分担研究報告書

アトピー性皮膚炎の発症・症状の制御および治療法の確立普及に関する研究
副題）痒み・掻破行動と皮膚炎増悪メカニズムに関するアトピーモデルを用いた研究

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研究要旨
タクロリムス（FK）は本邦で開発され、アトピー性皮膚炎治療に用いられているが、その抗かゆみメカニズムはまだ明らかでない。我々はステロイド他薬剤の比較実験から、FKの抗かゆみ効果が血清IgEや局所IL-17A産生とかかわりを持つことをしめした。
また、そのようなFKの抗かゆみ効果は外用終了3日程度持続し、その後次第に効果が薄れて、6日目には効果がなくなることを示した。これは我々のアトピー性皮膚炎におけるプロトピック軟膏を用いた抗かゆみ効果の実験結果と合致し、プロアクティブ療法の外用間隔を考えるうえで重要であると思われる。

A．研究目的

アトピー性皮膚炎（ＡＤ）の慢性皮膚病変部においては、表皮内知覚神経侵入・伸長とその執拗な痒みとの関連が指摘されている。マウスのTh2型慢性皮膚炎モデルにおいてもＡＤ治療外用薬であるタクロリムス(FK506；FK)は表皮内神経伸長を抑制し、掻破行動も抑制すると、関連が報告されている。我々はこれまでに、ハプテンおよびダニ抗原誘発性のアトピー性皮膚炎モデルにおいて、MAPK /ERK kinase1/2 (MEK1/2)阻害薬のひとつであるCX659S (CX) が、FKと同程度に表皮内神経伸長は抑制する一方,FKとは異なり掻破行動の抑制はしないことを確認した。

１．掻破行動を抑制したFKと抑制しなかったCXとの検討では、血清IgE値や局所でのIL-17A発現が掻破行動と何らかの関連を持つ可能性が考えられた。前回、抗IgE抗体を投与による治療を試みたが血清IgE値を軽度低下させたものの、完全なブロッキングができなかった。

２．また、FK外用の抗かゆみ効果が外用終了からどの程度継続するかは、臨床上の治療計画に非常に重要である。

B．研究方法

１．IgE抗体がシグナルを伝達する受容体であるFce受容体(FceRI)のノックアウト(KO)マウスを用いてIgE上昇と掻破行動をコントロールマウスと比較検討する。

２．C57BL/6マウスの剃毛した背部と耳に、0.5%ピクリルクロライド（ＰＣ）を週に２回、３週間塗布してＴｈ２型慢性皮膚炎を発症させ、同時に外用薬としてFKを用いて連日外用する。最終外用後、掻破行動、皮膚炎の状態、組織像、mRNA等を日ごとに計測した。

（倫理面への配慮）
これらの研究に関する動物実験プロトコールおよび遺伝子組み換え実験については施設倫理委員会で承認されている。

C．研究結果

１．FceRIKOマウスを購入し、施設内に清潔マウスへの人工授精によるクリーンアップ作業を行った。２度目で成功し、現在交配して少数のヘテロマウスを施設移入できた。

２．FK外用により、ハプテン反復塗布による慢性に皮膚炎におけるマウスの掻破行動はこれまで得られていた結果と同様に有意に抑制されていたが、外用終了1、2，3日後までは、ハプテンを再チャレンジしても有意に掻破行動を抑制していた。終了4日目からは次第に掻破行動の抑制効果が薄れ、6日後には無治療群と同レベルに戻った。

D．考察

1.FceRIKOマウスは現在KOコロニーをマウスの再樹立と繁殖を目指して交配中である。

２．今回、FK外用終了後、3日まではマウスの慢性皮膚炎の掻破行動が抑制されることがしめされた。これは臨床ではADの治療法を考えるうえで重要である。中等症、重症AD患者の病勢をコントロールする方法としてプロアクティブ外用療法が注目され、実際に大きな効果を上げている

が、病勢コントロール後、治療薬の外用間隔を空けていくことが患者の負担、副作用の低減上、重要である。我々は臨床研究で FK のプロアクティブ療法の効果を示しているが（Takeuchi S et al. Ann Dermatol, 2012）、かゆみが改善した後に外用を中止した群では、全例が痒みを再発し、その中央値が 3 日であったことを考えると、今回のマウスによる FK の抗かゆみ効果継続期間の検証結果はほぼ一致していると思われ、非常に興味深い。

E．結論

1．FceRIKO マウスで IgE 上昇と掻破行動の関連を近日中に検証できる見込みである。

2．今回 FK の抗かゆみ効果の継続が臨床研究だけでなく、客観的なマウスモデルでも示された。かゆみは掻破行動を介して AD 皮膚症状を悪化させるため、プロアクティブ療法における外用間隔としては、週に 2 日（約 3 日に 1 回外用）程度の外用が多くの人に無理なく継続できる外用間隔であるかもしれない。今後もマウスモデルを介して AD の病態、治療のメカニズムに迫りたい。

F．健康危険情報

なし。

G. 研究発表

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H. 知的財産権の出願・登録状況（予定を含む。）

1. 特許取得

なし。

2. 実用新案登録

なし。

3. その他

なし。

分担研究報告書

アトピー性皮膚炎の発症・症状の制御および治療法の確立普及に関する研究
1．脊髄内痒み神経の同定に関する研究　2. 石垣島コホート研究、
3．本土・琉球クラスターにおけるアトピー関連遺伝子の探索に関する研究

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研究要旨
1．アトピー性皮膚炎で重要なかゆみに対し、ラットモデルを用いてその痒み特異的神経の伝導路を明らかにした。より中枢でのかゆみ伝導路を見出し、かゆみの制御につなげたい。
2. 卵アレルギーの既往が乳幼児期のアトピー性皮膚炎の危険因子のひとつであり、また、アンケート上での卵アレルギーの既往やオボムコイドIgE 卵アレルギーの既往がアトピー性皮膚炎の危険因子のひとつであり、乳幼児期のアトピー性皮膚炎には卵アレルギーが深く関わっていることが示唆された。また、アンケート上での卵アレルギーの既往がある群がより重症であることが分かり、卵白への特異的血清 IgE 検査（オボムコイド）でも関与が示された。
3. GWAS の結果、過去に報告されたヨーロッパあるいは中国から報告された領域に加え、新たに8領域が同定された。GWAS は、アトピー性皮膚炎のメカニズム及び治療を切り開くうえで重要な強力な手法であると考える。また、石垣島コホートにおいて、フィラグリン遺伝子変異の有無がアトピー性皮膚炎の発症に必ずしも寄与しないことを見出した。

A．研究目的

1. 痒みという感覚は「掻きたいとう欲求を引き起こす不快な感覚」であり、皮膚における痒みを伝達する一次求心性神経線維に関しては C 線維がその役割を担っていることが知られている。一方で、C 線維は痛みや冷温覚をはじめとした痒みとは異なる感覚を伝達することも知られている。我々はこれまでに、痒み特異的な一次求心性神経線維の同定のため、ラットにセロトニン（5-HT）塗布による痒み知覚モデルを作製して脊髄後根神経節における電気生理学的記録を行い、5-HT による痒み刺激に特異的に反応する C 線維を同定した。本研究では後根神経節で記録した一次求心性神経線維が脊髄後角の入力部と、その後の神経伝導路を検討したい.

2. アトピー性皮膚炎の発症寄与因子の解明は重要な課題であるが、我が国におけるコホート研究は数少ない。我々は平成13年度より沖縄県石垣島の保育園児の集団検診と保護者へのアンケート調査、採血データの解析を開始し、発症頻度・IgE 値・感染症の合併・本症発症の寄与因子などを報告してきた。今回は、CCL17/TARC や CCL22/MDC などの Th2 ケモカインの推移とアトピー性皮膚炎の発症および卵アレルギーの関与を明らかにしたい。

3. アトピー性皮膚炎の有病率は上昇しており、現在、社会問題化している。本疾患の解明は未だ進んでおらず、分子生物学的アプローチを含めた、幅広い視点に立った病因の解明と新規治療薬の開発が切望されている。わが国でも IgE 産生能や気道過敏性に関する候補遺伝子や、最近ではフィラグリン遺伝子の転突然変異が高率に発見されるなどの報告がされつつあるが、未だ疾患を包括的に説明する決定的なものはない。より正確な候補遺伝子の探索のためには、ある地域に生活する集団を全体として解析するコホート解析を用いる必要があり、また複数の地域集団でゲノム解析を行うことでより関連の高い疾患候補遺伝子を同定できる。

我々は平成１３年度から沖縄県石垣島の保育園児の集団検診と採血、アンケート調査によるコホート解析を開始しているが、現在までにデータ固定されている 2011 までに延べ 7856 人の０－６歳時を診察し、平記入秒率は 6.3%であった。この検診は毎年継続されており、石垣島の乳幼児コホートの前向き調査を可能としている。H15-16 年にかけては最近保険適応となったアトピー性皮膚炎の重症度マーカー、TARC の血清値がアトピー患児での発症、持続、消退などの自然経過と強く関連することを見いだした。さらにアトピー疾患関連遺伝子の解明を進めるべくこのコホート群における遺伝

子解析を行うため、血液サンプルの採取を行っている。

最近、日本人がSNPタイピングにより大きくRyukyuとHondoクラスターの２つに分けられることが判明した。石垣島のコホート群における追跡調査、血液検査、遺伝子研究などで得られた成果 すなわち様々な臨床的アトピー関連因子や数々の候補遺伝子群やまた将来的にそれらを元にした病因の解明・新規治療などが日本人に広く応用可能であるかどうか、またはある特定の疾患サブグループや地域特異性（石垣島など）に認められる傾向にあるのかなどをより正確に検討するには、次なるステップとして同様の採血検査、遺伝子調査を本邦の他地域においても実施し、石垣島で得られた結果の有意性を確認する必要がある。そこで九州・山口地域一円からの人口流入地域である福岡と、全国からの人口流入地域である東京地域において得られる結果を、石垣スタディの結果と比較・検討したい。

B．研究方法

1. SDラットの胸髄を麻酔下に露出し、末梢に5-HTを塗布してパッチクランプ法にて脊髄後角部の膜電流を測定することで、脊髄後角における5-HTによる痒み刺激の入力部位を検討した。

2. 園児検診、アンケート結果、血清データ解析を行った。

3. 九州大学病院皮膚科、同総合診療部、慶応義塾大学病院皮膚科、東京大学にてアトピー性皮膚炎患者、健常者各1000人を目標に血液検体、アンケート調査票、ゲノムタイピングの結果を用いて解析する

（倫理面への配慮）

本研究での動物実験あるいは臨床研究は施設の倫理委員会にて承認されている。

C．研究結果

1. 5-HTによる痒み刺激に応答するC線維は、応答しないC線維と比較すると有意に脊髄後角の浅層に入力していた。また、5-HTによる痒み刺激に反応する脊髄後角ニューロンは、後根神経節ニューロンと同じく機械的刺激に反応する性質を持っていた。

2. データ固定された2011年までに、のべ7856人の園児を診察し、平均有病率は6.3％で男女差はなかった。また2009までの解析で、男児の血清IgEが高いこと、気管支喘息合併が高いことが判った。アンケート調査の多変量解析の結果、患児のアトピー性皮膚炎罹患の危険因子として、本人の気管支喘息とは卵アレルギーの既往、父親や同胞のアトピー性皮膚炎の既往が挙げられた。卵アレルギー歴のあるアトピー性皮膚炎園児は有意に血清TARC値が高く、またオボムコイド特異的IgE値高値（クラス２以上）のアトピー性皮膚炎園児で有意に血清TARC値が高かった。

3. GWASの結果、過去に報告されたヨーロッパあるいは中国から報告された領域に加え、新たに8領域が同定された。(Hirota T, et al. Genome-wide association study identifies eight new susceptibility loci for atopic dermatitis in the Japanese population. Nat Genet 2012;44(11):1222-6.)。また、本ゲノム解析で遺伝子多型とアトピー発症の関連が確認されたＴｈ２ケモカインＭＤＣは石垣島園児でアトピー性皮膚炎園児は対照群に比して有意に検査値が高いことがわかり、また健常人でも成人に比して非常に数値が高いことが判った。さらに、石垣島コホートにおいて、フィラグリン遺伝子変異の有無がアトピー性皮膚炎の発症に必ずしも寄与しないことを見出した。

D．考察

1. 5-HTによる痒み刺激に反応する1次求心性神経線維は、後根神経節においてC線維の中で機械的刺激にも反応する多様式なニューロンであり、脊髄後角においても同様の性質を持っていた。脊髄後角での入力部位に関しては浅層であることから、侵害刺激や温熱刺激の伝導路と同一部位であることが確認された。

2. 卵アレルギーの既往がアトピー性皮膚炎の危険因子のひとつであり、乳幼児期のアトピー性皮膚炎には卵アレルギーが深く関わっていることが示唆された。また、アンケート上での卵アレルギーの既往がある群が血清TARC値（アトピーの疾患重症度マーカー）が高値、すなわちより重症であることが判った。オボムコイド（卵白の主要アレルゲン）特異的IgE検査でも同様の知見が示されており、より確からしいことが判った。ミルクやコムギなどの3大食餌アレルゲンの他の抗原とやダニアレルギーと重症度との関連は見られず、興味深い。

3. 新たに同定された8領域は、気管支喘息におけるGWASと同様に好塩基球活性化因子を含んでおり、また免疫調節因子であるビタミンD代謝経路に関わる因子が含まれていた。実集団で関連を検証したい。

E．結論

1. より中枢における痒み特異的神経の伝導路に関する検討を行う必要がある。

2. 男児の気管支喘息の高合併率なども見られているため、食物アレルゲンに加え、カビなど他の環境因子とADか関連疾患の発症、重症度との関連解析が必要と思われる。

3. 今後、GWASで同定された因子についてさらなる検討が必要である。GWASの結果は、アトピー性皮膚炎のメカニズム及び治療を切り開くうえで重要な強力な手法であると考える。

F．健康危険情報

なし。

G. 研究発表

1. 論文発表

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1. 特許取得

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3.その他

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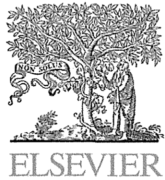

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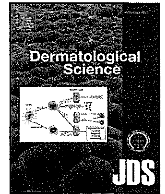

Reduction of CC-chemokine ligand 5 by aryl hydrocarbon receptor ligands

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SUMMARY

Background: The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that recognizes a large number of xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs), dioxins, and some endogenous ligands. Despite numerous investigations targeting AhR ligands, the precise physiological role of AhR remains unknown.
Objective: We explored novel AhR target genes, especially focused on inflammatory chemokine.
Methods: We treated (1) HaCaT, a human keratinocyte cell line, (2) normal human epidermal keratinocytes (NHEKs), and (3) mouse primary keratinocytes with AhR ligands, such as 6-formylindolo[3,2-b]carbazole (FICZ; endogenous ligand) and benzo[a]pyrene (BaP; exogenous ligand). Then, we detected mRNA and protein of chemokine using quantitative RT-PCR and ELISA. We next clarified the relationship between AhR and chemokine expression using AhR siRNA. In addition, we measured serum chemokine levels in patients with Yusho disease (oil disease), who were accidentally exposed to dioxins in the past.
Results: We identified CC-chemokine ligand 5 (CCL5), a key mediator in the development of inflammatory responses, as the AhR target gene. AhR ligands (FICZ and BaP) significantly reduced CCL5 mRNA and protein expression in HaCaT cells. These effects were observed in NHEKs and mouse primary keratinocytes. AhR knockdown with siRNA restored CCL5 inhibition by AhR ligands. In addition, AhR ligands exhibited a dose-dependent suppression of CCL5 production induced by Th1-derived cytokines. Finally, serum levels of CCL5 in patients with Yusho disease, were significantly lower than in controls.
Conclusion: Our findings indicate that CCL5 is a target gene for AhR, and might be associated with the pathology of dioxin exposure.
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1. Introduction

Dioxins and polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BaP) are environmental contaminants to which humans and wildlife are widely exposed. These pollutants tend to accumulate in higher animals and may interfere with the immune system [1,2]. Dioxin and PAH toxicity have been linked, at least in part, to activation of the aryl hydrocarbon receptor (AhR), a ligand-activated basic helix–loop–helix transcription factor. Upon ligand binding, cytoplasmic AhR translocates to the nucleus and dimerizes with the AhR nuclear translocator (ARNT). The ligand-activated AhR/ARNT complex then binds specific promoter elements called xenobiotic response elements, altering the expression of target genes presumed to contribute to dioxin and PAH toxicity [2]. Several reports have shown that dioxins and PAHs induce multiple inflammatory genes including cytokines and chemokines such as IL-1α, TNF-α, IL-8, and CC-chemokine ligand 1 (CCL1) and 2 in an AhR-dependent manner in vivo and in vitro [3–7]. Numerous other exogenous compounds, such as plant

Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; BaP, benzo[a]pyrene; CCL, CC-chemokine ligand; FICZ, 6-formylindolo[3,2-b]carbazole; NHEK, normal human epidermal keratinocyte; PAHs, polycyclic aromatic hydrocarbons; PCB, polychlorinated biphenyls; TCDD, 2,3,4,8-tetrachlorodibenzo-p-dioxin.

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polyphenols (e.g., resveratrol and curcumin) and synthetic drugs (e.g., ketoconazole) activate AhR, while some are thought to be AhR antagonists [8,9]. Although the physiological role of AhR remains largely unclear, endogenous AhR ligands have been proposed, such as 6-formylindolo[3,2-b]carbazole (FICZ) and bilirubin [10,11]. We previously reported that BaP induces IL-8 production from normal human epidermal keratinocytes (NHEKs) via the AhR signaling pathway, and that ketoconazole inhibits BaP-induced IL-8 production from NHEKs [7,9]. In this study, we further screened the production of various inflammatory chemokines in response to AhR ligands (FICZ and BaP) from human and mouse keratinocytes. We also determined serum chemokine levels in patients with Yusho disease (oil disease), who were accidentally exposed to dioxins in the past.

2. Materials and methods

2.1. Reagents and antibodies

FICZ was obtained from Enzo Life Sciences (Plymouth Meeting, PA); BaP and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich (St Louis, MO). Anti-AhR rabbit polyclonal antibody (H-211) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin rabbit polyclonal antibody (#4967) was purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were provided by Jackson ImmunoResearch Laboratories (West Grove, PA).

2.2. Cell culture

HaCaT cells, representing a human keratinocyte cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics. HaCaT cells were plated on 6-well plates, and at sub-confluence were treated with AhR ligands (FICZ (0.1, 1, 10, or 100 nM) or BaP (0.01, 0.1, 1, or 10 μM)) or vehicle (DMSO). NHEKs, obtained from Clonetics-BioWhittaker (San Diego, CA), were maintained in serum-free keratinocyte growth medium (Lonza, Walkersville, MD) supplemented with bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, transferrin, and epinephrine (Lonza, Walkersville, MD). NHEKs were plated on 24-well plates, and were treated with AhR ligands (FICZ (0.1, 1, 10, or 100 nM) or BaP (0.01, 0.1, 1, or 10 μM)) or vehicle (DMSO) at sub-confluence.

2.3. Preparation of mouse epidermal cell suspension

RPMI-1640 supplemented with 10% heat-inactivated FBS, antibiotics, and 50 μM 2-mercaptoethanol (Sigma–Aldrich) were used as complete medium. Ear skin specimens from female Balb/c mice (7–8 weeks old) were treated with 0.5% trypsin (Sigma–Aldrich) in phosphate-buffered saline (PBS; 30 min at 37 °C); this separated the epidermis from the dermal connective tissue. Epidermal sheets were agitated gently in complete medium with 0.05% deoxyribonuclease I (Sigma–Aldrich); the epidermal cell suspension was obtained following filtration through a cell strainer with a 40-μm pore size (BD Falcon, San Jose, CA). Mouse primary keratinocytes were plated onto 6-well plates, and at sub-confluence were treated with AhR ligands (10 nM FICZ or 1 μM BaP) or vehicle (DMSO). The protocol was approved by the Committee of Ethics on Animal Experiments in the Graduate School of Medical Sciences, Kyushu University.

2.4. Combined stimulation with TNF-α and IFN-γ

Cells were plated on 6-well plates, and at sub-confluence were exposed to TNF-α (10 ng/mL) and IFN-γ (10 ng/mL) in the presence or absence of AhR ligands (FICZ or BaP) for indicated times. After incubation, the medium was extracted for ELISA.

2.5. Real-time quantitative RT-PCR

Total RNA was isolated from HaCaT cells using the RNeasy Mini kit (Qiagen, Valencia, CA). Quantitative real-time RT-PCR was performed with PrimeScript RT reagent and SYBR Premix Ex Taq II (Takara Bio, Ohtsu, Japan) according to the manufacturer's instructions. PCR amplifications were performed with the following cycling conditions: 95 °C for 30 s, for 40 cycles at 95 °C for 5 s (denaturation step), at 60 °C for 20 s (annealing/extension steps). The cycle threshold (Ct) for each amplification was normalized to β-actin (internal control). Normalized gene expression was expressed as the relative quantity of gene-specific mRNA compared with control mRNA (fold induction). Oligonucleotide primers are listed in Table 1.

2.6. Immunoblotting

Protein lysates from HaCaT cells were isolated with lysis buffer (25 mM HEPES, 10 mM $Na_4P_2O_7 \cdot 10H_2O$, 100 mM NaF, 5 mM EDTA, 2 mM Na_3VO_4, 1% Triton X-100) and analyzed by SDS-PAGE on a 10% polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with specific antibodies. Immunological bands were identified with HRP-conjugated secondary antibodies followed by visualization with SuperSignal west pico chemiluminescence substrate (Pierce, Rockford, IL).

2.7. ELISA

Cell-culture supernatants were cleared by centrifugation and analyzed for the presence of immunoreactive CCL5 protein by using the Quantikine human or mouse CCL5/RANTES ELISA kit (R&D System, Minneapolis, MN) as directed by the manufacturer. Serum chemokine levels (CCL2, CCL5, CXCL9, and CXCL10) were also measured in 232 patients diagnosed with Yusho disease living in Fukuoka, Japan and 96 age- and

Table 1
Primer sequences used for real-time quantitative RT-PCR.

Gene (human)	Primer sequence (5′-3′)	
	Sense	Antisense
CYP1A1	TAGACACTGATCTGGCTGCAG	GGGAAGGCTCCATCAGCATC
CCL2	CCCCAGTCACCTGCTGTTAT	TGGAATCCTGAACCCACTTC
CCL5	TCTGCGCTCCTGCATCTG	GGGCAATGTAGGCAAAGCA
CXCL9	TTCCTCTTGGGCATCATCTTGCTGG	AGTCCCTTGGTTGGTGCTGATGCAG
CXCL10	CAAACTGCGATTCTGATTTGCTGCC	TGCTGATGCAGGTACAGCGTACGGT
β-Actin	ATTGCCGACAGGATGCAGA	GAGTACTTGCGCTCAGGAGGA

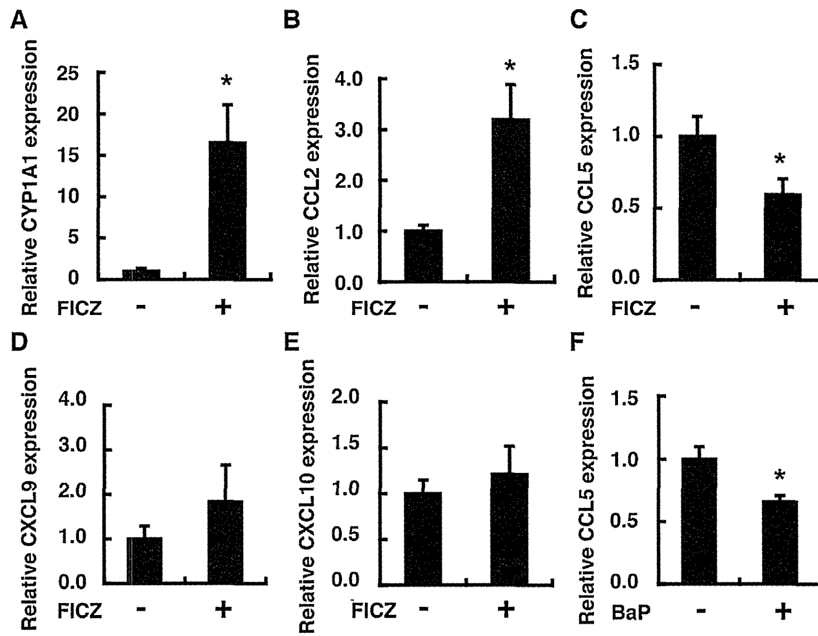

Fig. 1. AhR ligands reduced expression of CCL5. (A–E) HaCaT cells were treated with 10 nM FICZ for 3 h and total RNA was extracted. CYP1A1 (A), CCL2 (B), CCL5 (C), CXCL9 (D), and CXCL10 (E) mRNA expression was measured by real-time quantitative RT-PCR. mRNA levels were normalized to β-actin (internal control). (F) HaCaT cells were treated with 1 μM BaP for 3 h and total was RNA extracted. CCL5 mRNA expression was measured by real-time quantitative RT-PCR and normalized to β-actin. Data are presented as mean ± S.E. ($n = 4$ per group). $^{*}P < 0.05$ by unpaired t test.

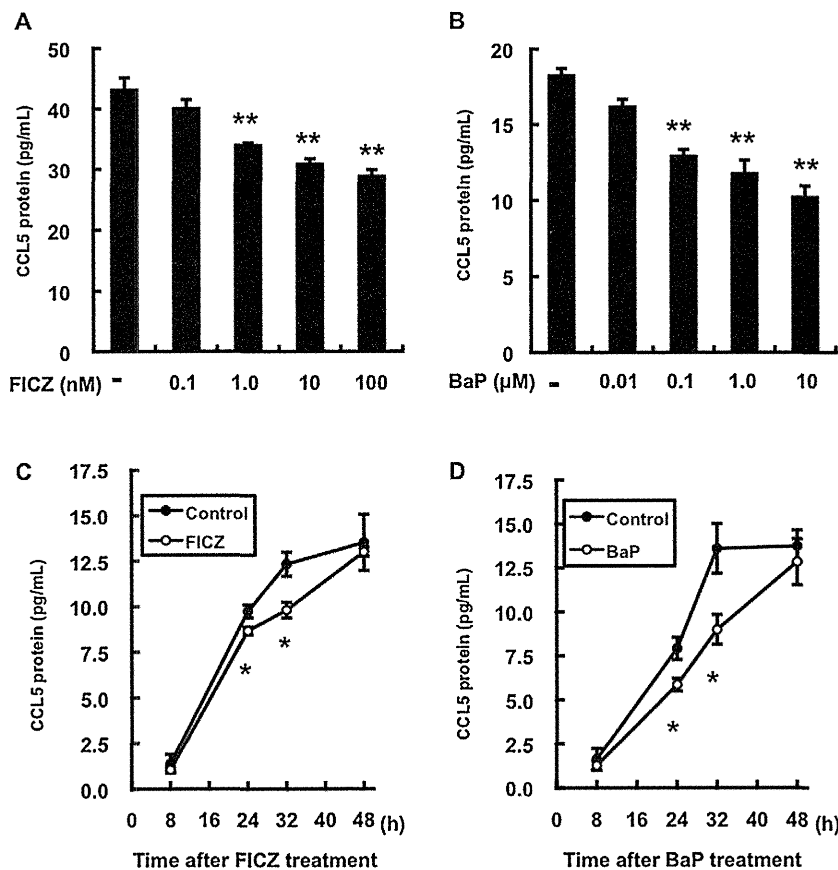

Fig. 2. Reduction of CCL5 protein levels in response to AhR ligands. (A and B) HaCaT cells were treated with FICZ (A) or BaP (B) for 24 h, and culture supernatants were collected. CCL5 protein was measured by ELISA. Data are presented as mean ± S.E. ($n = 4$ per group). $^{**}P < 0.01$ vs. control (DMSO only), assessed by one-way ANOVA (C and D) HaCaT cells were treated with 10 nM FICZ (C) or 1 μM BaP (D) for indicated time, and culture supernatants were collected. CCL5 protein was measured by ELISA. Data are presented as mean ± S.E. ($n = 4$ per group). $^{*}P < 0.05$ by unpaired t test.

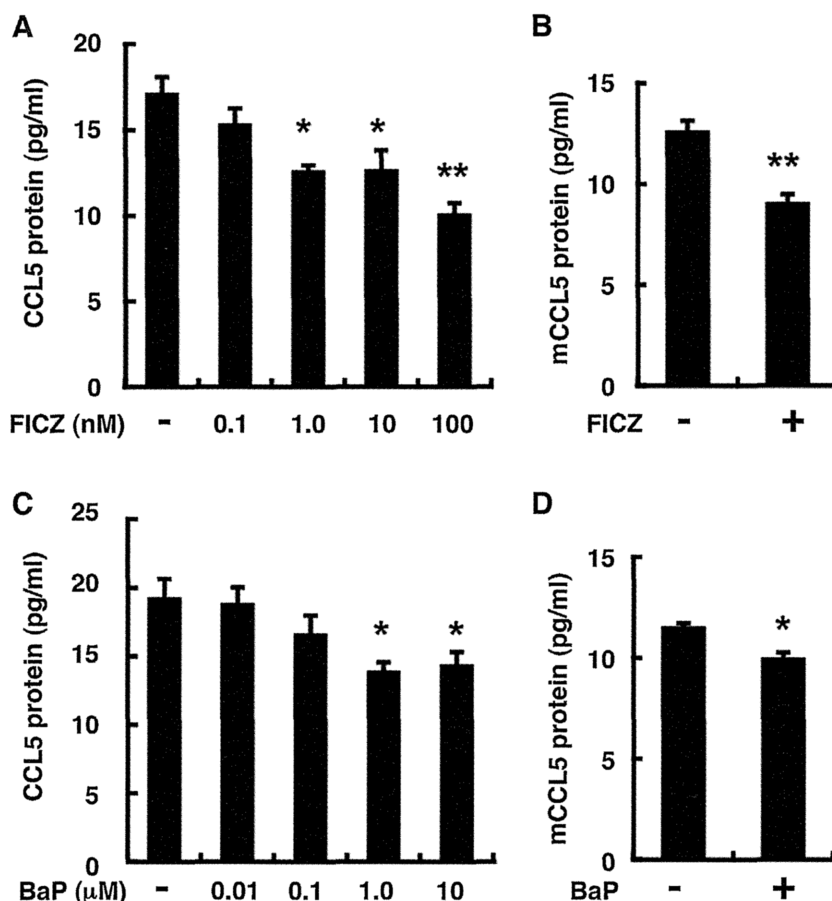

Fig. 3. Reduction of CCL5 protein levels in response to AhR ligands in NHEK and mouse primary keratinocytes. NHEKs were treated with FICZ (A) or BaP (C) for 24 h and culture supernatants were collected. CCL5 protein was measured by ELISA. Mouse primary keratinocytes were treated with 10 nM FICZ (B) or 1 μM BaP (D) for 24 h and culture supernatants collected. CCL5 protein was measured by ELISA. Data are presented as mean ± S.E. (n = 4 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (DMSO only), assessed by one-way ANOVA (A and C) or unpaired t test (B and D).

residency-matched normal individuals. Samples were acquired from 2005 to 2009 and stored at −80 °C to await analysis. Absorbance was measured using an iMark microplate absorbance reader (Bio-Rad, Hercules, CA), and the concentration of chemokines was determined in each sample by comparison to a standard curve. The study protocol was approved by the institutional ethics committee of Kyushu University Hospital and signed informed consent was obtained from each subject prior to study enrolment. Blood concentrations of dioxins in each patient were measured at the Fukuoka Institute of Health and Environmental Sciences (Fukuoka, Japan) as described elsewhere [12].

2.8. AhR siRNA transfection

AhR siRNA (s1200) and control siRNA (Negative Control #1) were purchased from Ambion (Austin, TX) and transfected as required into HaCaT cells using the HiPerFect Transfection kit (Qiagen) in accordance with the manufacturer's instructions.

2.9. Statistical analysis

Data are presented as mean ± S.E. Significance of the differences between groups was assessed using the Student's unpaired two-tailed t test (when 2 groups were analyzed), one-way ANOVA (for ≥3 groups), or the Mann–Whitney U-test (for human samples). A P value of <0.05 was considered statistically significant.

3. Results

3.1. AhR ligands reduce CCL5 expression in HaCaT cells

We verified the transcriptional activation of CYP1A1 (Fig. 1A) and CCL2 (Fig. 1B) by FICZ in HaCaT cells, a human keratinocyte cell line, as positive controls. We then assessed whether FICZ influenced the expression of other inflammation-related chemokines, such as CCL5 (CCR5 ligand) and CXCL9 and 10 (CXCR3 ligands). FICZ significantly reduced the expression of CCL5, but not that of CXCL9 and CXCL10 (Fig. 1C–E). BaP, another AhR ligand, also significantly reduced CCL5 mRNA expression (Fig. 1F). Thus, AhR ligands likely influence the transcription of CCL5.

3.2. AhR ligands inhibit CCL5 protein expression in HaCaT cells

We next examined of CCL5 protein levels in HaCaT cells treated with FICZ. Dose-dependent reduction of CCL5 protein was observed in HaCaT cells treated with FICZ (Fig. 2A) and BaP (Fig. 2B). To elucidate the time course of CCL5 suppression by AhR ligands, HaCaT cells were cultured in FICZ or BaP for up to 48 h. FICZ (Fig. 2C) or BaP (Fig. 2D) significantly decreased CCL5 protein in a time-dependent manner and restored it after 48 h. Our results show that AhR ligands, FICZ or BaP, reduce not only mRNA but also protein levels of CCL5 in HaCaT cells.

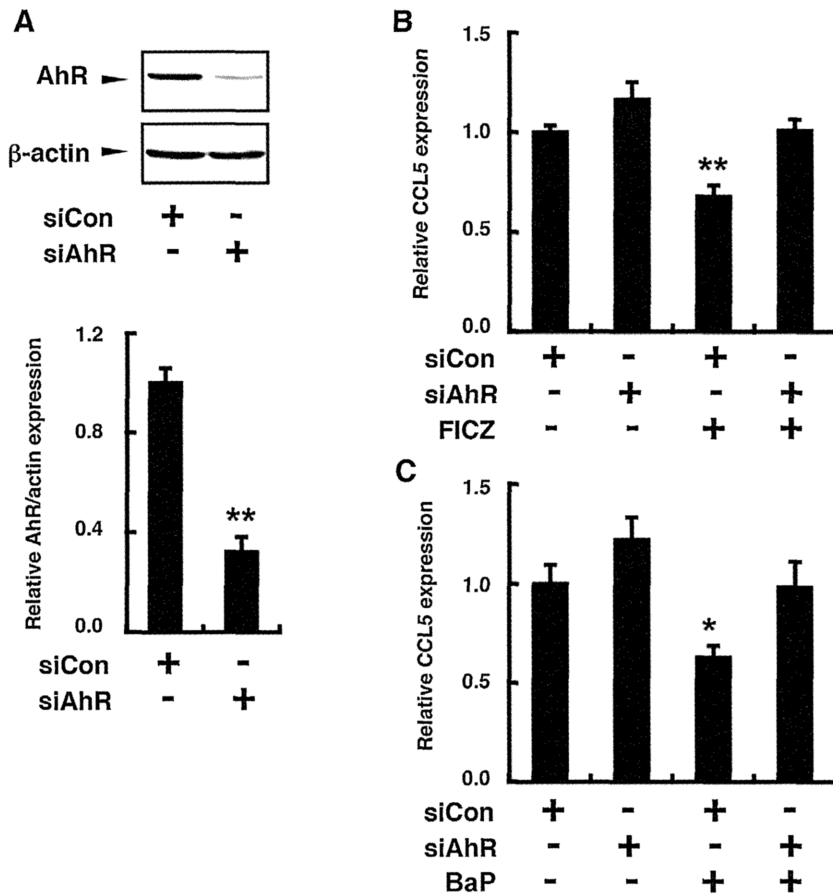

Fig. 4. AhR-dependent reduction of CCL5 expression in response to AhR ligands in HaCaT cells. (A) HaCaT cells were transiently transfected with AhR siRNA or control siRNA for 48 h and whole cell lysates were extracted. The relative amount of AhR was normalized to β-actin. Values were quantified using Image Gauge software. Data are presented as mean ± S.E. ($n = 3$ per group). $^{**}P < 0.01$ assessed by unpaired *t* test. (B and C) HaCaT cells were transiently transfected with AhR siRNA for 48 h, then treated with 10 nM FICZ (B) or 1 μM BaP (C) for 3 h. Total RNA was extracted and CCL5 mRNA expression measured by real-time quantitative RT-PCR. mRNA levels were normalized to β-actin. Data are presented as mean ± S.E. ($n = 4$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (control siRNA + DMSO), assessed by one-way ANOVA.

3.3. AhR ligands inhibit expression of CCL5 in NHEKs and mouse primary keratinocytes

To determine whether AhR ligands reduce CCL5 expression in other types of keratinocytes, we used NHEKs and mouse primary keratinocytes. FICZ significantly reduced CCL5 protein in a dose-dependent manner in NHEKs (Fig. 3A) and mouse primary keratinocytes (Fig. 3B). BaP also reduced CCL5 protein levels in NHEKs and mouse primary keratinocytes (Fig. 3C and D). Thus, AhR ligands suppressed CCL5 expression in keratinocytes.

3.4. AhR ligands reduce CCL5 expression via AhR

To investigate the involvement of AhR in FICZ or BaP-mediated inhibition of CCL5 expression, we employed AhR siRNA silencing. Reduction of AhR protein levels was confirmed in AhR

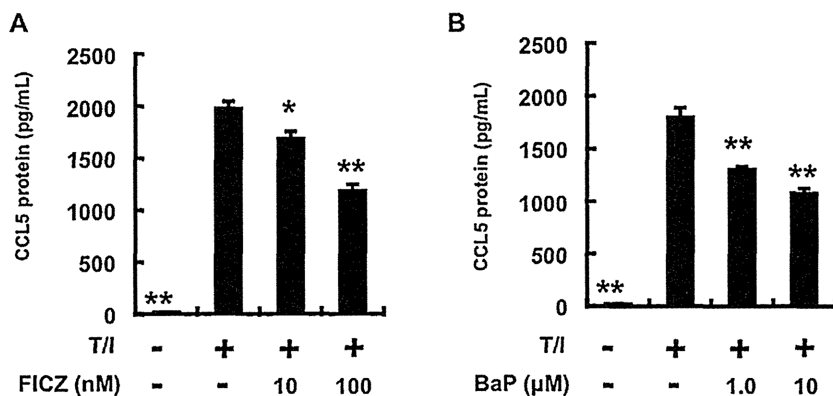

Fig. 5. AhR ligands inhibited CCL5 induced by the combined stimulation of TNF-α and INF-γ. HaCaT cells were stimulated with TNF-α and INF-γ (T/I) in the presence or absence of FICZ (A) or BaP (B) for 24 h, and culture supernatants were collected. CCL5 protein was measured by ELISA. Data are presented as mean ± S.E. ($n = 4$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. T/I only, assessed by one-way ANOVA.

Table 2
Serum levels of chemokines in dioxin-exposed and control samples.

	CCL5 (ng/mL)	CCL2 (pg/mL)	CXCL9 (pg/mL)	CXCL10 (pg/mL)
Yusho (n=232)	40.8 ± 1.5	388.2 ± 28.7	152.2 ± 8.7	125.0 ± 6.0
Control (n=96)	59.3 ± 3.2	321.1 ± 10.7	159.5 ± 14.1	121.5 ± 12.6
P	<0.0001	0.14	0.66	0.78

Data are presented as mean ± S.E.
P values were assessed by Mann–Whitney analysis.

siRNA-treated cells compared with control siRNA-treated cells (Fig. 4A). Intriguingly, AhR knockdown with siRNA abolished the suppression of CCL5 expression by either FICZ or BaP (Fig. 4B and C). These results suggest FICZ or BaP might reduce CCL5 expression via AhR.

3.5. AhR ligands inhibit TNF-α/IFN-γ-induced CCL5 expression

Keratinocytes can be induced to produce CCL5 protein when incubated with a combination of Th1-derived cytokines (TNF-α and IFN-γ). Therefore, we examined whether AhR ligands decrease the production of CCL5 in HaCaT cells that have been stimulated with TNF-α and IFN-γ. Consistent with previous report, stimulation with TNF-α and IFN-γ increased CCL5 protein in HaCaT cells (Fig. 5A and B). Interestingly, TNF-α/IFN-γ-induced CCL5 expression was inhibited by FICZ in a dose-dependent manner (Fig. 5A). BaP also suppressed CCL5 expression induced by TNF-α/IFN-γ (Fig. 5B).

3.6. Serum levels of CCL5 are reduced in dioxin-exposed human samples

Mean ages of the Yusho patients and controls were 70.8 ± 12.0 years and 66.1 ± 11.3. Blood levels of dioxins in Yusho patients, in particular 2,3,4,7,8-pentachlorodibenzofuran, were significantly higher than in controls (170.5 ± 16.5 pg/g lipid vs. 15.7 ± 0.9 pg/g lipid). Serum levels of CCL5 in Yusho patients were significantly lower than in controls (Table 2). Although not significant, serum levels of CCL2 in Yusho patients tended to be higher than in the controls. There was no difference in the serum concentrations of other chemokines between Yusho patients and controls.

4. Discussion

AhR ligands directly or indirectly modulate chemokine expression. For example, intraperitoneal administration of 2,3,4,8-tetrachlorodibenzo-p-dioxin (TCDD) in C57/BL6 mice induced expression of CXCL1 and CCL2 mRNAs in the liver, thymus, kidney, adipose tissue, and heart [13]. Furthermore, in vitro studies have shown that AhR ligands, such as TCDD, polychlorinated biphenyl 126 (PCB126), PCB77, or BaP, upregulate the expression of CCL1, CCL2, CXCL13, and IL-8 in various cell lines and primary cell cultures [3–7]. In contrast, CCL5 expression appears to be inhibited by AhR ligands. In utero exposure to TCDD in male rats reduced CCL5 expression in the testes [14]. Furthermore, gene array analysis revealed that CCL5 expression was inhibited in $CD4^+$ T cells isolated from TCDD-treated mice [15]. In this study, we also showed significantly reduced production of CCL5 protein in human and mouse keratinocytes stimulated with FICZ and BaP. These AhR ligands markedly suppressed CCL5 expression induced by Th1-derived cytokines (TNF-α and IFN-γ). In addition, as previously shown in our preliminary study [16], we demonstrated that serum levels of CCL5 in dioxin-exposed patients were significantly lower than in normal subjects. Yusho disease (oil disease) is a serious form of food poisoning caused by consumption of rice oil contaminated with dioxins and related organochlorines in western Japan in 1968, involving at least 1900 individuals [17]. Patients with Yusho disease have suffered from various symptoms such as general malaise, arthralgia, chloracne, and peripheral neuropathy. Blood levels of dioxins in these patients remain high [18].

CCL5 plays a pivotal role in maintenance of the inflammatory response through its ability to attract T lymphocytes, monocytes, natural killer cells, and basophils, which leads to temporo-spatial expansion of the inflammatory infiltrate [19]. CCL5 expression is involved in a variety of diseases including arthritis, SLE, diabetes, and glomerulonephritis [20–23]. We showed that AhR ligands suppressed Th1-derived cytokine-induced CCL5 production, suggesting that AhR ligands would inhibit to some extent the infiltration of inflammatory cells. On the other hand, low baseline CCL5 was reported to be an independent predictor of cardiac mortality in a cohort of male patients undergoing coronary angiography [24].

Numerous basic and epidemiological studies have shown that dioxins and related organochlorines may increase the risk for cardiovascular diseases. For example, chronic exposure to dioxins (TCDD or PCB126) led to a dose-dependent increase in the incidence of degenerative cardiovascular lesions in rats [25]. The International Agency for Research on Cancer Cohort, consisting of 36 cohorts from 13 countries, followed 21,863 dioxin-exposed workers for more than 20 years. A significant association between dioxin exposure and ischemic heart disease was detected in these workers (relative risk, 1.67; 95% CI, 1.23–2.26) [26]. Although further studies are needed to clarify the precise clinical significance of our data, the observed reduction of serum CCL5 in dioxin-exposed patients may be related to the increased incidence of cardiovascular diseases in such patients.

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Verbalizing Extremes of the Visual Analogue Scale for Pruritus: A Consensus Statement

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Itch (pruritus) is an unpleasant sensation that leads to the desire to scratch (1). Pruritus may compromise quality of life and sleep in affected individuals. Pruritus is similar to pain, in being a subjective symptom; assessment of its intensity is a key issue in evaluating severity and therapeutic outcome of patients with pruritic disorders of diverse origins (cutaneous, systemic, neuropathic, psychogenic) (2). Various types of rating scales have been used and validated in the study of clinical itch, including the visual analogue scale (VAS), numerical rating scale, verbal rating scale, and behavioural rating scale (2–4). Among these measurement tools, the VAS seems to be one of the most commonly used methods of assessing pruritus severity, as it provides an easy and rapid estimation of itch (3, 4). The VAS is a 10-cm long line, oriented horizontally or vertically, on which patients indicate the intensity of pruritus by marking the line at the point that corresponds to the severity of their pruritus, where the beginning of the scale refers to no pruritus (0 point) and the end of the scale to the most severe pruritus (10 points) (3, 4). On behalf of the International Forum for the Study of Itch (IFSI), we discussed methodological problems of the VAS in clinical settings. During our discussion we identified that it is necessary to clarify the verbal expression of the 10-point end, because it varies from study to study. It includes expressions such as "worst imaginable itch", "the most severe pruritus they can imagine", "most intense sensation imaginable", "maximal itch", "severe itching" and "unbearable pruritus" (3–8). In this report, we propose to consolidate the verbalization of extremes of VAS for "itch intensity" and "sleep disturbance (nocturnal itch)".

CONSENSUS STATEMENT

Members of the Japanese Society for Dermatoallergology and Contact Dermatitis (JSDACD) (MF, TE, AI, ST, YK, KT, TS, and HS) discussed possible core items for evaluating pruritus in clinical settings, including clinical trials, in Japan. Nine items were proposed and we evaluated the importance of each item by assigning a weight score (maximum points, 10) to each. The 2 highest-ranked items were "itch intensity" (score, 10 ± 0) and "sleep disturbance" (9.3 ± 0.8), followed by "maximum score of itch" (7.3 ± 2.6), "itch frequency" (6.1 ± 1.8), "itch duration" (6 ± 2), "number of itch sites" (4.3 ± 2.6), "quality of life" (4.3 ± 2.7), "itch site" (3.7 ± 1.4) and "nature of itch" (3.3 ± 1.5).

We then discussed measurement tools for the two highest rank items, "itch intensity" and "sleep disturbance." With respect to measuring "sleep disturbance (nocturnal itch intensity)," the discussion group reached an agreement that the VAS seemed to be a suitable scale, similar to the VAS for "itch intensity." As for the terminology to be used to describe the 10-point end of the VAS, our 8 JSDACD members preferred the expressions "worst itch imaginable" for scoring "itch intensity" and "I cannot sleep at all" for scoring "sleep disturbance (nocturnal itch)".

These results were presented at the 6th World Congress of Itch in Brest, France, 2011. After the Congress, e-mail voting was proposed for the terminology of the 10-point end of the VAS, and this proposal was accepted by the members of the IFSI special interest group (TE, MA, JS, AF, CB, NQP, GY, SS). Twenty-six IFSI members from outside Japan participated in e-mail voting. With regard to the expression of the 10-point end of the VAS for "itch intensity," "worst imaginable itch" was voted as the most suitable definition, including "worst itch imaginable" ($n=14$). With regard to the expression of the 10-point end of the VAS for "sleep disturbance", the expression "I cannot sleep at all" was selected as the suitable definition ($n=17$) (Fig. 1).

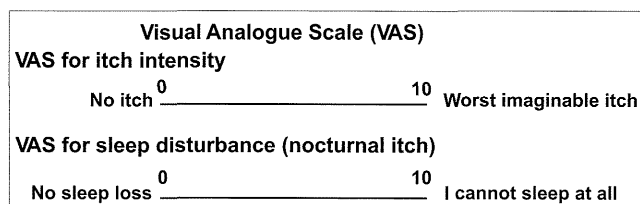

Fig. 1. Visual analogue scale (VAS).

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PERSPECTIVES

Our consensus is in line with the VAS for pain intensity, because recent publications suggest that both "worst imaginable pain" and "worst pain imaginable" seem to be frequently used expressions for the 10-point end of the VAS for pain intensity in various fields of medicine (9, 10). It is also in accordance with the labelled magnitude scale used in sensory psychophysics, where the end-point is "most intense sensation imaginable of any kind" (11). Further work is needed to test the comprehension and meaning of our proposed phrases against other contenders with patients and carers, and whether this varies across cultures and countries, especially when translated into other languages. In order to gain a wider consensus on our proposal, we intend to collaborate with other international groups, such as the Harmonising Outcome Measures for Eczema (HOME) initiative, which seeks to develop a core set of outcome measures for eczema that can be used in future clinical trials and record-keeping (12). Other topics to be further explored are: (*i*) Whether the VAS for "itch intensity" represents the mean itch intensity of only day-time, or both day- and night-time? (*ii*) Whether daily VAS assessments are more suitable than weekly ones? (*iii*) Should the subject be allowed to see his/her previous VAS score before deciding his/her current itch intensity? Based on the pain assessment study, Scott & Huskisson (13) recommended that prior scores should be made available to patients when serial measurements of pain are made in long-term experiments. Due to the presence of diverse clinical settings and trials, these items may be modified and adjusted appropriately by investigators.

The authors declare no conflicts of interest.

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Most Highly Cytokinergic IgEs Have Polyreactivity to Autoantigens

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Purpose: Monomeric IgE molecules, when bound to the high-affinity receptor, exhibit a vast heterogeneity in their ability to induce survival promotion and cytokine production in mast cells. At one end of this spectrum, highly cytokinergic (HC) IgEs can induce potent survival promotion, degranulation, cytokine production, migration, etc., whereas at the other end, poorly cytokinergic (PC) IgEs can do so inefficiently. In this study, we investigated whether IgEs recognize autoantigens and whether IgEs' binding of autoantigens correlates with difference s in HC versus PC properties. **Methods:** Enzyme-linked immunosorbent assays were performed to test whether IgEs bind antigens. Histamine-releasing factor in human sera was quantified by western blotting. Cultured mast cells derived from human cord blood were used to test the effects of human sera on cytokine production. **Results:** Most (7/8) of mouse monoclonal HC IgEs exhibited polyreactivity to double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), β-galactosidase, thyroglobulin and/or histamine-releasing factor. By contrast, mouse PC IgEs failed to react with these antigens. A human monoclonal HC IgE also showed polyreactivity to histamine-releasing factor, dsDNA and ssDNA. Interestingly, sera from atopic dermatitis patients showed increased reactivity to ssDNA and β-galactosidase and increased levels of histamine-releasing factor. Some atopic dermatitis patients, but not healthy individuals, had substantial serum levels of HRF-reactive IgE. Sera from atopic dermatitis patients with high titers of DNA-reactive IgE could induce several fold more IL-8 secretion in human mast cells than sera from healthy individuals. **Conclusions:** The results show that most HC, but not PC, IgEs exhibit polyreactivity to autoantigens, supporting the autoimmune mechanism in the pathogenesis of atopic dermatitis.

Key Words: Atopic dermatitis; mast cell; cytokine; IgE; autoantigen; histamine-releasing factor

INTRODUCTION

Mast cells are the key effector cell type in IgE-mediated immediate hypersensitivity and allergic disorders.[1,2] Mast cells bound by antigen-specific IgE via the high-affinity receptor for IgE (FcεRI) must encounter multivalent antigen for their activation.[3] Activated mast cells secrete preformed proinflammatory mediators, such as histamine, serotonin, nucleotides, proteases, and TNF-α, and synthesize and secrete lipid mediators (such as leukotrienes and prostaglandins), various cytokines and chemokines. In contrast to this traditional view, we and others showed that IgE binding to FcεRI in the absence of specific antigen engenders several biological outcomes in mast cells: upregulation of cell surface expression of FcεRI,[4-6] survival,[7,8] increase in histamine content,[9] histamine release, leukotriene release, receptor internalization, DNA synthesis,[10] increased responses to compound 48/80 and substance P,[11] increase in filamentous actin content,[12] membrane ruffling,[13] adhesion to fibronectin,[14] and migration.[15] These effects are due to binding to FcεRI by *monomeric*, but not aggregate, IgE molecules.[7,8,12] FcεRI cross-

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linking appears essential for mast cell activation induced by monomeric IgE,[16] similar to that by IgE+antigen or IgE+anti-IgE.[17,18]

Not all IgE molecules can induce all the activation events in the absence of antigen: IgEs display a wide spectrum of heterogeneity in their ability to induce the production and secretion of IL-6 and TNF-α, two tested cytokines produced by mouse mast cells, with HC IgEs at one extreme end and PC IgEs at the other.[10] More extensive receptor aggregation can be induced by HC IgEs than by PC IgEs. Consistent with such a difference, strong HC IgEs can induce each of the tested activation events, whereas PC IgEs induce only a limited set of activation events, and to a lesser extent.[19] Polyclonal IgE from mice and humans suffering from atopic dermatitis (AD) can enhance survival and cytokine production in mast cell cultures, indicating that monomeric IgE effects are operative in polyclonal situations as well.[20]

Compared to numerous reports on effects of monomeric IgE on mouse mast cells, effects of human IgE on human mast cells have been less studied. However, using human cord blood derived mast cells (CBMCs), Gilchrest et al.[21] reported that the chemokine I-309 RNA and protein levels were upregulated not only in response to IgE+anti-IgE stimulation but also by IgE alone and these responses were further augmented in the presence of IL-4. Cruse et al.[22] also reported that human monomeric myeloma IgE stimulated cultured human lung mast cells to release histamine, leukotriene C4, and IL-8 in the absence of a specific antigen. Matsuda et al.[16] showed that human monomeric IgE in the absence of specific antigen enhanced IL-8 and monocyte chemoattractant protein 1 (MCP-1) production in cultured human mast cells, and this response was augmented by preincubation of the cells in IL-4. However, it is still unclear whether the effects of monomeric HC versus PC IgEs exist in the human system. Since heterogeneity of human IgEs in the ability to prime basophils to stimulation with histamine-releasing factor (HRF) has been reported,[23] it is possible that some highly allergic patients might produce HC IgEs, which may activate mast cells or basophils in the absence of antigen.

Our recent study demonstrated that ~30% of the tested mouse IgE mAbs bind to HRF and that HRF+HRF-reactive IgE can activate mast cells.[24] Since HRF has two IgE-binding sites and can be present as a dimer, it is assumed that IgE-bound receptors can be cross-linked by HRF. Similar situations could occur if receptor-bound IgE react with multivalent (auto)antigen. Therefore, we investigated whether HC IgEs react with various antigens.

MATERIALS AND METHODS

IgE preparations

Mouse monoclonal IgEs were purified by ammonium sulfate precipitation from serum-free culture supernatants of hybridomas or peritoneal exudates of hybridoma-bearing mice, followed by DEAE column chromatography.[25] Some ELISA experiments were performed with hybridoma culture supernatants. Sources of IgEs: H1 DNP-ε-26 (26) and H1 DNP-ε-206 (206) from Fu-Tong Liu, University of California, Davis; AR40EA, BE1BD (BE1BD2D5), BE2, BE4 (BE4C2C3), DNP48BC, DNP48BD, HB30, and R25 from R.P.S.; C38-2, C48-2, and 27-74 from BD Biosciences; SPE-7 from Sigma-Aldrich; IGELa2 from American Type Culture Collection (ATCC) (TIB142).

Human IgEs: SKO-007 monoclonal IgE was purified by ammonium sulfate precipitation from serum-free culture supernatants of hybridomas (ATCC: CRL-8033-1). HE1 monoclonal IgE from a hybridoma was purchased from Diatec Monoclonals AS (Oslo, Norway). IgEs derived from multiple myeloma patients (BS-κ and ART) were purchased from The Binding Site Inc. (San Diego, CA, USA) or Athens Research & Technology (Athens, GA, USA). IgE from the multiple myeloma patient PS was a gift from Dr. Kimishige Ishizaka, LIAI. All IgE samples were dialyzed extensively against PBS, and ultracentrifuged at 100,000×*g* for 30 minutes before use to remove possible aggregates.

Sera from AD patients and normal subjects

After the informed consent was obtained, the sera of AD patients diagnosed according to the criteria of Hanifin and Rajka[26] were collected. Total numbers of AD patients and normal controls are 41 and 25, respectively. All studies involving human subjects were conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki and approved by the Institutional Review Boards of La Jolla Institute for Allergy and Immunology, Kyushu University, University of Occupational and Environmental Health and University of Yamanashi.

Enzyme-linked immunosorbent assay (ELISA)

Calf thymus double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA), β-galactosidase from bovine liver, thyroglobulin from bovine thyroid, insulin from bovine pancreas, LPS (all from Sigma-Aldrich) and recombinant HRF tagged with 6x histidine (all 10 μg/mL in PBS) were coated in 96-well plates for overnight at 4°C. After the wells were blocked with 10% FCS-PBS, mouse IgE molecules (10 μg/mL) were added. Bound mouse IgEs were detected by biotinylated anti-mouse IgE mAb (BD Bioscience PharMingen, San Diego, CA, USA), followed by streptavidin-HRP conjugates. Color was developed and absorbance at 450 nm was measured. In some experiments, human IgEs (10 μg/mL) or sera from healthy subjects and AD patients (1/10 dilution) were incubated in the well. Bound human IgE molecules were detected by biotinylated anti-human IgE mAb (BD Bioscience PharMingen), followed by streptavidin-HRP.

Human mast cell culture

Human CBMCs were generated as described.[27] Briefly, $CD34^+$ cells were purified from umbilical cord blood mononuclear cells

and cultured in serum-free Iscove's methylcellulose medium (IMDM: Stem Cell Technologies Inc., Vancouver, BC, Canada) containing 200 ng/mL SCF (PeproTech Inc., Rocky Hill, NJ, USA), 50 ng/mL IL-6 (PeproTech Inc.), 1 ng/mL IL-3 (PeproTech Inc.), 1% insulin-transferrin-selenium (Invitrogen, Carlsbad, CA, USA), 50 μM 2-mercaptoethanol, 1% penicillin-streptomycin (Invitroten), and 0.1% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA). On day 42 of culture, methylcellulose was dissolved in PBS and the cells were then suspended and cultured in IMDM (Invitrogen) supplemented with 100 ng/mL SCF, 50 ng/mL IL-6, and 5% fetal calf serum (Cansera, Rexdale, ON, Canada). After 15 weeks, the purity of the resulting CBMCs was ~98% ($c\text{-Kit}^+Fc\varepsilon RI^+$ by flow cytometry). The use of human cord blood was approved by the Institutional Review Board of Nihon University Graduate School of Medical Science.

Cytokine measurement

CBMCs were incubated with 10 ng/mL of IL-4 in the presence or absence of 1 μg/mL of IgE for sensitization. After 24 hours, the cells were washed and resuspended in culture medium. The cells (1×10^5/100 μL) were then stimulated with IgE molecules for the indicated times. After stimulation, supernatants and cell pellets were collected for analysis of IL-8 and IL-6 expression and production. IL-8 production was measured using a human IL-8 ELISA kit (BD PharMingen, San Diego, CA, USA).

Cell survival

CBMCs were incubated with IL-4 in the presence or absence of IgE for sensitization. After 24 hours, the cells were washed and resuspended in SCF-free culture medium. The cells (1×10^5/100 μL) were then stimulated with anti-IgE, SCF, or IgE molecules for 72 hours. After 72 hours, cell survival was analyzed after staining with Trypan blue.

Real-time RT-PCR

Total cellular RNA was isolated from CBMCs with an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. An equal amount of total RNA (100 ng) was used for reverse transcription. Real-time RT-PCR was performed as follows: cDNA (10 ng) was amplified in 20 μL in the presence of 1 μL of "Assay-on-Demand" primer sets for IL-8, IL-6, and GAPDH purchased from Applied Biosystems (Foster City, CA, USA) in 7700 ABI thermal cyclers (Applied Biosystems). Relative expression levels were determined using Ct method.

Measurement of serum HRF

Human sera (10 μL) were precipitated first with acetone. Precipitated materials were dissolved in SDS sample buffer and, together with dilution series of recombinant HRF-His6, analyzed by SDS-PAGE and blotted onto PVDF membranes. The blots were probed with anti-HRF antibody followed by HRP-conjugated secondary antibody. HRF bands were detected by ECL reagent (Perkin-Elmer). The concentrations of HRF in the samples were determined by densitometry.

Statistical analysis

Data are expressed as mean ± SD. Paired student *t* test was used for mean comparisons. Differences were considered statistically significant at P values <0.05.

RESULTS

Mouse HC, but not PC, IgE molecules react with various autoantigens

HC IgEs are defined operationally by the ability to induce secretion of IL-6 and/or TNF-α from BMMCs, whereas PC IgEs lack this ability.[10] We have recently shown that some of the tested IgEs interact with HRF.[24] We extended this line of study by testing whether mouse IgE mAbs interact with other antigens, such as dsDNA, ssDNA, β-galactosidase, thyroglobulin, insulin and LPS. ELISA data (Fig. 1) showed that most HC IgEs react with two or more of these antigens. For example, the most potent HC IgE SPE-7 reacted with dsDNA, ssDNA and thyroglobulin, in addition to DNP (hapten used to generate this mAb) and thioredoxin, which was previously shown to bind to SPE-7 IgE.[28] H1 DNP-ε-26 IgE reacted with dsDNA and ssDNA; C38-2 IgE reacted with HRF, dsDNA, ssDNA, β-galactosidase and thyroglobulin; IGELa2 IgE reacted with HRF, β-galactosidase and thyroglobulin. The possibility of non-specific binding was ruled out. For example, interactions of TNP-specific C38-2 and IGELa2 IgEs with HRF were inhibited by TNP-glycine,[24] suggesting that the HRF-binding site in IgE overlaps at least in part with the antigen-binding site. By contrast, none of the 13 tested PC IgE mAbs reacted with the antigens under the same conditions. Neither HC nor PC IgEs reacted with insulin or LPS. These experiments clearly demonstrate that most of mouse HC, but not PC, IgEs react with multiple autoantigens.

Human IgE molecules also show the HC vs. PC heterogeneity

Human CBMCs express low levels of FcεRI on their cell surface.[29] Previous studies showed that incubation of human CBMCs with IL-4 enhances FcεRI expression[29] and renders the cells sensitive to stimulation with human IgE, which induces the production of chemokines, IL-8 and monocyte chemoattractant protein 1.[16] Therefore, we used IL-4-preincubated human CBMCs in this study. To determine whether human IgE molecules show heterogeneity in inducing cytokine production in human mast cells, human CBMCs were incubated with 5 μg/mL of human monoclonal and myeloma-derived IgE molecules for 2 hours, to measure cytokine mRNAs and proteins. As shown previously,[16] quantitative RT-PCR showed that IL-8 mRNA expression is induced strongly by a monoclonal (HE1) IgE and weakly by another myeloma IgE, BS-κ (Fig. 2A). We could also show secretion of IL-8 protein induced by the HE1 IgE (Fig. 2B).

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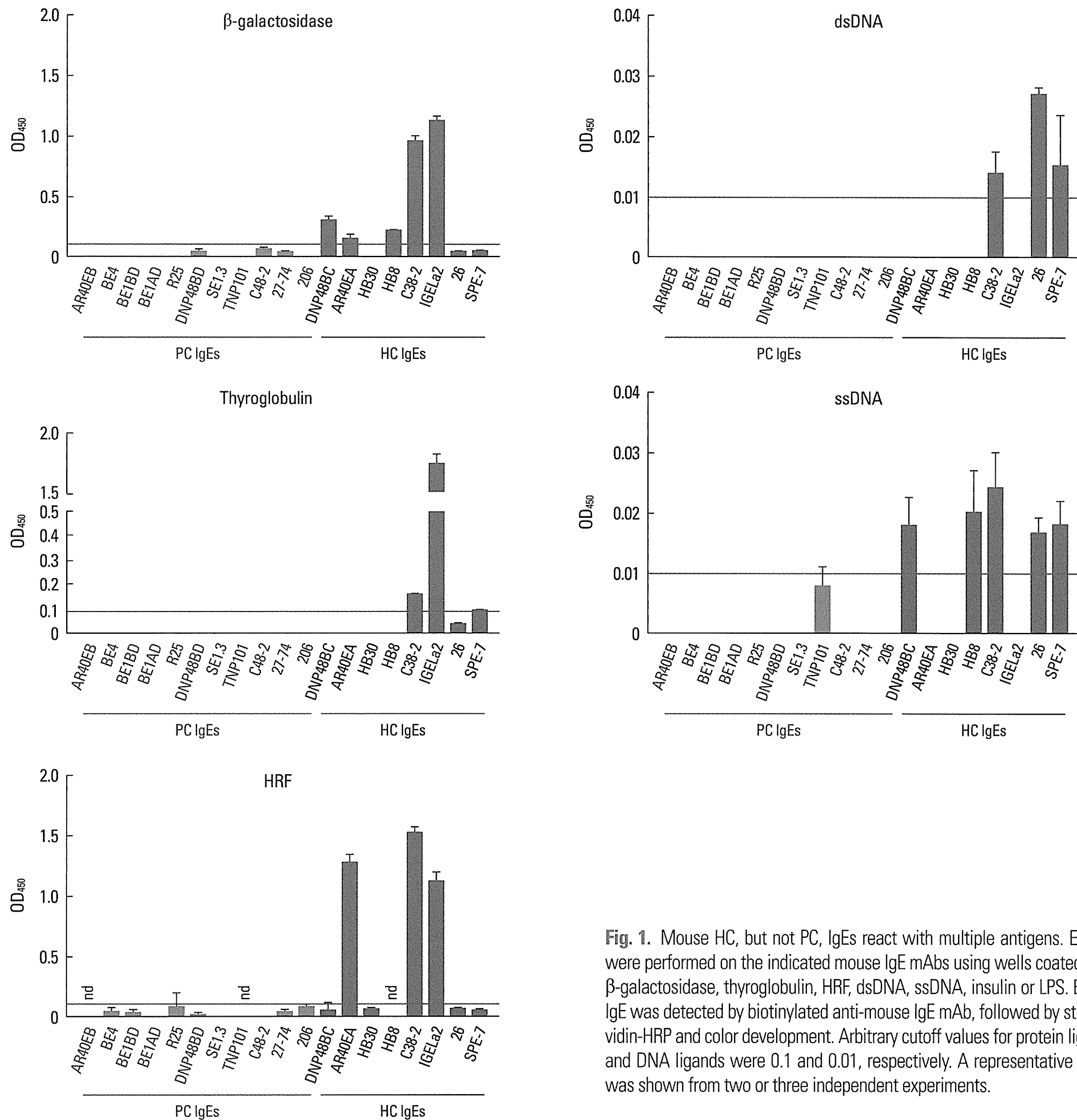

Fig. 1. Mouse HC, but not PC, IgEs react with multiple antigens. ELISAs were performed on the indicated mouse IgE mAbs using wells coated with β-galactosidase, thyroglobulin, HRF, dsDNA, ssDNA, insulin or LPS. Bound IgE was detected by biotinylated anti-mouse IgE mAb, followed by streptavidin-HRP and color development. Arbitrary cutoff values for protein ligands and DNA ligands were 0.1 and 0.01, respectively. A representative result was shown from two or three independent experiments.

However, the other tested monoclonal IgE preparations failed to induce IL-8 mRNA. Similarly, only HE1 IgE induced IL-6 mRNA (Fig. 2C). Secreted IL-6 levels could not be measured because CBMCs were cultured in IL-6-containing medium. HE1 IgE, but not SKO IgE, enhanced survival of CBMCs (Fig. 2D). Thus, these results indicate that HE1 is an HC IgE and others are PC IgEs.

A human HC IgE, but not PC IgEs, react with various autoantigens

We next performed ELISA to test whether human HC and PC IgEs react with dsDNA and ssDNAs. As shown in Fig. 3, HE1 IgE, which was previously shown to bind to HRF,[24] reacted with both dsDNA and ssDNA. By contrast, none of the human PC IgEs reacted with either DNA.

Sera from AD patients contain higher levels of HRF and ssDNA- or β-galactosidase-binding IgEs

The ability of some IgEs to bind to multivalent autoantigens such as HRF or DNA raised the possibility that mast cells and basophils can be activated by interacting with such an autoantigen via FcεRI-bound IgE. Given the importance of IgE in the