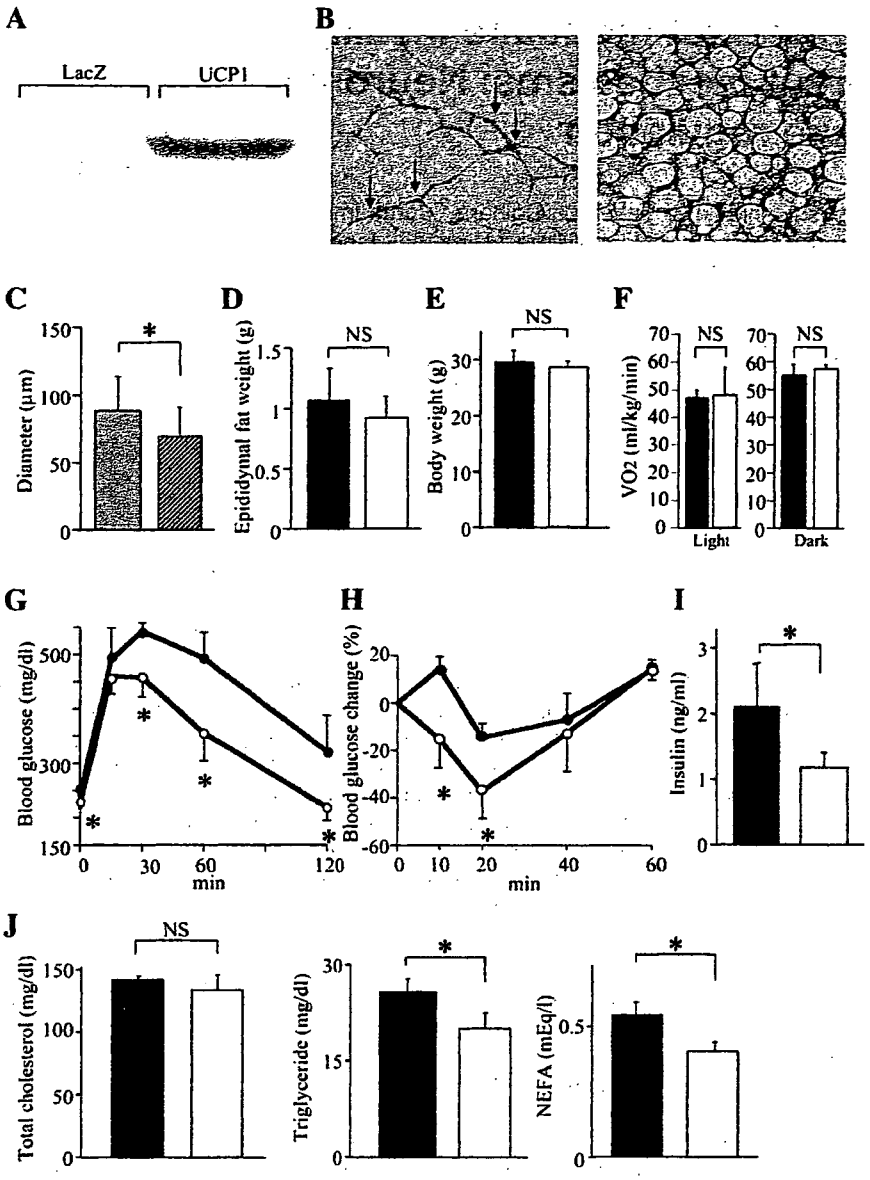

Figure 1. UCP1 expression in Epi improved glucose tolerance and insulin sensitivity

A) Immunoblotting, with anti-UCP1 antibody, of Epi extracts from LacZ and UCP1 mice on day 3 after adenoviral administration.

B) Immunohistochemistry, with anti-UCP1 antibody, of Epi (left panel) and BAT (right panel) sections from a UCP1 mouse on day 3 after adenoviral administration. These two samples were immunostained under the same conditions.

C) Diameters of UCP1-nonexpressing (gray bar) and UCP1-expressing (hatched bar) adipocytes in Epi from UCP1 mice on day 3 after adenoviral administration.

D–J) Epididymal fat weights (D), body weights (E), resting oxygen consumption during light and dark phase (F), and metabolic parameters (G–J) of LacZ mice (black bars) and UCP1 mice (white bars) on day 3 after adenoviral administration. Glucose-tolerance (G) and insulin-tolerance tests (H) were performed on day 3. Data in (H) are expressed as percentages of the blood glucose levels immediately before intraperitoneal insulin loading. Serum insulin levels (I) and serum lipid parameters (J); left: total cholesterol, middle: triglyceride, right: free fatty acids) were measured after a 10 hr fast (n = 6 per group). Data are presented as means ± SD (n = 6 per group). *$p < 0.05$ by unpaired t test.

We further confirmed enhanced metabolism by adenoviral UCP1 expression using 3T3-L1 adipocytes. UCP1 expression decreased intracellular ATP concentrations (Figure S1C) and increased levels of peroxisome proliferator-activated receptor γ coactivator (PGC) 1α and cytochrome c expression (Figure S1D). Thus, exogenous UCP1 was functionally active, resulting in increased mitochondrial biosynthesis in adipocytes.

However, neither total Epi weights nor body weights differed between LacZ and UCP1 mice on day 3 after adenoviral administration (Figures 1D and 1E). Oxygen consumption was not affected by UCP1 expression in Epi during either the light or the dark phase (Figure 1F), also reflecting the very limited UCP1 expression. Therefore, to avoid the secondary effects of body-weight change, we analyzed metabolic parameters on day 3. To our surprise, however, even very limited UCP1 expression in Epi resulted in marked changes in metabolic phenotype.

Glucose- and insulin-tolerance tests indicated marked improvements in glucose tolerance and insulin sensitivity (Figures 1G and 1H). Fasting blood glucose (Figure 1G) and insulin (Figure 1I) levels were significantly lower in UCP1 mice, further confirming improved insulin sensitivity. In addition, serum lipid parameters, including triglycerides and free fatty acids (Figure 1J), were also improved with UCP1 expression in Epi. Thus, limited regional expression of UCP1 in Epi markedly improved systemic insulin resistance, resulting in improvement of diabetes and dyslipidemia.

Next, we measured serum adipocytokine levels (Figure 2A). Adiponectin and tumor necrosis factor α levels were not significantly altered. In contrast, serum leptin was markedly decreased, by 46%, with UCP1 expression in Epi. Although intra-abdominal fat-tissue weights were unaltered or only very slightly decreased in UCP1 mice (Figure 1D and Figure S1E),