マスト細胞へのガレクチン９の作用

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目　　次

第１章　緒言　１頁

第２章　マウス骨髄由来培養マスト細胞へのガレクチン９の作用

第１節　背景　３頁

第２節　材料と方法　５頁

第３節　結果　８頁

第４節　考察　10頁

第５節　小括　13頁

第３章　ヒトマスト細胞株へのガレクチン９の作用

第１節　背景　14頁

第２節　材料と方法　16頁

第３節　結果　21頁

第４節　考察　24頁

第５節　小括　26頁

第４章　総括　27頁

謝辞　28頁

引用文献　29頁

図表　32頁

第 1 章　緒言

ガレクチン 9（Gal-9）は T 細胞の細胞上清より好酸球走化因子として初めて同定され[1-3]、その後消化管上皮やマクロファージ、好酸球、マスト細胞、線維芽細胞、滑膜細胞といった多様な細胞に発現していることが報告されている[4-7]。

Gal-9 は細胞凝集、接着、アポトーシス、生存、分化の誘導といった様々な機能を有する。これらの機能は Gal-9 が T-cell immunoglobulin and mucin containing-protein 3 (TIM-3)に結合することによって発揮される[5, 8, 9]（図 1）。TIM-3 も Helper T cell type 1 (Th1)細胞[9]、Cytotoxic T- lymphocyte (Tc1) 細胞[10]、Helper T cell type 17 (Th17)細胞[11]、Natural killer (NK)細胞[12]、Natural killer T (NKT)細胞[13, 14]、樹状細胞（dendritic cells , DC）[15]、マスト細胞[16, 17]といった多様な細胞に発現している。

Gal-9 は NK 細胞[18]と DC の成熟化による細胞傷害性 T 細胞の活性化により[19]、抗腫瘍活性を示す。さらに Gal-9 はメラノーマと乳癌細胞の凝集を誘導し、転移を抑制する[20, 21]。Gal-9 はマウスにおける実験的自己免疫性脳脊髄炎（experimental autoimmune encephalomyelitis 、EAE）とコラーゲン誘導関節炎（collagen-induced arthritis 、CIA）の症状を負に制御することも報告されている[9, 22]。抗 TIM-3 モノクローナル抗体（mAb）と同様、Gal-9 は TIM-3 を介して Th1 細胞のアポトーシスを誘導し、EAE の進展を抑制する[9]。また Gal-9 は Th17

細胞の分化を阻害し、制御性 T 細胞の分化を促進することで CIA の進展も抑制する[22]。

さらに Gal-9 と TIM-3 は齧歯類のアレルギー性気道炎症モデルにおける肺組織で発現が上昇しており[23-25]、気道炎症の誘導への関与が示唆されている。実際、Gal-9 を前投与したマウスでは、オボアルブミンとチリダニで誘導した気道炎症と過敏性が抑制されていることが報告されている[26]。

マスト細胞はウイルスや細菌などの病原体や花粉、ダニなどの外来抗原に暴露されている気道粘膜や皮膚などに多く存在し、IgE を介した脱顆粒やサイトカイン産生によりアレルギー炎症に関わり、その制御はアレルギー疾患の制御につながる。また自然免疫系を介した感染防御にもサイトカイン産生により関わっていることが近年明らかとなってきている[27]。

マスト細胞における Gal-9 の作用について、Gal-9 は IgE と抗原の複合体の形成を阻害することで、マスト細胞の細胞株の脱顆粒を抑制するという報告がある[28]。しかし Gal-9 によるマスト細胞からのサイトカイン産生や生存を検討した報告はなく、またヒトマスト細胞での作用を検討した報告もない。

そこで今回、マウス骨髄由来培養マスト細胞（bone marrow cell-derived cultured mast cells 、BMCMCs）（第 2 章）およびヒトマスト細胞株、HMC-1（第 3 章）を用いてサイトカイン産生や生存を含めた Gal-9 の作用について検討した。

第 2 章　マウス骨髄由来培養マスト細胞へのガレクチン 9 の作用

第 1 節　背景

Gal-9 は多様な細胞に発現し、細胞凝集、接着、アポトーシス、生存、分化の誘導といった様々な機能を有していることが報告されている[4-7]（図 1）。

マスト細胞における Gal-9 の作用について、Gal-9 は IgE と抗原の複合体の形成を阻害することで、マスト細胞の細胞株の脱顆粒を抑制するという報告がある[28]。しかし Gal-9 によるマスト細胞からのサイトカイン産生や生存を検討した報告はない。また抗 TIM-3 アゴニスト抗体（抗 TIM-3 ポリクローナル抗体）は BMCMCs のサイトカイン産生を促進するが、IgE を介した脱顆粒には影響を与えないことが報告されている[16]。Gal-9 は、はじめ TIM-3 のリガンドであると報告されたが[9]、その後 CD44[26]や IgE[28]といった TIM-3 以外の分子に結合して作用を発揮することが報告されており、Gal-9 によるマスト細胞からのサイトカイン産生や生存への作用が、抗 TIM-3 アゴニスト抗体と同じ結果となるかは不明である。

またマスト細胞は Toll like receptor（TLR）を介した刺激によって、炎症性サイトカインを産生し、自然免疫を介した宿主防御においても重要な役割を担っている[27]。しかし TLR を介したマスト細胞の活性化における Gal-9 の役割は不明である。

そこで今回、BMCMCsを用いてサイトカイン産生や生存を含めたGal-9の作用および自然免疫系を介した刺激によるマスト細胞の活性化におけるGal-9の役割について検討した。

第２節　材料と方法

細胞培養

マウス骨髄由来培養マスト細胞（BMCMCs）はC57BL/6マウス由来の骨髄細胞を10ng/ml recombinant mouse IL-3、10% fetal bovine serum （FBS）、0.1% 2-mercaptoethanol、100U/ml penicillin および100μg/ml streptomycin を添加した RPMI 1640 (Sigma Chemical Co, St. Louis, MO, USA) を用いて、5% CO_2 、37°Cの条件のもと6～12週間培養して誘導した。フローサイトメーターにて評価したマスト細胞（c-kit陽性、Fc ε RI陽性）98%以上のものを使用した。

ELISA法

BMCMCs (1x105 cells/well 、96 well plate使用)を1 μ g/ml anti-dinitrophenyl（DNP）IgE (SPE-7, Sigma Chemical Co, St. Louis, MO, USA)の存在下で37 °C 18 時間培養して IgE 感作し、次に図 2A に示した濃度の recombinant mouse galectin-9 (rmGal-9)(GalPhama Co. Ltd., Kagawa, Japan) で 1 時間前処置し、抗原として 20ng/ml の dinitrophenyl human serum albumin （DNP-HSA、Sigma Chemical Co, St. Louis, MO, USA) の存在下および非存在下で37 °C 18 時間培養した。

BMCMCs (1x10^5 cells/well 、96 well plate使用) を図2Bに示した濃度のrmGal-9で、100ng/ml サルモネラ由来 lipopolysaccharide (LPS) (Sigma Chemical Co, St.

Louis, MO, USA)の存在下および非存在下で37 °C 18 時間培養した。また40μg/ml のTIM-3 阻害抗体であるRMT3-23 (順天堂大学より供与)[16]および215015 (R&D Systems, Minneapolis, MN, USA)またはcontrol rat IgG 2a (eBioscience, San Diego, CA, USA) で37 °C 1時間、rmGal-9とLPS刺激前に培養した。

上清中のIL-6およびTNF-αはELISA kits (eBioscience, San Diego, CA, USA)を用いて製造会社の手順に従い測定した。

β-hexosaminidase release assay

BMCMCs を1μg/ml IgE (SPE-7) の存在下で37 °C 18 時間培養してIgE感作し、洗浄後、96well plate に1 x10^5 cells/wellで播種後、図3に示した濃度のrmGal-9 にて 37 °Cで1時間前処置し、その後100ng/ml DNP-HSA もしくは100ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co, St. Louis, MO, USA) と1 μg/ml ionomycin (Sigma Chemical Co.) を添加し37 °Cで30分刺激した後、細胞上清を回収した。細胞上清中のβ-hexosaminidase 活性の測定は先行文献を一部改変して行った[29]。簡潔に述べると、50 μlの検体を0.1M sodium citrate (pH 4.5)中の1.3 mg/ml p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Chemical Co.) 100μl に加えて96-wellプレートを用いて37 °C、1時間インキュベートした。その後、50 μl の0.4 M glycine (pH10.7)を各wellに添加して酵素反応を止めた。酵素活性(OD405)

はプレートリーダーを用いて測定した。 データについては、細胞中の総β-hexosaminidase 量に対する各条件下で放出されたβ-hexosaminidaseの割合をパーセント表示した。

細胞生存の評価

BMCMCs (4 x10^6 cells/ml)の細胞を10 ng/ml IL-3、2.5 μg/ml IgE (SPE-7)または1 μM rmGal-9 と100ng/ml LPSの存在下または非存在下 に37 °C で6日間培養し、経時的に評価した。その後 FITC-conjugated annexin-V とpropidium iodide (MEBCYTO-Apoptosis Kit; MBL Co. Ltd, Nagoya, Japan)にて染色し、propidium iodide陰性、Annexin-V陰性生存細胞の割合をFACSCaliburとそのソフトウェアCellQuest (BD, Dranklin Lakes, NJ, USA)を用いて測定した。

統計解析

全てのデータはmeans ± SDで表した. Unpaired Student's t-test（two-tailed）または ANOVAにて適宜、統計解析を行った。 $p < 0.05$ を統計学的有意とした。

第3節　結果

rmGal-9 の BMCMCs へのサイトカイン産生に対する作用

IgE は単独でマスト細胞からのサイトカイン産生を促すことが報告されている [30]。今回の検討でも IgE は単独でマスト細胞からのサイトカイン産生を有意に誘導することが確認された（IL-6、TNF-α ともに $p < 0.05$）。さらに rmGal-9 は IgE 存在下で BMCMCs からのサイトカイン産生（IL-6 および TNF-α）を 1μM で有意に促進することが、明らかとなった（図 2A)。一方で抗原による IgE の架橋下でのサイトカイン産生（IL-6 および TNF-α）は rmGal-9 非存在下と比較して、1μM の rmGal-9 で有意に抑制された（図 2A)。次に BMCMCs の IgE を介さない、自然免疫系を介したサイトカイン産生に対する作用を検討するため、LPS の存在下に rmGal-9 を添加した。rmGal-9 は LPS 共存下で BMCMCs からのサイトカイン産生を誘導することが示された（図 2B)。また TIM-3 阻害抗体は、rmGal-9 による LPS 共存下での BMCMCs からのサイトカイン産生誘導を阻害せず、このことから TIM-3 以外のリガンドの関与が示唆された（図 2C)。

rmGal-9 は **BMCMCs** おける脱顆粒を抑制する

rmGal-9 の BMCMCs における抗原による IgE の架橋による脱顆粒への効果を検討した。IgE で 18 時間感作した BMCMCs を rhGal-9 (0, 0.01, 0.1, 1 μM)にて 1

時間前処置し、抗原またはPMAとionomycinで刺激した。β-hexosaminidase release assayによって評価したBMCMCsからの脱顆粒は、1 μMの rmGal-9前処置にて有意に抑制されていた（図3）。以上より rmGal-9 はBMCMCsにおける抗原によるIgEの架橋による脱顆粒を阻害することが示された。

rmGal-9 の BMCMCs の生存に対する影響

次に rmGal-9 の BMCMCs のアポトーシスに対する作用について検討した。細胞を 10 ng/ml IL-3 または 1 μM rmGal-9 と 100ng/ml LPS の存在下または非存在下 に 6 日間培養し、経時的に評価した（1、2、4、6 日目）。生存細胞（annexin-V 陰性）の割合は、rmGal-9 と LPS の共存下で 2 日目に vehicle control、rmGal-9 単独刺激および LPS 単独刺激と比べて有意に減少していた（図 4）。以上から rmGal-9 はBMCMCs おいて、LPS の共存下でアポトーシスを増強することが示された。

第 4 節　考察

今回の検討でマウス Gal-9 はマウス骨髄由来培養マスト細胞（BMCMCs）に対し促進と制御、両面の機能を有することが示された。rmGal-9 は BMCMCs の生存と脱顆粒および抗原による IgE の架橋下でのサイトカイン産生を抑制したが、IgE または LPS 共存下でのサイトカイン産生は促進し、LPS 共存下でのサイトカイン産生誘導については、TIM-3 以外の分子の関与が示唆された。

今回の検討で、rmGal-9 は IgE 存在下では BMCMCs からのサイトカイン産生を促進したが、抗原による IgE の架橋下では抑制した（図 2A）。Gal-9 は IgE と抗原の複合体の形成を阻害することで、マウスのマスト細胞株の抗原による IgE の架橋下での脱顆粒を抑制すると報告されており [28]、本検討でも rmGal-9 が IgE と抗原の複合体の形成を阻害し、サイトカイン産生が抑制されたと考えられる。一方 IgE 単独存在下では、Gal-9 は抗原による IgE の架橋よりは低いもののサイトカイン産生を増強した。これは BMCMCs 上の IgE へ Gal-9 が非特異的に結合し、IgE を架橋した可能性がある。

一方で自然免疫系を介したサイトカイン産生に対しては、rmGal-9 は LPS の共存下に BMCMCs からのサイトカイン産生を誘導した（図 2B）。さらに TIM-3 阻害抗体は、rmGal-9 の LPS 共存下での BMCMCs からのサイトカイン産生誘導を阻害せず（図 2C）、TIM-3 以外の分子の関与が示唆された。Gal-9 はヒアルロン

酸のリガンドであるCD44に結合し、Th2の遊走を阻害することでオボアルブミンとチリダニで誘導した気道炎症を抑制することが報告されている[26]。これらのことからマスト細胞におけるrmGal-9による自然免疫等を介したサイトカイン産生においては、CD44もしくは未知の受容体の関与が考えられた。

次にBMCMCsにおけるIgEと抗原による脱顆粒に対して、rmGal-9は抑制効果を示すことが示唆された（図3）。この結果はGal-9がIgEと抗原の複合体の形成を阻害することで、マスト細胞と好塩基球の細胞株の脱顆粒を抑制するという報告[28]と同様の結果であった。これに対し抗TIM-3アゴニスト抗体は、BMCMCsのサイトカイン産生を促進するが、IgEを介した脱顆粒には影響を与えないことも報告されている[16]。よって今回の結果はrmGal-9のTIM-3を介した作用というより、IgEと抗原の複合体の形成阻害による作用である可能性が示唆された。

さらにrmGal-9はBMCMCsおいて、LPSの共存下でアポトーシス誘導を促進した（図4）。 Gal-9は単体でマウスの胸腺細胞、Th1、Th17やヒトのメラノーマ細胞株のアポトーシスを誘導するが[2, 7, 21, 22, 31]、それとは対照的に抗TIM-3抗体（抗TIM-3ポリクローナル抗体）はアゴニスト抗体として、マウス骨髄由来培養マスト細胞のアポトーシスを抑制することが報告されている[16]。今回の結果はTIM-3依存性の結果とは異なり、rmGal-9の細胞への作用はTIM-3非依存

性の可能性があることが示唆された。

今回の検討で、rmGal-9 はマスト細胞において促進と制御、両面の機能を有することが明らかとなった。rmGal-9 はマスト細胞からの脱顆粒を抑制する一方で、サイトカイン産生を促進し、アレルギー疾患の発症や進展に関わることが示唆された。よって rmGal-9 はマスト細胞からの脱顆粒に伴う即時型反応を主とする病態の治療薬となる可能性が示唆された。一方でサイトカイン産生は促進することから遅発型反応を増強する可能性があり、遅発型反応を抑制するステロイド薬との併用が必要となる可能性も示唆された。

第５節　小括

マウス Gal-9 はマウス骨髄由来培養マスト細胞（BMCMCs）の生存と脱顆粒および抗原による IgE の架橋下でのサイトカイン産生を抑制したが、IgE または LPS 共存下のサイトカイン産生は促進し、LPS 共存下のサイトカイン産生については、TIM-3 以外の分子の関与が示唆された。

第３章　ヒトマスト細胞株へのガレクチン９の作用

第１節　背景

Gal-9 は多様な細胞に発現し、細胞凝集、接着、アポトーシス、生存、分化の誘導といった様々な機能を有していることが報告されている[4-7]。これらの機能は Gal-9 が TIM-3 に結合することによって発揮される[5, 8, 9]（図 1）。

Gal-9 と TIM-3 は齧歯類のアレルギー性気道炎症モデルにおける肺組織で発現が上昇しており[23-25]、気道炎症の誘導への関与が示唆されている。実際、Gal-9 を前投与したマウスでは、オボアルブミンとチリダニで誘導した気道炎症と過敏性が抑制されていることが報告されている[26]。この系では Gal-9 はヒアルロン酸のリガンドである CD44 に結合し、Th2 の遊走を阻害することで抗原にて誘導した気道炎症の抑制効果を示す[26]。このように Gal-9 の作用は条件によっては TIM-3 非依存性の場合がある。それを支持する報告として、Gal-9 は IgE と抗原の複合体の形成を阻害することで、マウスのマスト細胞と好塩基球の細胞株の脱顆粒を抑制するという報告もある[28]。一方、抗 TIM-3 アゴニスト抗体（抗 TIM-3 ポリクローナル抗体）は、マウス骨髄由来培養マスト細胞（BMCMCs）のサイトカイン産生を促進するが、IgE を介した脱顆粒には影響を与えないことも報告されている[16]。Gal-9 は、はじめ TIM-3 のリガンドであると報告されたが[9]、その後 CD44[26]や IgE[28]といった TIM-3 以外の分子に結合して作用を発揮する

ことが報告されており、Gal-9 によるマスト細胞からのサイトカイン産生や生存への作用が、抗 TIM-3 アゴニスト抗体と同じ結果となるかは不明である。またこれまでマウスのマスト細胞の細胞株での検討[28]はあるものの、ヒトマスト細胞における Gal-9 の作用を検討した報告はなく、また Gal-9 の IgE を介さない作用についても明らかではない。そこで今回、IgE レセプターである FcεRI を発現しない、ヒトマスト細胞株、HMC-1 を用いて IgE 非刺激下での Gal-9 の作用について検討した。

第２節　材料と方法

細胞培養

ヒトマスト細胞株、HMC-1細胞は10% FBSと100U/ml penicillin および100μg/ml streptomycin を添加したα-minimum essential medium (Gibco BRL, Grand Island, NY) を用いて、5% CO_2 、37°Cの条件のもと培養した。培地は週2回、半量ずつ交換した。ヒト気管支上皮細胞（normal human bronchial epithelial cells ：NHBEs ）およびヒト冠動脈内皮細胞（human coronary artery endothelial cells：HCAECs ）の培養は先行文献を参考に行った[32]。

定量的 **PCR**

総RNAの抽出は HMC-1 、NHBEsおよび HCAECs からRNeasy (Qiagen, Valencia, CA, USA) を用いて製造会社の手順に従って行った。Human universal reference (HUR) RNA (BD Biosciences, Palo Alto, CA, USA) を陽性コントロールとして使用した。 次に回収したRNAからのcDNA合成をiScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA)を用いて行った。 TIMs のプライマーは以下の通りである。 TIM-1 (sense, 5'- TGT TCC TCC AAT GCC TTT GC -3'; antisense, 5'- TTG CTC CCT GCA GTG TCG TA -3'), TIM-3 (sense, 5'- CAA TGC CAT AGA TCC AAC CAC C -3'; antisense, 5'- GCA GTG GAC AGA ACC TCC AAA A-3'),

TIM-4 (sense, 5'-TCC TGC TGA CAT CCA AAG CA -3'; antisense, 5'- TGG GAG ATG GGC ATT TCA TT -3'), and GAPDH (sense, 5'- GAA GGT GAA GGT CGG AGT C -3'; antisense, 5'- GAA GAT GGT GAT GGG ATT TC -3')。TIM遺伝子の定量をするため，標準濃度のTIM1, TIM3, TIM4 および GAPDH を段階希釈し、検量標準に用いた。総RNA検体の5 ng に相当するcDNA を定量し、メッセンジャーRNA 発現レベルはGAPDH のレベルで補正した。

Western blotting 法

HMC-1 細胞 ($5x10^5$ cells/well 、24 well plate使用) を 1 μM recombinant human galectin-9 (rhGal-9)(GalPhama Co. Ltd., Kagawa, Japan)で、図6に示した時間刺激した。その後回収した細胞を、5% 2 mercaptoethanolを添加したNuPAGE sample buffer (Invitrogen, Carlsbad, CA) 200 μl にて融解し超音波処理を行った。SDS-PAGE (5–15% Ready Gels J; Bio-Rad, Hercules, CA) を用いた電気泳動により細胞融解液のタンパクを分離し，ニトロセルロース膜 (iBlot Gel Transfer Stacks, mini; Invitrogen)に転写した。イムノブロッティングはrabbit anti-phospho-p44/42 MAPK (Erk1/2) mAb (clone D13.14.4E; Cell Signaling Technology, Danvers, MA) と rabbit anti-p44/42 MAPK (Erk1/2) mAb (clone 137F5; Cell Signaling Technology) を１次抗体とし、horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling

Technology) を２次抗体として使用した。 標的のバンドをPierce Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA)を用いて検出した。

細胞生存の評価

HMC-1細胞を5 μg/ml のmitomycin-C (Sigma Chemical Co, St. Louis, MO, USA) で2時間前処置した。洗浄後、4x10^5 cells/mlの細胞を0.25, 0.5 ,または1 μM の rhGal-9 の存在下または非存在下 37 °C で0, 24 または48時間培養した。その後 trypan blue 染色を用いて、生存細胞を顕微鏡下に計測した。またFITC-conjugated annexin-V とpropidium iodideにて染色し、propidium iodide陰性、annexin-V陽性アポトーシス細胞の割合をFACSCanto IIとそのソフトウェア Diva Software (BD, Dranklin Lakes, NJ, USA)を用いて測定した。

β-hexosaminidase release assay

HMC-1細胞(1x10^5 cells/well 、96well plate使用) をrhGal-9 (0, 0.25, 0.5 and 1 μM) にて 37 °Cで30分前処置し、その後100ng/ml PMAと1 μg/ml ionomycinを添加し 37 °Cで30分刺激した後、細胞上清を回収した。細胞上清中のβ-hexosaminidase 活性の測定は先行文献を一部改変して行った[29)]。簡潔に述べると、50 μlの検体を 0.1M sodium citrate (pH 4.5)中の1.3 mg/ml p-nitrophenyl-N-acetyl-β-D-glucosaminide

(Sigma Chemical Co.) 100μl に加えて96-wellプレートを用いて37 °C、１時間インキュベートした。その後50 μl の0.4 M glycine (pH10.7)を各wellに添加して酵素反応を止めた。酵素活性(OD405) はプレートリーダーを用いて測定した。 データについては、細胞中の総β-hexosaminidase 量に対する各条件下で放出されたβ-hexosaminidaseの割合をパーセント表示した。

ELISA法

HMC-1 細胞(1x10^5 cells/well 、96 well plate 使用) を図 9B に示した濃度のrhGal-9 で 20 mM lactose (Nacalai Tesque, Kyoto, Japan) または sucrose (Wako, Osaka, Japan) の存在下で37 °C 18 時間培養した。また図9Dに示した濃度のERK inhibitor (PD98059; Calbiochem, La Jolla, CA)または ERK inhibitor control (SB202474; Calbiochem) および solvent (0.1% (v/v) DMSO) 単体で 37 °C 30 分、もしくは図 9C に示した濃度の rhTIM-3-Fc (R&D Systems, Minneapolis, MN, USA) またはhuman IgG (Sigma Chemical Co, St. Louis, MO, USA) で 37 °C １時間、HMC-1 細胞をそれぞれ rhGal-9 刺激前に培養した。上清中の IL-6、IL-8 、MCP-1 はELISA kits (R&D Systems, Minneapolis, MN, USA) を用いて製造会社の手順に従い測定した。

統計解析

全てのデータはmeans ± SDで表した. Unpaired Student's t-test（two-tailed）または ANOVAにて適宜、統計解析を行った。 $p < 0.05$ を統計学的有意とした。

第3節　結果

ヒトマスト細胞株における TIM ファミリーの発現

はじめに HMC-1 細胞とヒト初代細胞における TIM ファミリー(TIM-1、TIM-3 および TIM-4)の発現を定量的 PCR にて調べた。HMC-1 細胞に TIM-1 と TIM-3 のメッセンジャーRNA が発現していることを確認したが、NHBE と HCAEC には発現していなかった（図 5）。一方 TIM-4 のメッセンジャーRNA はいずれの細胞にもほとんど発現していなかった。

rhGal-9 は HMC-1 細胞において Erk1/2 のリン酸化を誘導する

HMC-1 細胞において、rhGal-9 に結合する TIM-3 のメッセンジャーRNA の発現を確認したので、次に rhGal-9 のシグナル伝達分子のリン酸化について Western blotting 法を用いて検討した。rhGal-9 は HMC-1 細胞において Erk1/2 のリン酸化を誘導するが p38 MAPK のリン酸化は誘導しなかった（図 6)。rhGal-9 は HMC-1 細胞において、RAF/MEK/ERK の増殖因子受容体シグナル伝達経路を介して活性化することが示された。

rhGal-9 は HMC-1 細胞においてアポトーシスを誘導する

次に rhGal-9 の HMC-1 細胞の生存に対する影響について検討した。HMC-1

細胞を mitomycin-C 処置後、0.25, 0.5 ,または 1 μM の rhGal-9 の存在下または非存在下で 0, 24, 48 時間培養した。trypan blue 染色陰性の生存細胞は、0.5μM および 1 μM の rhGal-9 存在下で vehicle control (0 μM rhGal-9)と比較して有意に減少していた。これと一致して propidium iodide 陰性、 annexin-V 陽性のアポトーシス細胞の割合は 1 μM の rhGal-9 存在下で vehicle control (0 μM rhGal-9)と比較して有意に上昇していた（図 7）。以上から Gal-9 は HMC-1 細胞においてアポトーシスを誘導することが示された。

rhGal-9 は HMC-1 細胞において脱顆粒を抑制する

rhGal-9 の HMC-1 細胞における IgE を介さない脱顆粒への効果を検討した。HMC-1 細胞を rhGal-9 (0, 0.25, 0.5 and 1 μM)にて前処置し、PMA と ionomycin で刺激した。β-hexosaminidase 放出によって評価した HMC-1 細胞からの脱顆粒は、0.5 μM の rhGal-9 前処置にて有意に抑制されていた（図 8）。一方 1 μM の rhGal-9 前処置では、抑制傾向ではあったものの有意ではなかった。以上より Gal-9 は HMC-1 細胞における脱顆粒を至適濃度で IgE 非依存的に阻害することが示された。

rhGal-9 は HMC-1 細胞におけるサイトカイン産生を促進する

脱顆粒における抑制効果とは対照的に、rhGal-9 は HMC-1 細胞におけるサイトカイン産生（IL-6、 IL-8 および MCP-1）を濃度依存的に促進した（図 9A）。ガレクチンの作用の多くはその lectin 活性に依存していることが知られている[5]。rhGal-9 によって誘導される HMC-1 細胞からの IL-6 の産生も lactose の存在下で抑制され、sucrose では抑制されないことから lectin 活性に依存していることが示された（図 9B）。さらに rhGal-9 によって誘導される IL-6 の産生は分泌型の TIM-3 (rhTIM-3/Fc) の存在下で抑制され、コントロールの human IgG では抑制されないことから TIM-3 を介している可能性が示唆された（図 9C）。また rhGal-9 によって誘導される IL-6 の産生は ERK1/2 阻害薬（PD98059）の存在下で抑制され、そのコントロール（SB202474）では抑制されないこと（図 9D）から、Gal-9 による ERK1/2 の活性化が HMC-1 細胞におけるサイトカイン産生の促進に必要であることが示された。

第 4 節　考察

今回の検討でヒト Gal-9 はヒトマスト細胞株、HMC-1 細胞に対し促進と制御、両面の機能を有することが示された。Gal-9 は HMC-1 細胞の生存と脱顆粒を抑制したが、サイトカイン産生は ERK1/2 の活性化により促進した。

Gal-9 は HMC-1 細胞において Erk1/2 のリン酸化を誘導するが p38 MAPK のリン酸化は誘導しなかった（図 6）。一方でヒト単球由来の DCs の成熟は、Erk1/2 ではなく p38 MAPK の活性化によって誘導される [33]。よって Gal-9 のシグナル伝達経路は細胞の種類によって異なることが示唆された。DC と HMC-1 細胞との違いは、介在する分子の違いの可能性もある。実際、lectin 活性は rhGal-9 によって誘導される HMC-1 細胞からの IL-6 の産生に必要であったが（図 9B）、ヒト DC では必要なかった [33]。さらに Gal-9 は HMC-1 細胞においてアポトーシスをマウスの胸腺細胞、Th1、Th17 やヒトのメラノーマ細胞株と同様に誘導した [2, 7, 21, 22, 31]。それとは対照的に抗 Tim-3 抗体はアゴニスト抗体として、マウス骨髄由来培養マスト細胞の IgE を介したサイトカイン産生を促進するが、アポトーシスを抑制することが報告されている [16]。以上のことから Gal-9 の細胞への作用は、TIM-3 依存性、非依存性の両方があることが示唆された。

Gal-9 は IgE と抗原の複合体の形成を阻害することで、マウスのマスト細胞株の IgE を介した脱顆粒を抑制すると報告されている [28]。今回の検討では、

FcεRI を発現しない HMC-1 細胞[34]を用いて PMA/ionomycin による脱顆粒に対する Gal-9 の作用について検討した。その結果、Gal-9 は HMC-1 からの脱顆粒を抑制することが明らかとなり、IgE を介した脱顆粒の抑制とは異なるメカニズムがあることが示唆された。

今回の検討で、Gal-9 はマスト細胞において促進と制御、両面の機能を有することが明らかとなった。Gal-9 はマスト細胞からの脱顆粒を抑制し、アポトーシスを誘導するが、サイトカイン産生は促進し、アレルギー疾患の発症や進展に関わることが示唆された。よって Gal-9 は、マスト細胞からの脱顆粒に伴う即時型反応を主とする病態の治療薬となる可能性が示唆される一方で、サイトカイン産生を促進することから遅発型反応を増強する可能性があり、遅発型反応を抑制するステロイド薬との併用が必要となる可能性も示唆された。

第５節　小括

ヒト Gal-9 はヒトマスト細胞株、HMC-1 細胞の生存と脱顆粒を抑制したが、サイトカイン産生は ERK1/2 の活性化により促進した。

第 4 章　総括

今回の検討で、Gal-9 はマウスおよびヒトマスト細胞において促進と制御、両面の機能を有することが明らかとなった。

Gal-9 を前投与したマウスでは、オボアルブミンとチリダニで誘導した気道炎症と過敏性が抑制されていることが報告されている [26, 28]。喘息モデルマウスにおける Gal-9 による症状の抑制効果は、マスト細胞からのサイトカイン産生の誘導よりむしろ脱顆粒の抑制によるところが大きい [28]。しかしながら、抗原負荷後の Gal-9 処置はマスト細胞からのサイトカイン産生を増強し、また局所への好酸球遊走を促し、遅発型反応の増悪につながる可能性がある [2]。

Gal-9 はマスト細胞からの脱顆粒を抑制し、アポトーシスを誘導するが、サイトカイン産生は促進し、アレルギー疾患の発症や進展に関わることが示唆された。よって Gal-9 はマスト細胞からの脱顆粒に伴う即時型反応を主とする病態の治療薬となる可能性が示唆された。一方でサイトカイン産生は促進することから好酸球を主体とする遅発型反応を増強する可能性があり、遅発型反応を抑制するステロイド薬との併用が必要となる可能性も示唆された（図 10）。

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図表

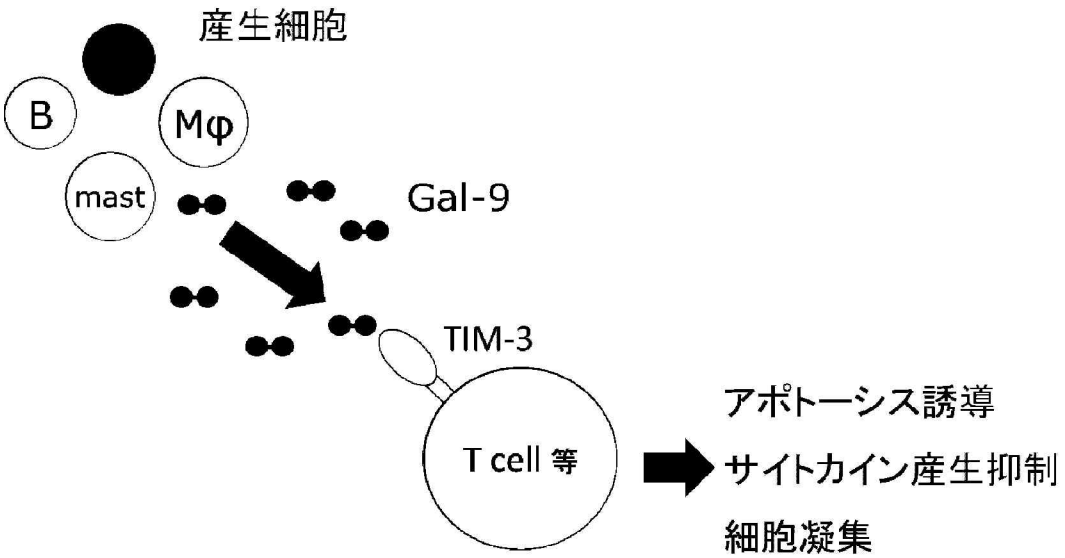
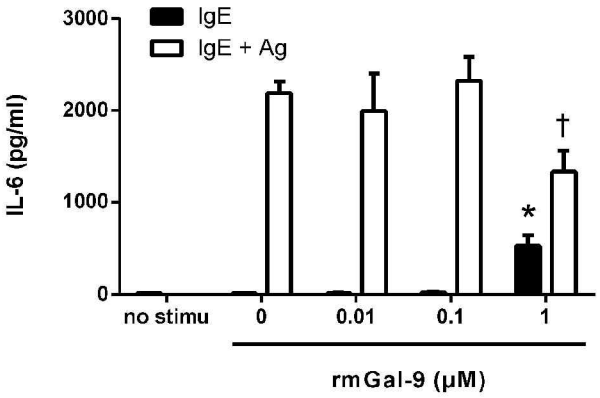


図 1　Gal-9 は TIM-3 を介して多彩な作用を発揮する

Gal-9 は主に T 細胞などの白血球より産生され、TIM-3 を細胞表面に発現している T 細胞などに TIM-3 を介して結合し、アポトーシスの誘導やサイトカイン産生の抑制、細胞凝集など多彩な作用を発揮する。一方、Gal-9 は CD44 や IgE など、TIM-3 以外の分子にも結合して作用を発揮する。



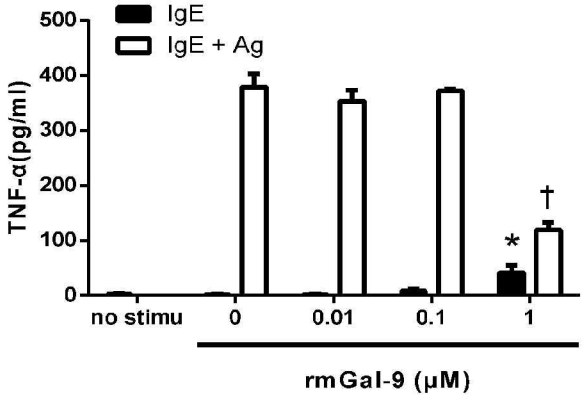


図 2A　recombinant mouse galectin-9 (rmGal-9) は IgE 存在下で BMCMCs からのサイトカイン産生を促進するが、抗原による IgE の架橋下ではサイトカイン産生を抑制する

BMCMCs を 18 時間 IgE 感作し、次に図に示した濃度の rmGal-9 で 1 時間前処置し、抗原の存在下および非存在下で 18 時間培養した。上清中のサイトカイン（IL-6 および TNF-α）を ELISA 法にて測定した。結果は 3 検体の mean ± SD を示し、また 2 回の独立した実験の内、代表的なものを示す。* p <0.05 および †p <0.05 はそれぞれ各条件の 0 μM rmGal-9 との比較を示す。

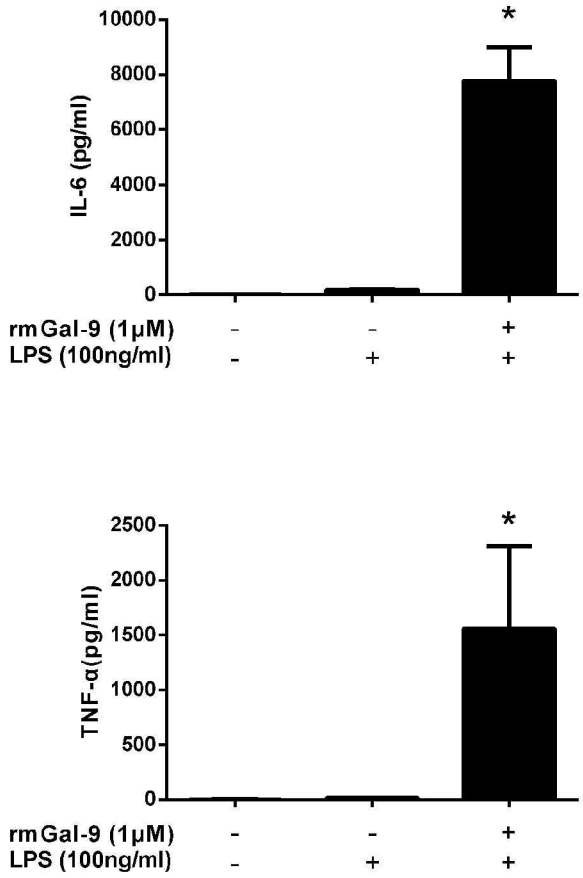
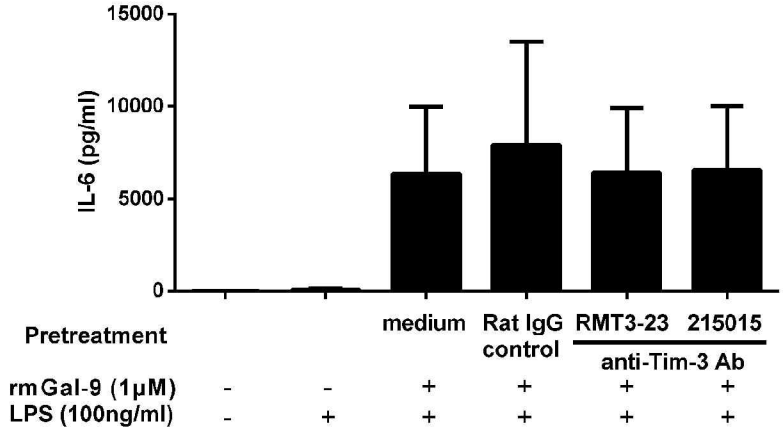


図 2B　rmGal-9 は LPS 共存下に BMCMCs からのサイトカイン産生を誘導する

BMCMCs を図に示した濃度の rmGal-9 で、100ng/ml LPS の存在下で 37 °C 18 時間培養した。上清中のサイトカイン（IL-6 および TNF-α）を ELISA 法にて測定した。結果は 3 検体の mean ± SD を示し、また 2 回の独立した実験の内、代表的なものを示す。* p <0.05 は 0 μM rmGal-9 との比較を示す。



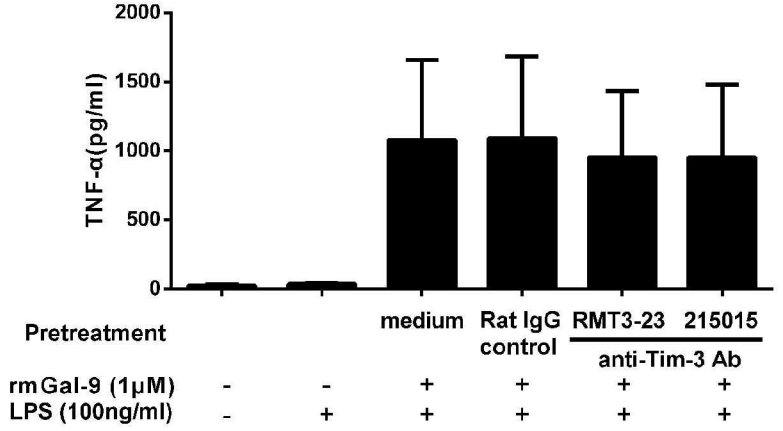


図 2C　TIM-3 阻害抗体は rmGal-9 の LPS 共存下における BMCMCs からのサイトカイン産生誘導を阻害しない

TIM-3 阻害抗体（RMT3-23、215015 ）または control rat IgG 2a にて 1 時間前処置し、図に示した濃度の rmGal-9 で、100ng/ml LPS の存在下で 18 時間培養した。上清中のサイトカイン（IL-6 および TNF-α）を ELISA 法にて測定した。結果は 3 検体の mean ± SD を示し、また 2 回の独立した実験の内、代表的なものを示す。

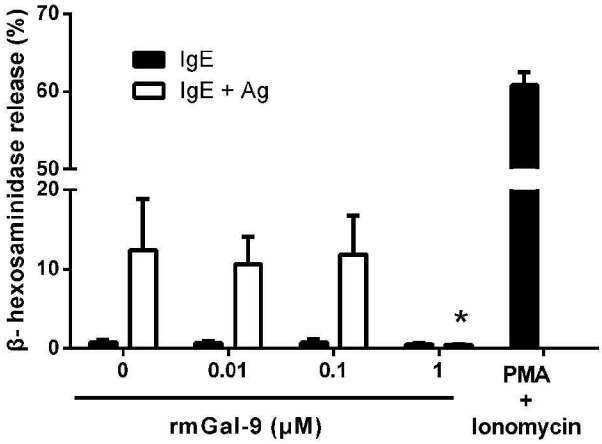


図 3　rmGal-9 は BMCMCs において脱顆粒を抑制する

IgE にて 18 時間感作した BMCMCs を rmGal-9 (0, 0.01, 0.1 and 1 μM)にて 1 時間前処置し、抗原または PMA と ionomycin で刺激した。脱顆粒は上清中の β-hexosaminidase 放出によって評価した。結果は 4 検体の mean ± SD を示し、また 2 回の独立した実験の内、代表的なものを示す。* p <0.05 は 0 μM rmGal-9 との比較を示す。

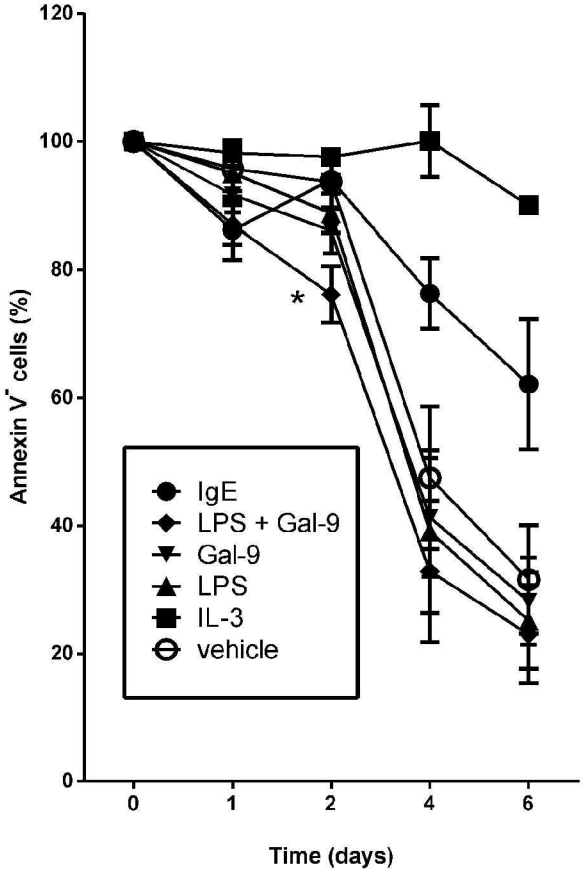


図 4　rmGal-9 は BMCMCs において LPS の共存下でアポトーシスを増強する

BMCMCs を 10 ng/ml IL-3 または 1 μM rmGal-9 と 100ng/ml LPS の存在下または非存在下に 6 日間培養し、経時的に評価した。生存細胞（propidium iodide 陰性、annexin-V 陰性）の割合をフローサイトメーターにて評価した。結果は 3 検体の mean ± SD を示し、2 回の独立した実験の内、代表的な結果を示す。* p <0.05 は vehicle control、rmGal-9 単独刺激および LPS 単独刺激との比較を示す。

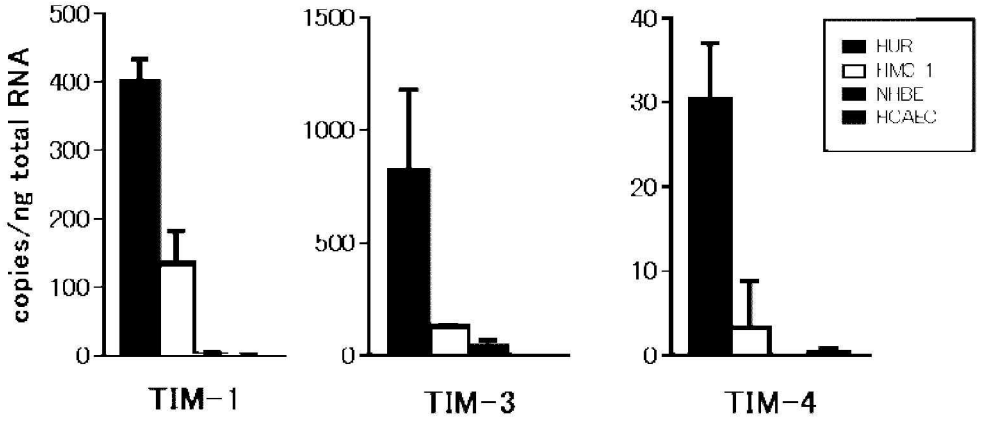


図５　ヒトマスト細胞株における TIM ファミリーの発現

ヒトマスト細胞株、HMC-1 とヒト初代細胞（ヒト気管支上皮細胞、NHBEs、ヒト冠動脈血管内皮細胞、HCAECs）における TIM ファミリーのメッセンジャーRNA の発現を定量的 PCR にて調べた。Human universal reference (HUR) RNA をコントロールとして使用した。3 回の独立した実験の内、代表的な結果を示す。

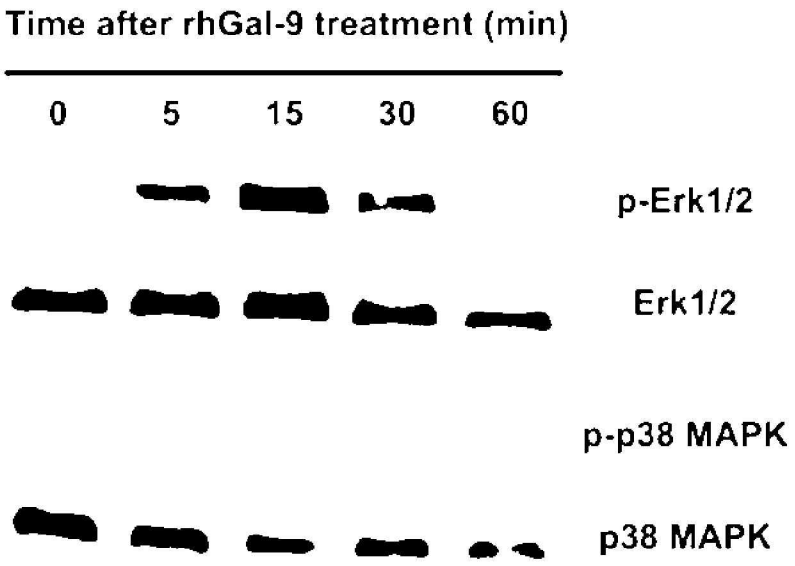


図 6　recombinant human galectin-9 (rhGal-9) は HMC-1 細胞において Erk1/2 のリン酸化を誘導する

HMC-1 細胞を rhGal-9 の存在下に図に示した時間培養した。次に Erk1/2 および p38 MAPK のリン酸化を Western blotting 法を用いて検討した。3 回の独立した実験の内、代表的な結果を示す。

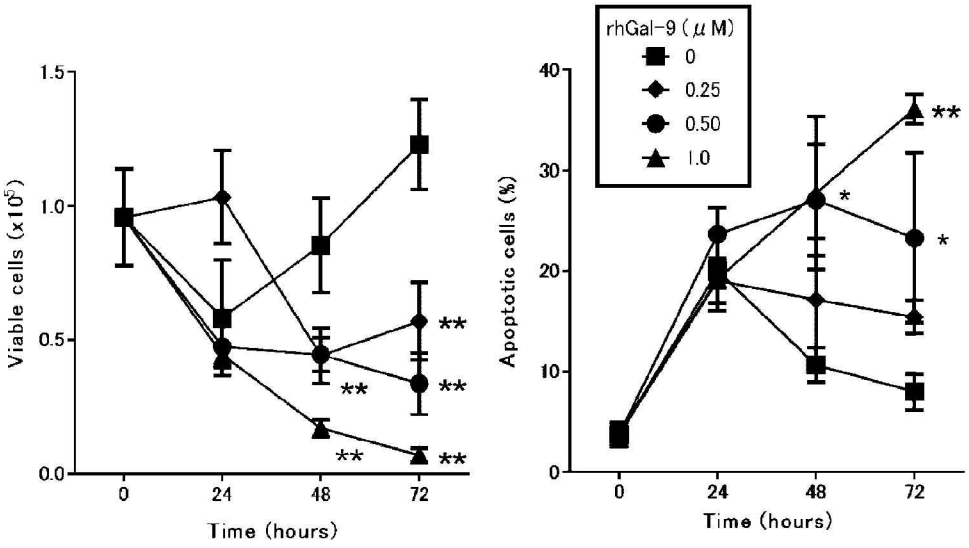


図 7　rhGal-9 は HMC-1 細胞においてアポトーシスを誘導する

HMC-1 細胞を 0.25, 0.5 ,または 1 μM の rhGal-9 の存在下または非存在下に図に示した時間培養した。生存細胞は trypan blue 染色にて評価し、propidium iodide 陰性、 annexin-V 陽性のアポトーシス細胞はフローサイトメーターにて評価した。結果は 3 検体の mean ± SD を示し、また 3 回の独立した実験の内、代表的なものを示す。* p <0.05 と ** p <0.01 は vehicle control（0μM rhGal-9）との比較を示す。

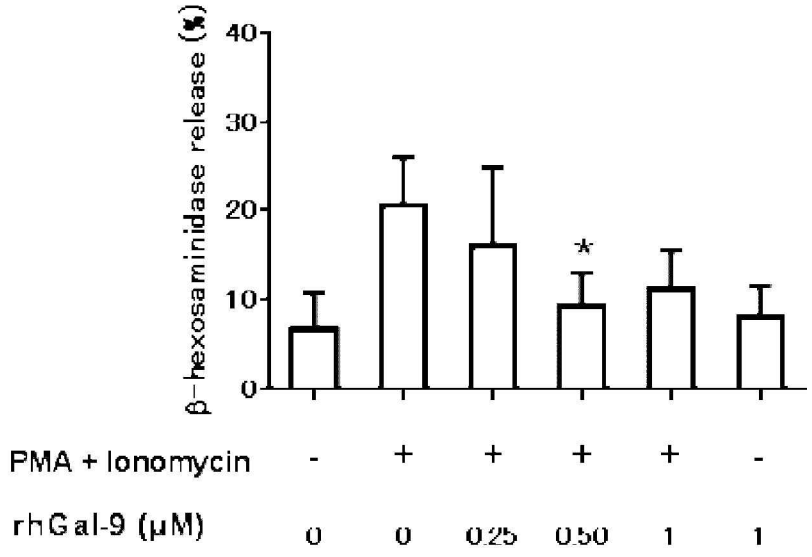


図 8　rhGal-9 は HMC-1 細胞において脱顆粒を抑制する

HMC-1 細胞を rhGal-9 (0, 0.25, 0.5 and 1 μM)にて 30 分間前処置し、PMA と ionomycin で 30 分間前刺激した。脱顆粒は上清中の β-hexosaminidase 放出によって評価した。結果は 6 検体の mean ± SD を示し、また 3 回の独立した実験の内、代表的なものを示す。* p <0.05 は PMA と ionomycin 刺激のみとの比較を示す。

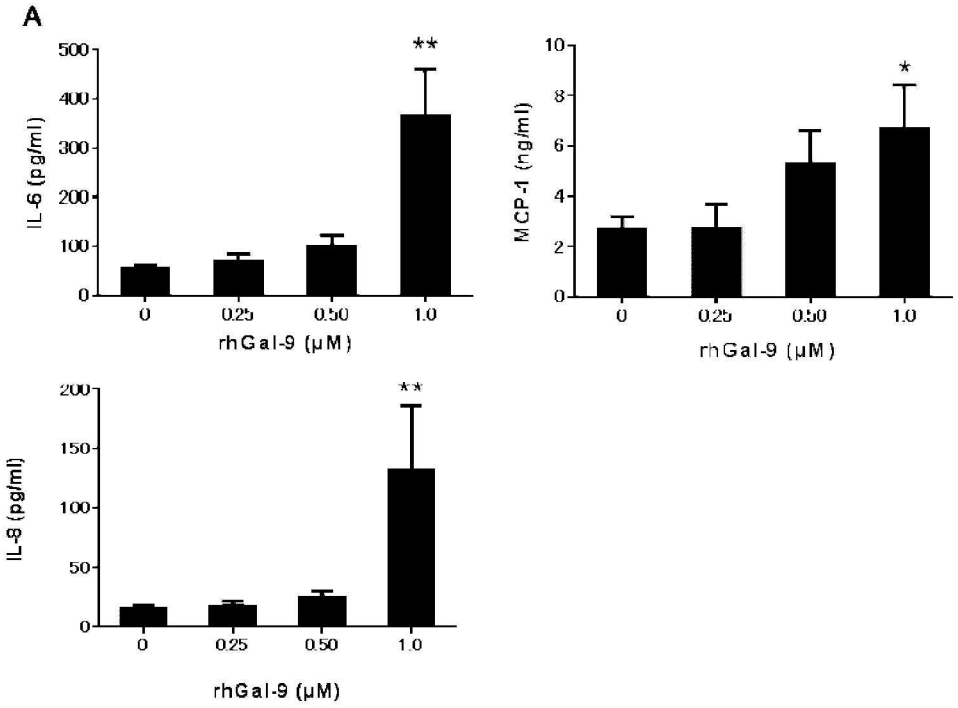


図 9A　rhGal-9 は HMC-1 細胞からのサイトカイン産生を誘導する

HMC-1 細胞を図に示した濃度の rhGal-9 で 18 時間培養し、上清中のサイトカイン（IL-6、 IL-8 および MCP-1）を ELISA 法にて測定した。結果は 3 検体の mean ± SD を示し、また 3 回の独立した実験の内、代表的なものを示す。* p <0.05 と** p <0.01 は 0 μM rhGal-9 との比較を示す。

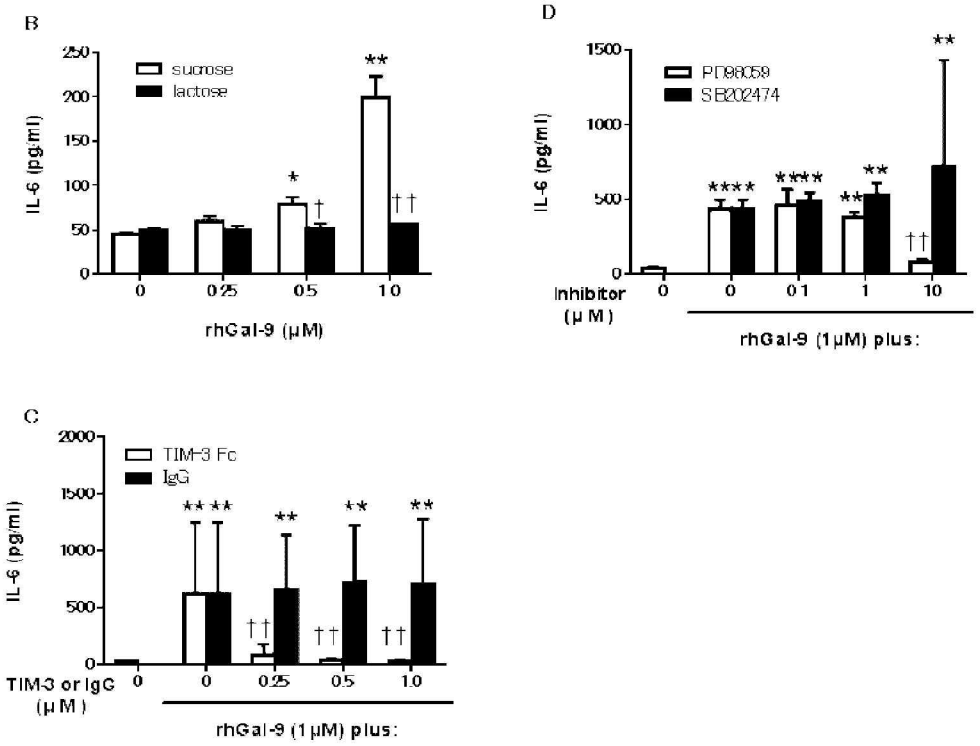


図 9B~D　rhGal-9 による HMC-1 細胞からのサイトカイン産生誘導は TIM-3 を介する可能性があり、ERK1/2 の活性化と lectin 活性に依存する

HMC-1 細胞を図に示した濃度の rhGal-9 で 20 mM lactose または sucrose の存在下に 18 時間培養した。（図 9B）また ERK inhibitor (PD98059)または ERK inhibitor control (SB202474) および solvent (DMSO) 単体で（図 9C）、もしくは rhTIM-3-Fc または control human IgG で（図 9D）1 時間、HMC-1 細胞をそれぞれ rhGal-9 刺激前に培養した。上清中のサイトカイン（IL-6）を ELISA 法にて測定した。結果は 3~6 検体の mean ± SD を示し、また 3 回の独立した実験の内、代表的なものを示す。* p <0.05 と** p <0.01 は 0 μM rhGal-9 との比較を示す。

†p <0.05 と††p <0.01 はそれぞれ sucrose (B)、 control human IgG (C)、 ERK inhibitor control (D) との比較を示す。

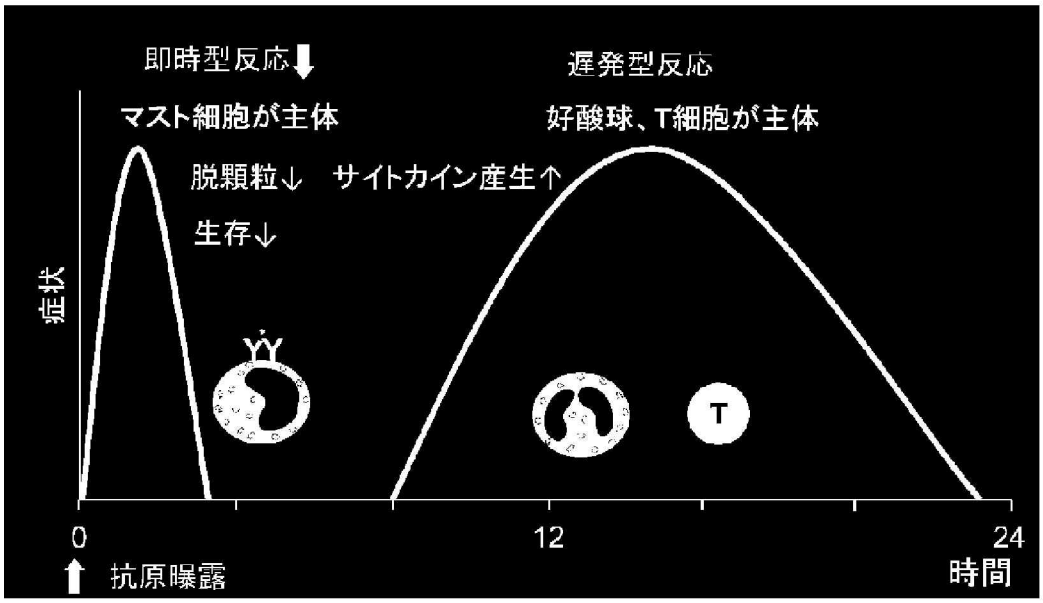


図 10　Gal-9 のマスト細胞への作用

Gal-9 はマスト細胞からの脱顆粒を抑制し、アポトーシスを誘導するが、サイトカイン産生は促進した。よって Gal-9 はマスト細胞が主体的役割を示す即時型反応は抑制するが、サイトカイン産生は促進し好酸球を主体とする遅発型反応は増強する可能性がある。

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Salivary Cortisol Response to Stress in Young Children with Atopic Dermatitis

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Abstract: Poor responsiveness of the hypothalamic–pituitary–adrenal (HPA) axis under stress may be one explanation for stress-induced exacerbation of atopic dermatitis (AD) symptoms. In previous studies, children and adults with AD showed attenuated salivary cortisol responses to psychosocial stress, suggesting hyporesponsiveness of the HPA axis, but few studies have been conducted in young children, who are vulnerable to systemic side effects of topical corticosteroid (TCS) therapy. We evaluated whether salivary cortisol responses to the stress of venipuncture in young children with AD were related to the severity of AD or performance of TCS therapy. We studied 38 young children with AD (median age 16.5 mos, range 3–66 mos) being treated at our outpatient unit. Patients were divided into three groups according to the scoring of atopic dermatitis index: mild (n = 12), moderate (n = 14), and severe (n = 12). To evaluate the responsiveness of the HPA axis to stress, salivary cortisol was determined before and after venipuncture. Salivary cortisol responsiveness to stress correlated negatively with severity of AD ($p = 0.048$) but not with previous use of TCS ($p = 0.43$) in young children with AD. Our findings suggest that the disease activity of AD, rather than TCS use, is responsible for HPA axis dysfunction in children with AD.

Clinical observations and experimental findings have emphasized that exacerbation of atopic dermatitis (AD) symptoms is closely related to psychosocial stress (1,2). Stress itself can reportedly cause epidermal barrier dysfunction and mast cell activation through release of neuropeptides, which in turn facilitates exacerbation of allergic inflammation (reviewed in reference 1). Stress also activates the hypothalamic–pituitary–adrenal (HPA) axis to release cortisol, a potent attenuator of inflammatory reactions in general, although previous studies indicated that children and adults with AD showed attenuated salivary cortisol responses to psychosocial stress, suggesting hyporesponsiveness of the HPA axis (3,4). Therefore, poor responsiveness of

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the HPA axis under stress may be one explanation of stress-induced exacerbation of AD symptoms. Similar endocrine irregularities of the HPA axis have also been reported after pharmacologic challenge in patients with AD (5,6). It may be argued that hyporesponsiveness of the HPA axis in patients with AD is simply a side effect of topical corticosteroid (TCS) therapy, which may lead to steroid phobia (fear of TCS use) in patients with AD and even in health care professionals (7). Previous reports have investigated the influence of TCS on the HPA axis in children with AD, with contradictory results (8–10). Other studies have suggested that AD disease activity, rather than the use of TCS, is responsible for changes in the HPA axis in patients with severe AD (5,11). Young children are believed to be at special risk for systemic effects of TCS because of high percutaneous absorption of TCS due to their relatively large body surface area per weight. Therefore, it is important to investigate whether HPA dysfunction is observed in young children with AD and is related to TCS therapy.

Blood cortisol concentration increases in response to unpredictable, uncontrollable, and novel situations (12,13). Salivary cortisol levels correlate with plasma unbound cortisol levels (14). Moreover, because saliva samples can be obtained without stress, salivary cortisol assessment is a reliable tool for investigating HPA axis function, especially in young children (13). Previous studies reported a significantly attenuated cortisol response to standardized laboratory psychological stress, which consisted mainly of public speaking and mental arithmetic tasks in front of an audience, in children and adults with AD (3,4), but those procedures are difficult to implement in children younger than 6 years of age. Moreover, venipuncture is an unpredictable, uncontrollable, novel situation for young children, so it itself might be a significant stressor resulting in activation of the HPA axis in young children, detected as high cortisol levels. We used venipuncture as the acute stressor to investigate changes in the level of salivary cortisol.

We evaluated whether impairment of the salivary cortisol response to the stress of venipuncture in young children with AD was related to the severity of the AD symptoms and whether TCS use affected the response.

METHODS

Subjects

We studied 38 young children (24 boys and 14 girls) with AD (median age 16.5 months, range 3–66 months) being treated at our outpatient unit. Patients who had received regular TCS therapy were included, but those who had received inhaled or oral corticosteroids in the preceding 6 months were excluded. All patients were Japanese whose parents were of middle socioeconomic status. An allergist made the diagnosis of AD in accordance with the clinical criteria defined by the Japanese Dermatological Association (15). The severity of AD in each patient was assessed using the scoring of atopic dermatitis (SCORAD) index (16). The severity of AD was defined according to the SCORAD index (mild < 25, moderate 25–50, severe ≥50).

Previous use of TCS was assessed by a modification of the previously defined score, the treatment score (Table 1) (10), which was based on the potency of the preparation used (TCS preparations were grouped according to clinical potency, as described in the Japanese Therapeutic Guideline for AD (17)), percentage of body surface area to which it was applied, the duration of treatment in the last month. The hospital ethics committee of the National Center for Child Health and Development approved this study. Informed consent was obtained from all caregivers.

Procedures

To evaluate the responsiveness of the HPA axis to stress, salivary cortisol concentrations were determined before and after venipuncture. Venipuncture for clinical purposes and saliva sampling were performed between 10:00 a.m. and 3:00 p.m. to avoid any possible inhibitory effect of a high morning basal cortisol level on further cortisol release (18). To determine free cortisol concentration, saliva samples were collected 5 minutes before and 15 to 20 minutes after the venipuncture was completed, because that is when salivary cortisol level peaks (13).

Cortisol Measurement

Saliva was obtained using a Sorbette sampling device (Salimetrics, State College, PA), which consists of a

TABLE 1. Score for Topical Corticosteroid Treatment (Ref. 10)

Score (total 10)	0	1	2	3	4
Potency of preparation (0 to 4)	None	Mild	Strong	Very strong	Strongest
Body surface area treated, % (0 to 3)		< 9		> 36	
Treatment duration* (0 to 3)		< 7	≥7	Continuous	

*Days in the past month.

TABLE 2. Clinical Characteristics

Characteristic	Mild n = 12	Moderate n = 14	Severe n = 12	p-value*
Sex, male/female, n	6/6	9/5	9/3	0.44
Age, months, median (range)	19 (4–66)	21 (3–60)	10.5 (3–57)	0.41
SCORAD, median (range)	16 (8–25)	40 (26–48)	64.5 (51–86)	< 0.001
Score for TCS treatment, median (range)	4.5 (4–7)	5 (0–7)	0 (0–7)	0.38
Non-TCS users, n	0	2	7	0.002
Number of venipunctures, median (range)	2 (1–3)	1 (1–4)	1 (1–3)	0.10
Total immunoglobulin E, IU/mL, median (range)	163 (5.2–4,562)	212 (12.9–9,500)	1,093.5 (55.6–4,161)	0.12

TCS, topical corticosteroid.
*Kruskal–Wallis test or chi-square test.

microsponge with a short plastic shaft as a handle. After the microsponge had been put under the tongue for 1 minute, the Sorbette was placed in a plastic tube and centrifuged for 15 minutes at 1,800 g, resulting in a clear, watery supernatant. The samples were stored at) 30°C until analysis. For cortisol determination, 50 l L of saliva was used for duplicate analysis with a salivary cortisol enzyme-linked immunosorbent assay kit (Salimetrics), according to the manufacturer's protocol.

Statistical Analysis

Salivary cortisol concentrations did not show a normal distribution and were therefore log-transformed for analysis. Group variables were compared using the Mann–Whitney U test, the Kruskal–Wallis test, or the chi-square test. The paired t test was used to compare salivary cortisol response to the stressor (venipuncture). Spearman rank correlation was used to investigate the relationship between the parameters of the salivary cortisol response to the stressor and the variables (disease severity and TCS treatment). Data were analyzed using STATA software (Windows version 8.0, Stata Corp., College Station, TX). $P < 0.05$ was considered to indicate statistical significance in all comparisons.

RESULTS

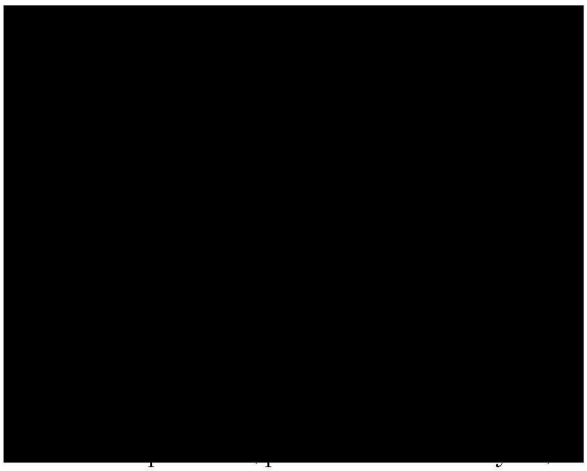
Clinical Characteristics

Clinical characteristics of the patients are summarized in Table 2. The patients were grouped according to severity of AD (mild [12 patients], moderate [14 patients], and severe [12 patients]). There were no significant differences between the AD severity groups in terms of the age, sex, score for TCS treatment, number of venipunctures, or total immunoglobulin (Ig)E level (IU/mL), but there was a statistically significant difference in the number of patients not treated with TCS ($p = 0.002$).

Salivary Cortisol Response to Venipuncture

First, we evaluated whether venipuncture could be a stressor that induced significantly higher salivary cortisol levels in young children with AD. The number of venipunctures and the change in salivary cortisol level were investigated for possible association in patients in whom—for technical reasons—several venipunctures were needed to achieve blood sampling. The change in salivary cortisol level was calculated by dividing the postvenipuncture cortisol concentration by the prevenipuncture cortisol concentration, yielding a ratio. When subjects were stratified according to number of venipunctures, the increase in salivary cortisol level depended on the number of venipunctures ($p = 0.04$; Kruskal–Wallis test, Fig. 1). This result suggested that venipuncture was a sufficient stressor to induce a change in cortisol level in children younger than 6 years of age.

There were no significant differences in prevenipuncture and postvenipuncture salivary cortisol levels between the three groups, but the salivary cortisol level increased significantly after venipuncture in all groups ($p < 0.05$; paired t test, Fig. 2).



Correlation Between Salivary Cortisol Response and Severity of AD or TCS Treatment

The change in salivary cortisol level correlated significantly with the pre- and postvenipuncture salivary cortisol levels ($r =) 0.50$, $p = 0.01$; $r = 0.61$, $p < 0.001$, respectively; data not shown). Conversely, it correlated negatively with severity of AD ($r =) 0.32$, $p = 0.048$; Fig. 3). No significant correlation was found between change in salivary cortisol level and score for TCS treatment ($p = 0.43$; Fig. 4), age, sex, or total IgE (data not shown).

DISCUSSION

In previous studies, children and adults with AD showed attenuated salivary cortisol responses to psychosocial stressors, suggesting hyporesponsiveness of the HPA axis, which may be one explanation for stress-induced exacerbation of AD symptoms (3,4), but few studies have been performed in young children, who are vulnerable to systemic side effects of TCS therapy. In this study, we found that salivary cortisol response to the stress of venipuncture in young children with AD correlated negatively with the severity of symptoms.

We first evaluated whether venipuncture could be a stressor in children younger than 6 years of age. Although previous studies reported that changes in cortisol level after venipuncture were not significant in children aged 6 and older (19,20), we found that venipuncture is a stressor in children younger than 6 years of age. This difference might be because children have more occasions to undergo venipuncture and become used to it as they get older. Although venipuncture is difficult to standardize, and the individual characteristics of the children would influence its role as a stressor, salivary cortisol level rose dependent on the number of venipunctures. These results suggest that venipuncture is a sufficient stressor to induce a change in cortisol level in children younger than 6 years of age. The number of venipunctures did not differ significantly between the three groups of patients with different AD severity (mild 1.75 ± 0.72, moderate 1.29 ± 0.80, severe 1.42 ± 0.64, mean $\pm$SD; $p = 0.10$), suggesting that patients in all three groups received virtually the same level of stress.

The salivary cortisol response to venipuncture as a stressor correlated negatively with the severity of AD. This result is in line with previous studies that found a weaker cortisol response to stressors in adults and children with AD (3,4). Meanwhile, there were no significant

differences in prevenipuncture cortisol levels between the groups with various AD severity, which is also in line with previous studies (3,4). Dysfunction of the HPA axis in individuals with AD may become apparent only when a stress stimulus is present.

According to a previous study (21), the normal salivary cortisol level in young children was approximately 0.16 to 0.36 l g/dL at baseline. The cortisol level in each group of subjects before venipuncture was compatible with that range, and there were no significant differences between the groups. Therefore, we think that our time points for performing venipuncture (10:00 a.m. and 3:00 p.m.) were sufficient for detecting any elevation in cortisol level, regardless of whether severe pruritus might have somewhat altered the circadian rhythm of cortisol release.

The underlying psychobiologic mechanisms of hyporeactivity of the HPA axis in patients with AD are not fully understood. Historically, these changes have been interpreted as a consequence of an ongoing chronic allergic inflammatory process, which releases pro-inflammatory cytokines (22). Some studies reported that an atopic disposition in neonates is associated with greater responsiveness of the HPA axis to stressors, which may promote the development of AD in later life (21,23). It remains to be determined whether these changes in HPA function precede or follow the onset of AD.

Most individuals with AD have high serum concentrations of total and allergen-specific IgE, and the severity of AD is known to be weakly associated with serum IgE levels and degree of Th2-type immune predisposition (24). In the present study, we also found that serum IgE levels were higher in those with more severe AD, but the differences were not statistically significant, presumably because of limited power or the fact that we studied very young infants, but further study is needed to elucidate whether hyporeactivity of the HPA axis is simply a consequence of the chronic inflammatory process or is specific to Th2-type immune responses.

The effects of TCS on HPA function in children with AD have been studied using various methods but with contradictory results (8–10). Our present study found no significant correlation between salivary cortisol level and previous TCS treatment, suggesting that there are other factors related to the disease. Our results are in line with previous findings showing that patients with AD and not treated with TCS had a weaker cortisol response than control subjects (3,4). Moreover, some studies showed that a significant decrease in the disease activity of AD after intensive treatment with large amounts of a potent TCS during hospitalization was associated with normalization of the basal serum cortisol level compared with levels at admission (5,11). These results suggest that the disease activity of AD, rather than TCS use, is responsible for dysfunction of the HPA axis in patients with severe AD.

In children with AD, percutaneous absorption of TCS was proven to be significantly lower in the convalescent phase of the disease than in the acute phase, probably because of the restoration of the skin barrier (25). Although percutaneous absorption of a potent TCS is likely to occur, especially during the acute phase of severe AD, the positive effect of adequate disease control seems to clinically outweigh the suppressive effect on adrenal gland function. Therefore, early restoration of the skin barrier by appropriate TCS therapy might contribute to reducing any undesirable effect of TCS on the HPA axis in individuals with AD in the long term. Good control of AD would improve HPA axis function, which might reduce stress-induced exacerbation of AD symptoms.

In addition, to clarify the effect of TCS on the HPA axis in patients with AD, we would like to compare the cortisol responses of patients with similar severity of AD but different TCS usage. We believe that appropriate use of TCS may improve the skin condition and quality of life of patients and in turn improve the HPA axis response. Suppression of the HPA axis can be seen only in patients with extremely severe AD or inappropriate administration of TCS. Further large-scale, longitudinal studies should be undertaken to elucidate the underlying mechanisms of HPA axis hyporeactivity in patients with AD. Limitations of this study are that the number of subjects was not sufficiently large and the sleep patterns of the patients were not closely determined, but a strength of the study is that we can evaluate HPA axis function to stress non-invasively in young children with AD.

Salivary cortisol responsiveness to the stress of venipuncture correlated negatively with the severity of AD but showed no correlation with previous use of TCS in young children with AD. These findings have major implications for daily practice when treating young patients with moderate to severe AD and steroid phobia.

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Factors Associated with Steroid Phobia in Caregivers of Children with Atopic Dermatitis

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Abstract: Topical corticosteroids (TCS) are first-line therapeutic agents for atopic dermatitis (AD). Some patients express irrational fear and anxiety about using TCS, which leads to poor outcomes for AD. Although it is important to understand the factors underlying steroid phobia so that its effects can be minimized, few studies have addressed this subject. Here, we used a questionnaire to investigate predictive factors for steroid phobia in the caregivers (usually mothers) of children with AD. We studied 436 children with AD (mean age 47.6 mos, range 2–236 mos) who newly visited our AD outpatient unit. The caregivers were asked to complete a medical history questionnaire regarding AD. Steroid phobia was analyzed for correlations with other patient and caregiver variables. Overall, 38.3% of the caregivers were reluctant to use TCS on their children s skin. Patient characteristics female sex (odds ratio [OR] = 1.85 vs male; p = 0.005), child's paternal history of AD (OR = 1.94; p = 0.03), and frequent changing of clinics (OR = 1.25; p = 0.03) were predictive factors for steroid phobia. AD severity did not correlate with steroid phobia. Our findings suggest that greater attention to the patient s sex and clinical background of patients with AD is important to the success of AD therapy, regardless of AD severity.

Topical corticosteroids (TCS) are first-line therapeutic agents for atopic dermatitis (AD) (1). In daily clinical practice, it is common for patients to express irrational fear and anxiety about using TCS (steroid phobia) (2–6). Steroid phobia may lead to poor patient adherence to TCS therapy, resulting in poor control of AD (3,5), and poor control of AD may lead to physical, psychological, and social isolation, including sleep disturbance, teasing, and school refusal, which are thought to be more serious problems than the adverse effects of TCS (3,7).

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Furthermore, those problems result in lower quality of life, not only for children with AD, but also for their families (8), sometimes even leading to family disruption (3). Some patients and caregivers with steroid phobia choose alternative and unproven therapies, which may cause exacerbation of AD (9). Others choose strict dietary therapy that results in malnutrition or failure to thrive (10).

In Japan, steroid phobia is widespread because of confusion and misinformation regarding AD therapies, including strict dietary restriction therapy, in the 1980s and negative media publicity that has exaggerated the adverse effects of TCS since the 1990s (11). Moreover, alternative and unproven therapies have gained popularity as an "atopy industry" (12). Incorrect information has often been disseminated over the Internet (13). In addition to these problems, Japan has experienced scams and lawsuits regarding alternative therapies, deaths due to malnutrition caused by extreme dietary restriction therapy, teasing-linked suicides, and at worst, family suicides (13).

Although steroid phobia is a serious problem, few studies have investigated the factors associated with it (14). The present study was designed to shed light on this, with the ultimate objective of improving the treatment of AD.

MATERIALS AND METHODS

Patients

The present study was conducted on new outpatients (and their caregivers) with AD aged 1 month to 20 years of age who visited the Outpatient Unit of the Division of Allergy, National Center for Child Health and Development, Tokyo, from April 2003 to June 2006. Patients were excluded if they had other forms of inflammatory dermatitis. The hospital ethics committee of the National Center for Child Health and Development approved this study, and it was conducted in accordance with the principles outlined in the Declaration of Helsinki.

Clinical Background and Data

Allergists diagnosed AD. The severity of AD in each patient was assessed using the scoring of atopic dermatitis (SCORAD) index (15) and then classified into one of four categories according to that index (remission 0, mild 1–25, moderate 25–49, and severe ≥50) (16). Age, sex, age at onset of AD, duration of eczema, parental history of AD, current usage of TCS, and results of blood tests (eosinophil and total immunoglobulin E [IgE] levels) were confirmed from the medical records for each patient.

Questionnaire

The caregiver attending a child patient—in most cases (approximately 95%) the mother—was asked to complete a questionnaire before the doctor examined the child. The questionnaire consisted of items concerning perceptions regarding TCS and the history of AD therapy. The items regarding perceptions of TCS included caregiver's steroid phobia, adverse effects, and perceived image of TCS therapy. Steroid phobia was assessed by asking "Would you agree to use TCS on your child's skin?" Those who answered "never" or "no, I'd rather not use TCS if I can avoid it" were defined as having steroid phobia. Preconceptions regarding adverse effects and the image of TCS therapy were ascertained by asking open-ended questions. The history of AD therapy was also ascertained using open-ended questions, and frequency of changing clinics, use of alternative therapies, and negative experiences with doctors were determined. Caregivers were also asked about the history of nonadherence to TCS therapy, how they applied TCS to the child, and the use of soap to bathe the child (Table 2).

Statistical Analyses

To check the validity of the single-item question about steroid phobia, the chi-square test was used to analyze for associations between the question "Would you agree to use TCS on your child's skin?" and the caregiver's image of TCS, history of nonadherence to TCS therapy, the caregiver's preconceptions regarding adverse effects, application of TCS, and use of alternative care. Then we performed bivariate logistic analysis of steroid phobia against patient sex, age, onset age, duration of eczema, severity of AD, parental history of AD, eosinophil count, total IgE level, frequency of changing clinics, and negative experience with doctors. Finally, we performed multiple logistic analysis to identify factors associated with steroid phobia, using as explanatory variables the factors that showed a marginal association with steroid phobia with $p < 0.4$ in the bivariate analysis. Data were analyzed using STATA software (Windows version 8.0; StataCorp, College Station, TX). $p < 0.05$ was considered to indicate statistical significance in all comparisons. The postestimated goodness-of-fit (Hosmer-Lemeshow) was confirmed for logistic regression analysis.

RESULTS

Patients

Four hundred forty-eight caregivers completed the questionnaire. We excluded 12 patients who had other

forms of inflammatory dermatitis, leaving 436 patients for the analyses: 286 male (65.6%) and 150 female (34.4%). Their mean age was 47.6 ± 48.9 months (range 2–236 mos). The characteristics of the patients are summarized in Table 1.

Confirmation of the Single-Item Question on Steroid Phobia

The incidence of steroid phobia measured using a single-item question was 38.3% of all caregivers (Table 1) and 58.7% of those with a history of nonadherence to TCS therapy (Table 2). We used the chi-square test to confirm the validity of the single-item question about steroid phobia, "Would you agree to use TCS on your child's skin?" There were strong correlations between negative perceptions regarding TCS (negative image of TCS, history of nonadherence to TCS therapy, strong apprehension regarding adverse effects of TCS, not a current user of TCS, and preference for alternative care) and the question (Table 2). Because these negative perceptions of TCS are a crucial component of steroid phobia, we considered this single-item question about steroid phobia to be useful.

Bivariate Analyses

In the bivariate analyses, female sex (odds ratio [OR] = 1.59, 95% confidence interval [CI] = 1.06–2.39), duration of eczema (OR = 0.93, 95% CI = 0.88–0.99), and paternal history of AD (OR = 1.91, 95% CI = 1.06–3.44) were significantly associated with steroid phobia; severity of AD and blood sample data were not (Table 3).

Multivariate Analyses

To evaluate the effects of confounding factors, a logistic regression model was adjusted for patient sex, age, duration of eczema, parental history of AD, frequency of changing clinics, and negative experience with doctors. In the multivariate analyses, female sex (adjusted OR [aOR] = 1.85, 95% CI = 1.20–2.85), child's paternal history of AD (aOR = 1.94, 95% CI = 1.03–3.58), and frequent changing of clinics (aOR= 1.25, 95% CI= 1.03–1.53) were significantly associated with steroid phobia (Table 4).

DISCUSSION

We found that the predictive factors for steroid phobia in caregivers of children with AD are patient female sex,

TABLE 1. Characteristics of Patients

	Mean (range) or	SD or %
Demographic		
Patient's sex M/F	286/150	65.6/34.4
Patient's age (months)	47.6 (2–236)	48.9
< 12	120	27.5
12–72	217	49.8
> 72	99	22.7
Clinical characteristic		
Onset age	10.4 (1–191)	21.3
< 12	338	77.5
12 < < 36	74	17.0
36<	16	3.7
Duration of eczema	36.8 (0–224)	43.3
Severity of (SCORAD) index		
Remission (0)	12	2.8
Mild (1–25)	159	36.5
Moderate (> 25)	175	40.1
Severe (> 50)	90	20.6
Parental history of	102	23.4
Mother	60	13.8
Father	51	11.7
Both	9	2.1
Eosinophil count (/1 L)	662.3 (0–5,592.1)	689.5
< 294	93	
294–467	93	
467–793	93	
> 793	93	
Eosinophil count (%)	6.74 (0–34.8)	4.86
< 3.55	93	
3.55 < < 5.7	95	
5.7 < < 8.7	92	
8.7<	92	
Total IgE (IU/mL)	2259.2 (2 97,600)	8692
< 53.9	93	
53.9 < < 310	93	
310 < < 1,141	93	
1,141<	94	
Steroid phobia		
Caregivers who were reluctant to use	167	38.3
Past consultation for AD		
Frequency of changing	2.1 (0–6)	1.2
Caregivers who had negative experiences with	80	18.4
Application of TCS		
Use of TCS		
Non-current	24	5.5
Past user	64	14.7
Current user	334	76.6
Caregivers who apply TCS sparingly at doctor's	171	39.2
Caregivers who apply TCS sparingly at own	81	18.8
Caregivers who apply TCS liberally at doctor's	58	13.3
Caregivers who apply TCS liberally at own	35	8
Alternative		
Caregivers who preferred alternative	71	16.3
Caregivers who don't use soap to bathe the child	25	5.7

AD, atopic dermatitis; SCORAD, Scoring Atopic Dermatitis; TCS, topical corticosteroids.

TABLE 2. Confirmation of Single-item Question About Steroid Phobia

Category	Steroid phobia (+) (N = 167)	Steroid phobia (-) (N = 262)	p-Value†
Image of TCS held by caregiver			
Negative	96 (62.3)	88 (37.1)	< 0.001*
Positive	7 (4.5)	65 (27.4)	
Both negative and positive	50 (32.5)	75 (31.6)	
Other	1 (0.6)	9 (3.8)	
History of non-adherence with TCS (by caregiver)			
Yes	90 (57.7)	63 (25.0)	< 0.001*
No	66 (42.3)	189 (75.0)	
Preconceptions regarding adverse effects of TCS (by caregiver)			
Adverse effects on the skin (including skin thinning and darkening)			
Yes	112 (67.1)	128 (48.9)	< 0.001*
No	55 (32.9)	134 (51.1)	
Skin thinning			
Yes	54 (32.3)	59 (22.5)	0.024*
No	113 (67.7)	203 (77.5)	
Skin darkening			
Yes	35 (21.0)	28 (10.7)	0.003*
No	132 (79.0)	234 (89.3)	
Systemic adverse effects of TCS			
Yes	57 (34.1)	53 (20.2)	0.001*
No	110 (65.9)	209 (79.8)	
Application of TCS			
Use of TCS			
Non-current user	11 (6.7)	10 (4.0)	< 0.001*
Past user	41 (25.2)	23 (9.1)	
Current user	111 (68.1)	219 (86.9)	
Caregivers who apply TCS sparingly at doctor's instruction	66 (50.0)	105 (49.3)	0.16
Caregivers who apply TCS sparingly at own judgment	38 (28.8)	43 (20.2)	
Caregivers who apply TCS liberally at doctor's instruction	**17 (12.9)**	**41 (19.2)**	
Caregivers who apply TCS liberally at own judgment	11 (8.3)	24 (11.3)	
Alternative care			
Caregivers who preferred alternative care			
Yes	37 (22.2)	34 (13.0)	0.013*
No	130 (77.8)	228 (87.0)	
Caregivers who don't use soap to bathe the child			
Yes	8 (4.9)	17 (6.6)	0.46
No	156 (95.1)	240 (93.4)	

*$p < 0.05$.
†Chi-square test.
Data given in parentheses are expressed as percentage.

child's paternal history of AD, and frequent changing of clinics for the patient but not severity of AD. These results suggest that greater attention to the clinical background of patients with AD is important in addressing steroid phobia, regardless of the severity of AD.

The incidence of steroid phobia among caregivers was 38.3% (Table 1), which is consistent with previous studies. A report from Hong Kong showed that 40% of patients with moderate and 60% of patients with severe AD expressed concern about using TCS, but there was no association between steroid phobia and severity of AD (6). In the United Kingdom, 72.5% of patients with AD worried about using TCS, and 36.5% of those had been nonadherent to TCS therapy (4). Of caregivers of children with AD in Australia, 40% answered that TCS was dangerous, and 20% said it was too dangerous to use on their child's skin (17). In France, 80.7% of the parents of children with AD and people with AD reported having fears about TCS, and 36% admitted nonadherence to treatment (18). Although methodologic differences make it difficult to compare these percentages directly, approximately one-third of parents are reluctant to use TCS on their children. Medical care providers need to be sensitive to this anxiety about using TCS in their daily clinical practice.

We also confirmed the validity of the single question about steroid phobia, "Would you agree to use TCS on your child's skin?" by finding strong correlations with negative perceptions regarding TCS (negative image of TCS, history of nonadherence to TCS therapy, strong apprehension regarding adverse effects of TCS, not a current user of TCS and preference for alternative care) (Table 2). This result was similar to a recent study that reported a correlation between steroid phobia and the need for reassurance, the belief that topical corticosteroids

TABLE 3. Correlation with Steroid Phobia (Bivariate)

	Bivariate OR	95% CI	p-Value
Patient's sex			
Male	Reference		0.025*
Female	1.59	1.06–2.39	
Patient's age (mos)			
< 12	Reference		0.027*
12–72	1.06	0.67–1.67	
> 72	0.54	0.30–0.96	
Onset age (mos)			
< 12	Reference		0.87
12–36	1.14	0.69–1.90	
> 36	1.05	0.38–2.88	
Duration of eczema (yrs)	0.93	0.88–0.99	0.018
Severity of AD (SCORAD index)			
Remission (0)	Reference		0.8
Mild (1–25)	0.59	0.18–1.90	
Moderate (25–50)	0.63	0.20–2.04	
Severe (> 50)	0.7	0.21–2.33	
Parental history of AD			
Mother			
No	Reference		0.39
Yes	1.28	0.73–2.22	
Father			
No	Reference		0.031*
Yes	1.91	1.06–3.44	
Eosinophil count (/1 L)			
< 294	Reference		0.82
294–467	0.89	0.49–1.63	
467–793	0.86	0.47–1.58	
793<	1.12	0.62–2.03	
Eosinophil count (%)			
< 3.55	Reference		0.51
3.55 5.7	0.75	0.40 1.38	
5.7–8.7	1.06	0.58–1.92	
> 8.7	1.16	0.64–2.10	
Total IgE (IU/ml)			
< 53.9	Reference		0.81
53.9–310	0.75	0.41–1.37	
310–1,141	0.93	0.51–1.68	
> 1,141	0.91	0.50 1.65	
Past consultation for AD			
Frequency of changing clinics	1.14	0.96–1.34	0.13
Caregivers with negative experience with doctors			
No	Reference		0.14
Yes	1.45	0.89–2.37	

*p < 0.05.

TABLE 4. Correlation with Steroid Phobia (Multivariate)

	†Multivariate OR	95% CI	p-Value
Patient's sex			
Male	Reference		0.005*
Female	1.85	1.20–2.85	
Patient's age (mos)			
< 12	Reference		
12–72	1.03	0.62–0.73	0.9
> 72	0.55	0.20–1.53	0.25
Duration of eczema (years)	0.96	0.86–1.07	0.45
Parental history of AD			
Mother			
No	Reference		0.39
Yes	1.15	0.64–2.05	
Father			
No	Reference		0.034*
Yes	1.94	1.03–3.58	
Past consultation for AD			
Frequency of changing clinics	1.25	1.03–1.53	0.026*
Caregivers with negative experience with doctors			
No	Reference		0.78
Yes	1.08	0.63–1.85	

*p < 0.05.
†Adjusted for all values listed above.

pass through the skin into the bloodstream, a prior adverse event, inconsistent information about the quantity of cream to apply, a desire to self-treat for the shortest possible time, or poor treatment adherence (18). Our observation suggests that appropriate education of patients to remedy negative perceptions of TCS would be one good strategy for addressing steroid phobia, as others have noted (2,4,19).

In this study, most patients and caregivers (91.3%) had used TCS, at least in the past (Table 1), which suggests that only a few caregivers had totally rejected TCS therapy for their child at the onset of AD. This implies that, whether the caregivers of children with AD become steroid phobic or not depends on the experience during AD therapy that they have received. An earlier study on factors related to steroid phobia suggested that a preconception of ineffectiveness or adverse effects of TCS was associated with steroid phobia (14).

Our results indicate that the patient's sex, a paternal history of AD, and frequent changing of clinics for the patient were associated with steroid phobia (Table 4).

There have been no studies focusing on patient sex as an association factor for steroid phobia. In our study, we found that misinterpretation of skin darkening as a TCS side effect was significantly associated with steroid phobia (Table 3). Light skin, bright eyes, and black hair have long been considered essential factors for beauty in Japanese girls (20). Thus, we assume that our finding may be due to a cultural factor in Japan that—together with the misinterpretation—leads the parents of girls to be reluctant to use TCS for their children.

We found that the child's paternal—but not maternal—history of AD was associated with steroid phobia (Tables 3 and 4). If parents had experienced treatment failure in the past, they tended to feel steroid phobia for their children. On the other hand, parental history of AD could be a remedy for steroid phobia because they may have been advised about or read correct knowledge regarding TCS during their previous experience with TCS therapy. We should have asked whether the parents had experienced treatment failure in the past, but in Japan, steroid phobia was widespread in 1980s (12). Therefore some parents with AD had not received

appropriate or successful TCS therapy and might have negative perceptions of TCS. Although our results showed that one of the predictive factors for steroid phobia was child's paternal history of AD, this might be because the caregivers of most Japanese children with AD are their mothers. Mothers would have more chances to acquire the correct knowledge regarding TCS from doctors, and any steroid phobia they might have might be relieved. On the other hand, fathers would have less opportunity to be in contact with their children's doctor, and their steroid phobia might remain. Family or relatives have also been reported to be major sources of steroid phobia (4,6,9). Thus, a father with a history of AD might cause steroid phobia in the mother–caregiver. We believe that these results indicate that correct information regarding TCS is needed not only for mother–caregivers, but also for father–partners.

Frequent changing of clinics suggests a history of treatment failure or distrust of medical care services, because in Japan patients can go to clinics without any referral. This finding of frequent changing of clinics might indicate that negative experiences with doctors, including ineffectiveness of TCS, are associated with steroid phobia.

On the other hand, AD severity was not associated with steroid phobia, which is consistent with previous studies (6,18). Furthermore, as others have noted (14,21), in our study, personal negative experiences and attitudes such as a preconception of ineffectiveness might have been more important as factors underlying steroid phobia than objective factors such as AD severity evaluated by a doctor. This finding suggests that AD severity should not be a factor in evaluating whether a patient has steroid phobia.

This study has a number of limitations. First, it was conducted at a national medical center where most patients would know that doctors would use TCS for AD therapy. Therefore, strongly phobic patients might have been underrepresented. Further studies of such patients should be undertaken to compare with our results. Second, the questionnaire about steroid phobia was not validated in other studies (4,6,17,18), although we confirmed that a single-item question, "Would you agree to use TCS on your child's skin?" was associated with negative perceptions of TCS (Table 2). Although we tried to identify negative experiences with doctors using open-ended questions regarding history of AD therapy, a future study should have multiple-choice questions and focus on a history of treatment failure and the patient–doctor relationship. Third, because of the cross-sectional study design, the presumed cause-and-effect relationship between predictive factors and steroid phobia may be the reverse; for example, steroid phobia may have been the reason for frequent changing of clinics. Fourth, other unmeasured factors, such as parental level of education, social status, and personality, might have confounded the results. Further studies should be done regarding these factors in steroid phobia, although medical expense would not be burden for Japanese caregivers because there are medical care subsidies for children in Japan.

In conclusion, our study suggests that greater attention to patient sex, paternal history of AD, and frequency of changing clinics for the patient will aid physicians in addressing steroid phobia, whereas AD severity does not play any role. It is necessary to devote sufficient time to careful elucidation of the clinical background of patients with AD from their caregivers. This would help physicians understand any steroid phobia of caregivers and contribute to overcoming steroid phobia in patients with AD and their caregivers.

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Galectin-9 Enhances Cytokine Secretion, but Suppresses Survival and Degranulation, in Human Mast Cell Line

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Competing Interests: Toshiro Niki and Mitsuomi Hirashima are board members of GalPharma Co., Ltd. The authors have the following patents related to material pertinent to this article: "Novel modified galectin 9 proteins and use thereof" which is applied by GalPharma and issued in Japan (4792390), the USA (8,268,324), EPC (1736541), Canada (2,561,696), India (239130), and Korea ((10-1222281) as of 2013.12.2). The authors have the following products related to material pertinent to this article: stable-form Gal-9. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Galectin-9 (Gal-9) was first identified as a chemoattractant and activating factor for eosinophils. [1–3] It is abundantly expressed in various tissues, especially the epithelium of the gastrointestinal tract, and in a variety of cells such as macrophages, eosinophils, mast cells, fibroblasts and synovial cells. [4–7].

Gal-9 influences various biological functions such as cell aggregation, adhesion, apoptosis, survival, activation and differentiation by binding to T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3). [5,8,9] Like Gal-9, Tim-3 is also expressed on various types of cells, including Th1 cells, [9] Tc1 cells, [10] Th17 cells, [11] NK cells, [12] NKT cells, [13,14] dendritic cells (DC) [15] and mast cells (MCs). [16,17] It is known that Gal-9 has anti-tumor activity by promoting activation of NK cells [18] and cytotoxic T lymphocytes by enhancing DC maturation. [19] Moreover, Gal-9 induces aggregation of melanoma and breast cancer cell lines and suppresses metastasis. [20–22] It was suggested that Gal-9 is a negative regulator of development of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) in mice. Indeed, like anti-TIM-3 mAb, [10] Gal-9 can suppress development of EAE by inducing Th1 cell apoptosis via TIM-3. [9] Gal-9 can also attenuate development of CIA by inhibiting differentiation of Th17 cells while enhancing differentiation of regulatory T cells. [23] Moreover, expression of each of Gal-9 and TIM-3 was shown to be increased in the lungs of rodents during allergic airway inflammation, [24–26] suggesting roles for Gal-9 and TIM-3 in induction of that disease. Indeed, Gal-9 administration to mice suppressed ovalbumin- and house dust mite antigen-induced airway inflammation and hypersensitivity. [27] In the setting, Gal-9 bound to CD44, interfering with binding of hyaluronan, a known ligand for CD44, and resulting in inhibition of Th2 cell recruitment through CD44-hyaluronan

interaction. [27] On the other hand, the role of TIM-3 in development of ovalbumin-induced airway inflammation and hypersensitivity is controversial. That is, the response was attenuated in mice treated with anti-TIM-3 mAb, [24] but normal in TIM-3-deficient mice. [28] Although the reason for that apparent discrepancy is unclear, the report using anti-TIM-3 mAbs did not fully characterize them, i.e., whether they were agonistic, blocking or depletion Abs. These observations suggest that the biological function of Gal-9 may be mediated independently of TIM-3 in certain settings. In support of this, binding of Gal-9 to IgE blocks IgE/Ag complex formation and thus inhibits IgE/Ag-FceRI crosslinking-induced degranulation of mast cell/basophilic cell lines. [29] In contrast, we showed that anti-TIM-3 agonistic antibody promoted cytokine secretion, but did not influence degranulation, by mouse bone marrow cell-derived cultured mast cells (BMCMCs) after IgE/Ag-FceRI crosslinking. [16] On the other hand, the role of Gal-9 in mast cell function in the absence of IgE remains unclear. Therefore, in the present study we examined the role of Gal-9 in the functions of a human mast cell line, HMC-1, which does not express FceRI, in the absence of IgE/Ag stimulation. We found that human Gal-9 enhanced cytokine secretion, but suppressed survival and degranulation, of HMC-1. These observations suggest that Gal-9 has dual properties as a regulator and activator of mast cells.

Materials and Methods

Cell Culture

HMC-1 cells (a human mast cell line) [30] were cultured in a-minimum essential medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin under a humidified atmosphere of 5% CO_2 at 37uC. Half of the medium was replaced twice per week. Normal human bronchial epithelial cells (NHBEs), normal human coronary artery endothelial cells (HCAECs) and normal human lung fibroblasts (NHLF) were obtained from Lonza (Wakersville, MD, USA) and were cultured as described elsewhere. [31].

Quantitative PCR

Total RNA samples were isolated from HMC-1 cells, NHBEs and HCAECs using RNeasy (Qiagen, Valencia, CA, USA) and digested with RNase-free DNase I (Qiagen) in accordance with the manufacturer's instructions. Human universal reference (HUR) RNA (BD Biosciences, Palo Alto, CA, USA) was used as a positive control. Then first-strand cDNA was synthesized from the isolated RNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Primers for TIMs and GAPDH were designed as follows: TIM-1 (sense, 59-TGT TCC TCC AAT GCC TTT GC-39 antisense, 59-TTG CTC CCT GCA GTG TCG TA-39), TIM-3 (sense, 59-CAA TGC CAT AGA TCC AAC CAC C-39 antisense, 59-GCA GTG GAC AGA ACC TCC AAA A-39), TIM-4 (sense, 59-TCC TGC TGA CAT CCA AAG CA-39 antisense, 59-TGG GAG ATG GGC ATT TCA TT-39) and GAPDH (sense, 59-GAA GGT GAA GGT CGG AGT C-39 antisense, 59-GAA GAT GGT GAT GGG ATT TC-39). To determine the exact copy numbers of the target genes, quantified concentrations of the purified PCR products of TIM-1, TIM-3, TIM-4 and GAPDH were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 5 ng of the total RNA samples were used for each quantitative PCR. The mRNA expression levels were normalized to the GAPDH level in each sample.

Flow Cytometry

Cells (HMC-1 cells and PBMCs) were incubated with human AB serum (Lonza) at 4uC for 5 min, and then stained with PE-conjugated anti-human TIM-1 mAb (Clone Name 1D12, BioLegend, San Diego, CA, USA), PE-conjugated anti-human TIM-3 mAb (Clone Name F38-2E2, BioLegend), PE-conjugated anti-human TIM-4 mAb (Clone Name 9F4, BioLegend) and PE-conjugated mouse IgG1 (Clone Name MOPC-21, BioLegend) at 4uC for 30 min. The expression of TIMs on the cells was determined with a FACS Canto II using Diva Software (BD Biosciences, San Jose, CA, USA).

Western Blotting

HMC-1 cells (56 10^5 cells/well in a 24-well plate) were treated with 1 nM recombinant human galectin-9 (rhGal-9) (GalPhama Co., Ltd., Kagawa, Japan) at 37uC for the indicated time periods. Then the cells were lysed and sonicated in 200 ml of NuPAGE sample buffer (Invitrogen, Carlsbad, CA, USA) containing 5% 2-mercaptoethanol. Proteins in the whole-cell lysates were separated by SDS-PAGE (5–15% Ready Gels J Bio-Rad) gel electrophoresis and transferred to nitrocellulose membranes (iBlot Gel Transfer Stacks, mini; Invitrogen). Immunoblotting was performed using rabbit anti-phospho-p44/42 MAPK (Erk1/2) mAb (clone D13.14.4E; Cell Signaling Technology, Danvers, MA) and rabbit anti-p44/42 MAPK (Erk1/2) mAb (clone 137F5; Cell Signaling Technology) as the 1st Abs and horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) as the 2nd Ab. The protein bands were visualized by enhanced Pierce Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA).

Cell Survival

HMC-1 cells were pretreated with or without 5 mg/ml mitomycin-C (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) for 2 hours. After washing, the cells (46 10^5 cells/ml) were cultured in the presence and absence of 0.25, 0.5 and 1 nM rhGal-9 at 37uC for 0, 24 or 48 h. Live cells were counted under a microscope after trypan blue staining. The cells were incubated with FITC-conjugated annexin V and propidium iodide (MEBCYTO-Apoptosis Kit; MBL Co., Ltd., Nagoya, Japan), and the percentage of propidium iodide-negative and annexin V-positive apoptotic cells was determined using a FACSCanto II with Diva Software.

Caspase Activity

HMC-1 cells (16 10^5 cells/ml) were cultured in the presence and absence of 0.5 nM rhGal-9 or 0.1 nM staurosporine (Cayman Chemical Company, Ann Arbor, MI, USA) at 37uC for 16 hours. Then the caspase-3/7 activities in the cells were determined by Caspase-Glo 3/7 assay (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. The luminescence (Relative Light Unit [RLU]) of each sample was measured with a fluorescence plate reader (ARVO X5, PerkinElmer, Waltham, MA, USA) at 490/535 nm.

β-hexosaminidase Release Assay

HMC-1 cells (16 10^5 cells/well in a 96-well plate; not treated with mitomycin C) were pre-treated with rhGal-9 (0, 0.25, 0.5 and 1 nM) at 37uC for 30 min, and then stimulated with 0.1 mg/ml PMA (Sigma Chemical Co.) and 1 mg/ml ionomycin (Sigma Chemical Co.) at 37uC for 30 min. The culture supernatants were collected, and the activity of β-hexosaminidase in each was determined as described previously, with minor modification. [32] In brief, 50-ml samples were incubated with 100 ml of 1.3 mg/ml

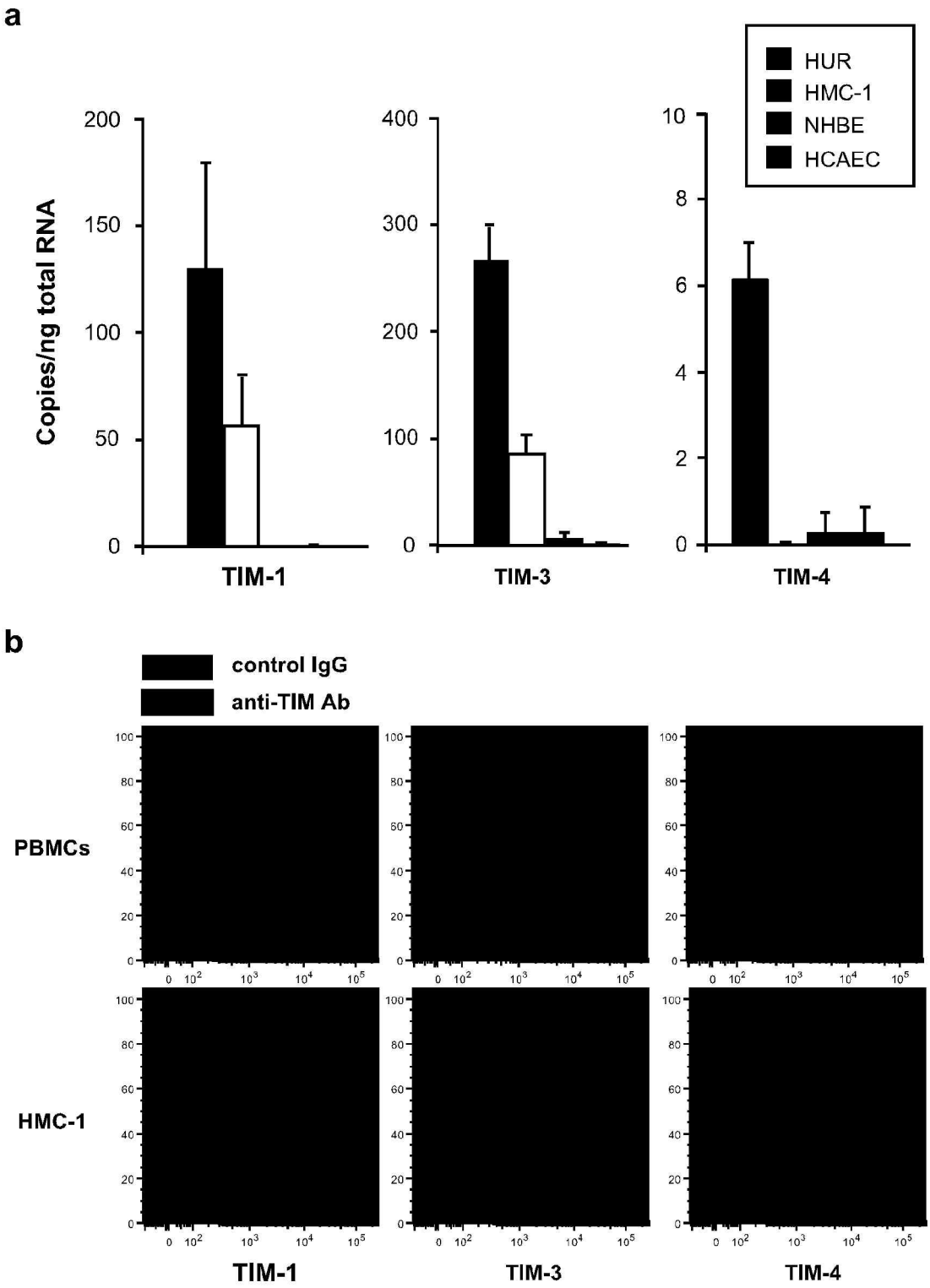

Figure 1. Expression of TIM family members in cultured human mast cell line and primary cells. (a) The mRNA expression for TIM family members (TIM-1, TIM-3 and TIM-4) in a human mast cell line, HMC-1, normal human bronchial epithelial cells (NHBEs) and normal human coronary artery endothelial cells (HCAECs) was determined by quantitative PCR. Human universal reference (HUR) RNA was used as a control. (b) The cell surface expression of TIM family members (TIM-1, TIM-3 and TIM-4) on HMC-1 cells and PBMCs was determined by flow cytometry. Shaded areas = isotype-matched control IgG staining, and bold lines = anti-TIM mAb staining. Data show a representative result of two independent experiments.

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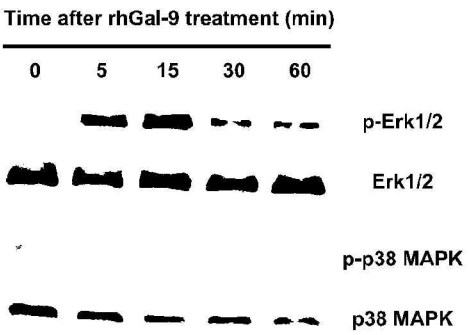

Figure 2. Galectin-9 induces phosphorylation of Erk1/2, but not p38 MAPK, in HMC-1 cells. HMC-1 cells were cultured in the presence of 1 mM recombinant human galectin-9 (rhGal-9) for the indicated times. Then the levels of phosphorylation of Erk1/2 and p38 MAPKin the cells were determined by western blot analysis. Data show a representative result of three independent experiments.
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p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Chemical Co.) in 0.1 M sodium citrate (pH 4.5) in a 96-well microtiter plate at 37uC for 1 h. The enzymatic reaction was stopped by addition of 50 ml of 0.4 M glycine (pH 10.7) to each well. Enzymatic activities (OD405) were measured using a plate reader. Data show the percent release of β-hexosaminidase under various conditions of stimulation relative to the total amount of β-hexosaminidase in the cells, as measured in the supernatants of frozen and thawed cells.

ELISA

HMC-1 cells (16 10^5 cells/ well in a 96-well plate) were treated with various concentrations of rhGal-9 in the presence and absence of 20 mM lactose (Nacalai Tesque, Kyoto, Japan) or sucrose (Wako, Osaka, Japan) at 37uC for 18 h. In some cases, HMC-1 cells were treated with ERK inhibitor (PD98059; Calbiochem, La Jolla, CA, USA), ERK inhibitor control (SB202474; Calbiochem) or solvent (0.1% (v/v) DMSO) alone at 37uC for 30 min, and with rhTIM-3-Fc (R&D Systems, Minneapolis, MN, USA) or human IgG (Sigma Chemical Co.) at 37uC for 1 hour before exposure to rhGal-9. The levels of IL-6, IL-8 and MCP-1 in the culture supernatants were determined with ELISA kits (R&D Systems) in accordance with the manufacturer's instructions.

Statistics

All data are expressed as means 6 SD. The unpaired Student's t-test, two-tailed, or ANOVA, as appropriate, was used for statistical evaluation of the results. P, 0.05 was considered statistically significant.

Results

Expression of TIM Family Members' mRNA in Human Mast Cell Line

As in our earlier study using mouse mast cells (16), we first examined the expression of mRNA for TIM family members (TIM-1, TIM-3 and TIM-4) in HMC-1 cells and other human primary cells (NHBE and HCAEC, as negative controls) by quantitative PCR. We found constitutive expression of mRNA for both TIM-1 and TIM-3 in HMC-1 cells, but not in NHBE or HCAEC (Figure 1a). On the other hand, expression of TIM-4 mRNA was barely detectable in these cells. Next, we determined the surface protein expression of the TIM family members (TIM-1, TIM-3 and TIM-4) in HMC-1 cells and PBMCs by flow cytometry. In contrast to mRNA expression, TIM-1 and TIM-3 as well as TIM-4 were barely detectable on either HMC-1 cells or PBMCs (Figure 1b).

Gal-9 Induces Phosphorylation of Erk1/2 in HMC-1 Cells

We previously demonstrated that IL-33 can induce cytokine secretion by human mast cells, although surface expression of ST2, a component of IL-33R, is barely detectable on these cells by flow cytometry [33]. Likewise, although surface expression of TIM-3 is barely detectable on HMC-1 cells, Gal-9, which is a ligand for TIM-3, may play some role in activation and/or regulation of HMC-1 cells. Thus, we examined the effect of rhGal-9 on the phosphorylation of signaling molecules in those cells by immunoblot analysis. We found that rhGal-9 induced phosphorylation of Erk1/2, but not p38 MAPK, in HMC-1 cells (Figure 2), suggesting that rhGal-9 may influence the function of HMC-1 cells.

Gal-9 Induces Apoptosis of HMC-1 Cells

We next examined the effects of Gal-9 on HMC-1 cell survival. After treatment with or without mitomycin C, HMC-1 cells were cultured in the presence and absence of 0.25, 0.5 and 1 mM rhGal-9 for 24, 48 and 72 hours. The number of trypan blue-negative viable cells was significantly decreased by 1 mM rhGal-9, while the percentage of propidium iodide-negative and annexin V-positive apoptotic cells was significantly increased irrespective of mitomycin C treatment (Figure 3a and 3b). In association with this, as in the case of staurosporine treatment, the levels of caspase-3/7 activity in HMC-1 cells were also significantly increased after rhGal-9 treatment (Figure 3c). These findings indicate that rhGal-9 induces apoptosis of HMC-1 cells.

Gal-9 Inhibits Degranulation of HMC-1 Cells

We next evaluated the effect of rhGal-9 on degranulation of HMC-1 cells. HMC-1 cells were treated with 0, 0.25, 0.5 and 1 mM of rhGal-9 for 30 min prior to stimulation with PMA+ionomycin. The level of degranulation, assessed by the release of β-hexosaminidase from the cells, was significantly suppressed by pretreatment with the optimal dose (0.5 mM) of rhGal-9 (Figure 4a). In the setting, pre-treatment with 0.5 mM rhGal-9 resulted in inhibition of cell survival (assessed as trypan blue-negative cells, and propidium iodide-negative and annexin V-positive apoptotic cells by flow cytometry) as well as degranulation after stimulation with PMA+ionomycin (Figure 4b–d), suggesting that the reduced degranulation of HMC-1 cells may be due to apoptosis of the cells after rhGal-9 treatment. On the other hand, the relative levels of degranulation per live HMC-1 cells were significantly reduced by pre-treatment with rhGal-9 (Figure 4e), suggesting that Gal-9 also inhibited PMA- and ionomycin-induced degranulation of HMC-1 cells independently of Gal-9-mediated apoptosis. Thus, these observations suggest that Gal-9 can inhibit PMA- and ionomycin-induced degranulation of HMC-1 cells, both directly and indirectly.

Gal-9 Induces Cytokine Production by HMC-1 Cells

In contrast to the inhibitory effect of rhGal-9 on degranulation, we found that IL-6, IL-8 and MCP-1 production by HMC-1 cells was dose-dependently induced by rhGal-9 (Figure 5a). It is known that most biological effects of galectins are mediated by their carbohydrate-binding activities. [5] In support of that, rhGal-9-mediated IL-6 production by HMC-1 cells was strongly suppressed by addition of an excessive amount of lactose but not sucrose (Figure 5b). Moreover, rhGal-9-mediated IL-6 production

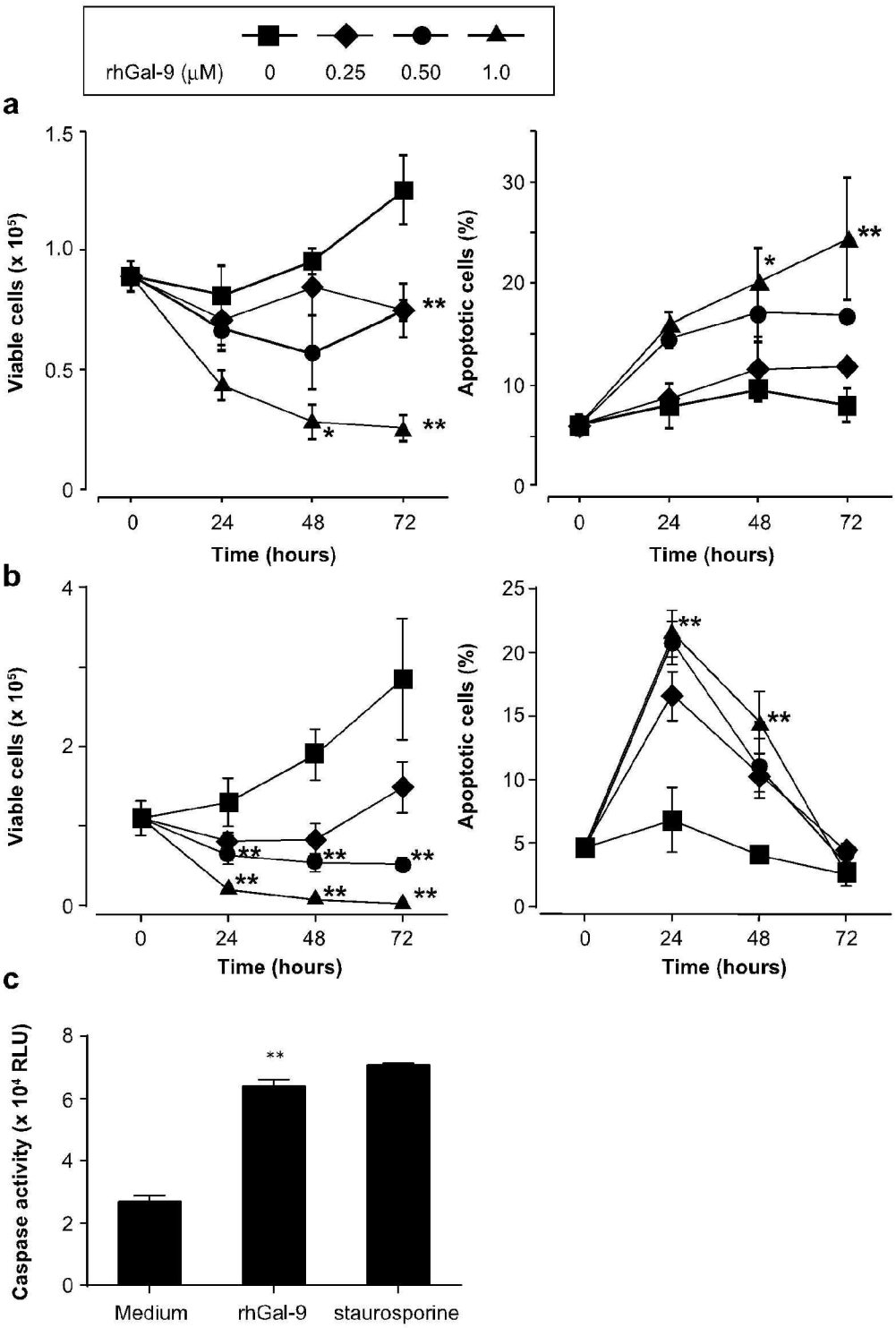

Figure 3. Galectin-9 induces apoptosis of HMC-1 cells. (a, b) HMC-1 cells pre-treated with (a) or without mitomycin C (b) were cultured in the presence of 0, 0.25, 0.5 or 1 mM recombinant human galectin-9 (rhGal-9) for the indicated time periods. The number of viable cells was determined by trypan blue staining. The proportion of propidium iodide-negative and annexin V-positive apoptotic cells was assessed by flow cytometry. (c) HMC-1 cells (no mitomycin C pre-treatment) were cultured in the presence and absence of 0.5 mM rhGal-9 or 0.1 mM staurosporine for 16 hours. Then the levels of caspase activity in the cells were determined. Data show the mean 6 SD of triplicate samples and are a representative result of three independent experiments. *p, 0.05 and **p, 0.01 versus the corresponding values for the vehicle control.
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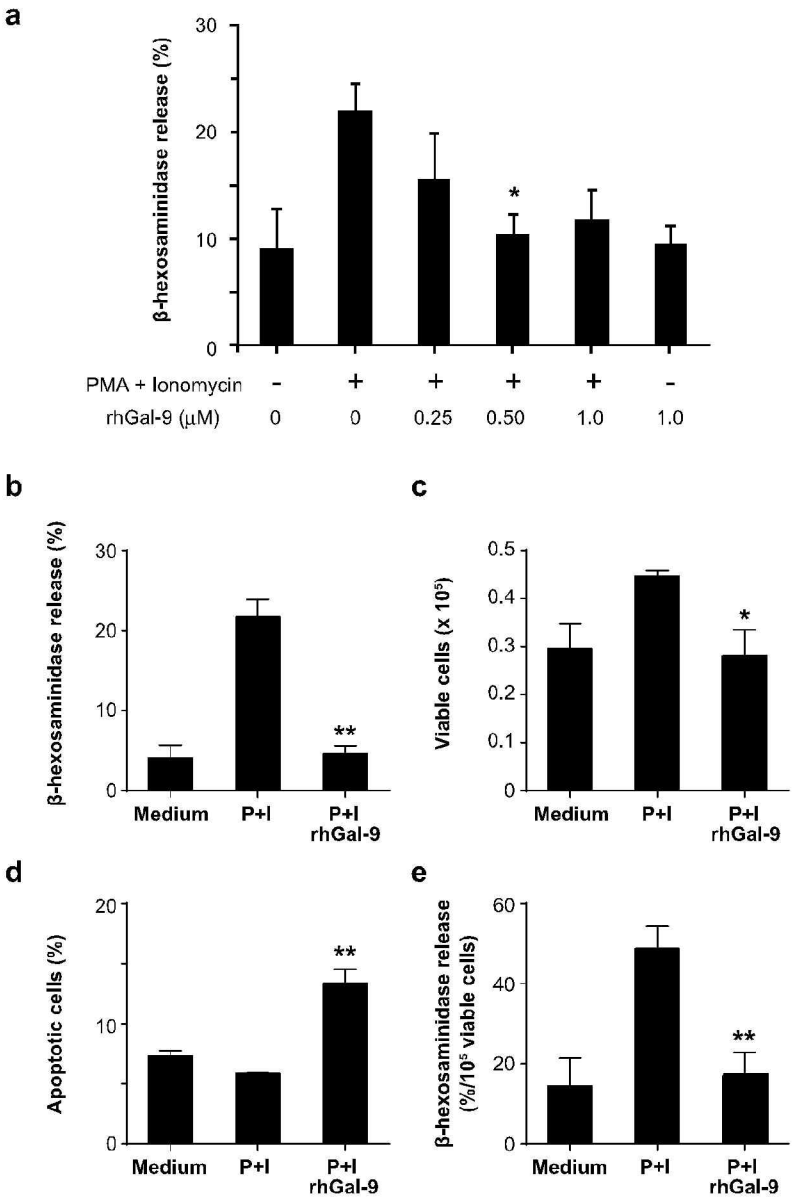

Figure 4. Galectin-9 inhibits PMA- and ionomycin-dependent degranulation of HMC-1 cells. (a, b) HMC-1 cells were treated with 0, 0.25, 0.5 or 1 mM (a) and 0 or 0.5 mM (b) recombinant human galectin-9 (rhGal-9) for 30 min. The cells were then stimulated with 0.1 mg/ml PMA +1 mg/ml ionomycin for 30 min. The level of degranulation was assessed from the activity of β-hexosaminidase in the culture supernatant and plotted as the percent release. (c) The number of viable cells in (b) was determined by trypan blue staining. (d) The proportion of propidium iodide-negative and annexin V-positive apoptotic cells in (b) was assessed by flow cytometry. (e) The relative level of degranulation per live HMC-1 cells was determined as (b)/(c). Data show the mean 6 SD of triplicate samples and are a representative result of three (a) or two (b–e) independent experiments. *p, 0.05, **p, 0.01 versus PMA+ionomycin alone.
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by HMC-1 cells was inhibited by addition of a soluble form of TIM-3 (rhTIM-3/Fc) but not control human IgG (Figure 5c), and by pre-treatment with PD98059 (an ERK1/2 inhibitor) but not SB202474 (control for the ERK1/2 inhibitor) (Figure 5d), suggesting that Gal-9-mediated ERK1/2 activation is required for cytokine production by HMC-1 cells.

Discussion

In the present study, we demonstrated that Gal-9 has dual roles in the functions of a human mast cell line, HMC-1. That is, Gal-9 reduced survival by inducing apoptosis and suppressed degranulation in HMC-1 cells, while it induced cytokine and chemokine production by these cells by activating ERK1/2.

We show that Gal-9 induced phosphorylation of Erk1/2, but not p38 MAPK, in HMC-1 cells (Figure 2). On the other hand, however, Gal-9 induced maturation of human monocyte-derived DCs through activation of p38 MAPK, but not ERK1/2. [34] These observations suggest that the Gal-9-mediated signaling pathway may be different in distinct types of cells. Alternatively, the difference between DCs and HMC-1 cells may be mediated by

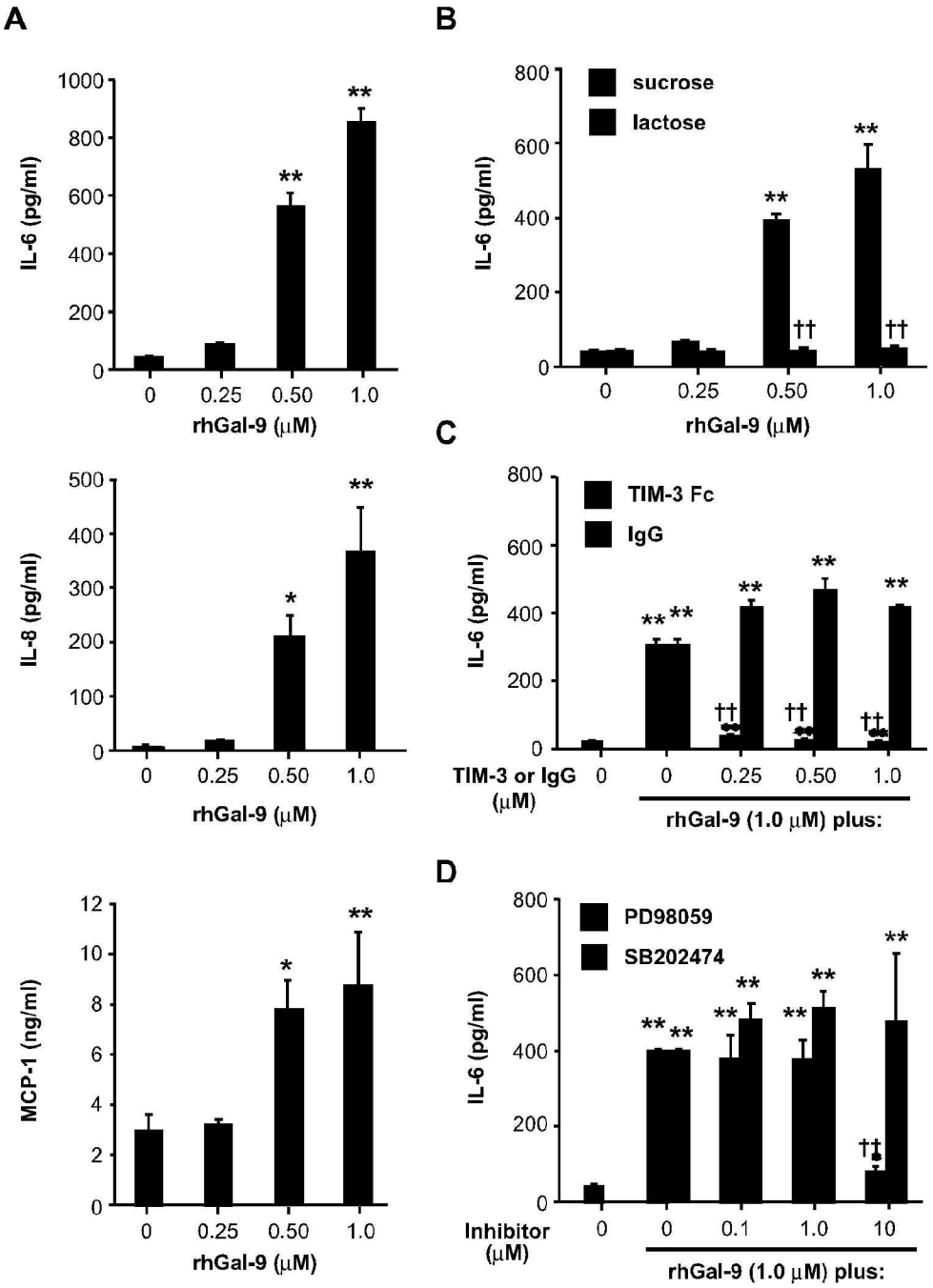

Figure 5. Gal-9 induces cytokine and chemokine production by HMC-1 cells. ELISA was performed to determine the levels of IL-6, IL-8 and MCP-1 in the culture supernatants of HMC-1 cells (a), HMC-1 cells pre-treated with 20 mM lactose or sucrose (b), HMC-1 cells pre-treated with recombinant human TIM-3/Fc (rhTIM-3/Fc) or control human IgG (human IgG) (c) and HMC-1 cells pre-treated with ERK inhibitor (PD98059) or its control (SB202474) (d) after 18 hours' stimulation with 0, 0.25, 0.5 or 1 mM recombinant human Galectin-9 (rhGal-9). Data show the mean 6 SD of triplicate samples and are a representative result of three independent experiments. *p, 0.05 and/or **p, 0.01 versus 0 mM rhGal-9 (a–d), and {p, 0.05 and/or {{p, 0.01 versus sucrose (b), control human IgG (c) or ERK inhibitor control (d).
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distinct receptors such as TIM-3 and unknown molecules that interact with Gal-9. Indeed, the lectin property of Gal-9 was required for Gal-9-mediated cytokine production by HMC-1 cells (Figure 5b), but not by human DCs. [34] In addition, Gal-9 induced apoptosis of HMC-1 cells (Figure 3) as well as thymocytes, Th1 cells and Th17 cells in mice, and human melanoma cell lines. [2,7,21–23,35] In contrast, we previously demonstrated that anti-TIM-3 antibody, which enhanced IgE/Ag-mediated cytokine production as an agonistic antibody, suppressed apoptosis of IL-3-induced mouse bone marrow cell-derived cultured mast cells. [16]

These observations suggest that Gal-9-mediated responses may be dependent or independent of TIM-3, in different cells, since TIM-3 is also known to bind to phosphatidylserine. [36].

It was shown that Gal-9 bound IgE, resulting in inhibition of IgE/antigen-FcεRI-mediated degranulation in mouse mast cell lines by preventing IgE/antigen complex formation. [29] In the present study, because HMC-1 cells do not express FcεRI, [37] we assessed the effect of Gal-9 on PMA/ionomycin-mediated degranulation of HMC-1 cells. Figure 4 shows that Gal-9 suppressed that degranulation both directly and indirectly, suggesting that there might be distinct mechanisms underlying the inhibitory effects of Gal-9 on IgE/antigen-FcεRI-mediated and PMA/ionomycin-mediated mast cell degranulation.

Studies in rodents found that treatment with Gal-9 before antigen challenge resulted in attenuation of ovalbumin- and mite allergen-induced allergic airway inflammation as well as passive cutaneous anaphylaxis after antigen challenge. [27,29] Gal-9's attenuation of such disorders in mast cells [29] was due to suppression of degranulation, rather than induction of cytokines and chemokines, probably independent of TIM-3, since TIM-3-deficient mice normally developed allergic airway inflammation. [28] However, treatment with Gal-9 after antigen challenge may exacerbate inflammation in the late phase of allergic diseases by enhancing cytokine and chemokine production by mast cells and recruiting eosinophils to local inflammatory sites.

In conclusion, Gal-9 appears to play dual roles in the function of human mast cell line. Our results suggest that Gal-9 may modulate the induction and progression of allergic diseases by suppressing degranulation and enhancing cytokine and chemokine production of mast cells. In addition, Gal-9 may be a potential therapeutic target for immediate allergic reactions induced by mast cell degranulation.

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Author Contributions

Conceived and designed the experiments: TO MI S. Nakae. Performed the experiments: RK TO. Analyzed the data: RK TO S. Nakae. Contributed reagents/materials/analysis tools: MI TN MH KI HT S. Nonoyama AM HS KM. Wrote the paper: RK TO S. Nakae.

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