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講演録

国際共同治験の現状と海外データの評価

宇山　佳明

1. はじめに

本邦では，2007年に「国際共同治験に関する基本的考え方」（以下，「基本的考え方」）が通知され，複数の地域で同一の治験実施計画書を用いて実施される国際共同治験が積極的に実施されるようになり，国際共同治験が主要な臨床試験と位置づけられている臨床データパッケージに基づき承認される医薬品が増加しつつあります．本日は，国際共同治験の事例の共有と，今後の国際共同治験のあり方について，現在検討中の事項を含めてお話しさせていただきます．

2. 基本的考え方

ご存知のとおり，1998年に「外国臨床データを受け入れる際に考慮すべき民族的要因について」（以下，ICH-E5）が，2007年に「基本的考え方」が通知されています．ICH-E5のブリッジングの考え方は国際共同治験においても有用であり，民族的要因について十分考慮したうえで国際共同治験を計画すべきです．また，適切な評価を行うためには，十分な日本人症例を組み入れることが適切と考えます．なぜなら，一貫した結果が得られなかった場合に，それが症例数が少なかったことによる偶然の結果なのか，真に民族的差異による結果なのかを区別できる様に計画しておくことが重要であるからです．また，国際共同治験への日本人症例の組み入れは，検証試験だけでなく，探索的な開発早期の臨床試験から組み入れることが適切と考えます．なぜなら，検証試験だけでなく，複数の試験で日本人症例における医薬品の有効性及び安全性が確認されることにより，日本人におけるより頑健なエビデンスが得られるからです．

3. 国際共同治験実施状況

2007年の「基本的考え方」の通知以降，日本を含む国際共同治験が増加し，2011年には臨床試験の約15－20％が国際共同治験として実施されています（図１）．また，既承認の医薬品の臨床データパッケージで見ても，2007年の「基本的な考え方」の通知以降，外国で実施された臨床試験を含む臨床データパッケージの構築は，ブリッジング戦略から国際共同治験へと推移しています．事実，ブリッジング戦略に基づく臨床データパッケージでの医薬品の承認は，2005年では全承認の約25％を占めましたが，2011年では約２％まで減少しています．一方，国際共同治験が主要な臨床試験である臨床データパッケージでの医薬品の承認は，2011年では約10％まで増加し，今後も増加することが想定されます（図２）．また，東アジア地域での国際共同治験に基づく承認事例も増加しています．これまでは，欧米で実施される臨床試験に日本が参加するか否かが議論の中心でしたが，近年，臨床試験が多様化しつつある中で，東アジア地域の重要性が認識されつつあると推測されます（表１）．なお，国際共同治験による承認

独立行政法人　医薬品医療機器総合機構
〒110-0013　東京都千代田区霞が関3-3-2　新霞が関ビル
第２回レギュラトリーサイエンス学会学術大会シンポジウム「国際共同開発によるNDA－有効性・安全性評価はどうあるべきか？－」　2012年９月２日，講演録．

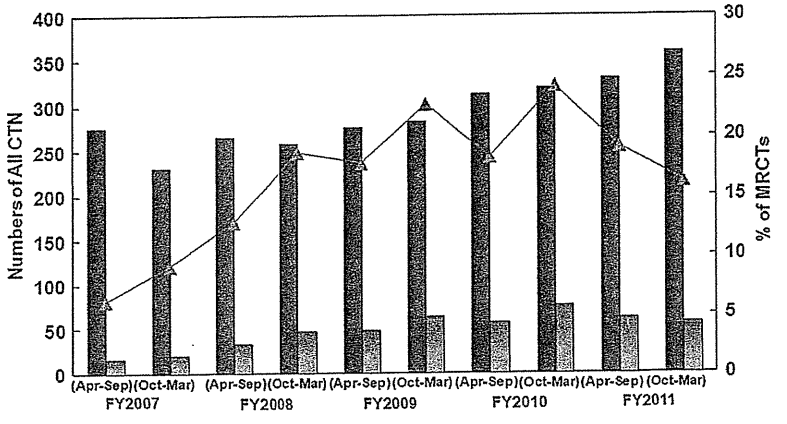


図1 日本を含む国際共同治験の実施状況

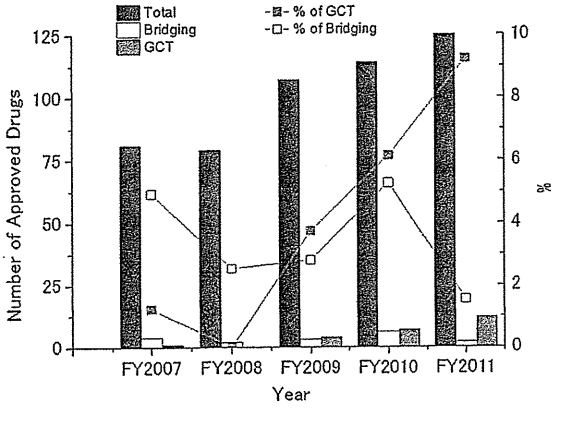


図2 既承認医薬品の臨床データパッケージ

事例は，現時点で，癌疾患領域が最も多く，次いで，循環器疾患領域が多く報告されています．

4. 国際共同治験に基づき承認された医薬品の事例

例1）アジア地域での国際共同治験：アリピプラゾール

アジア地域での国際共同治験の事例として，2012年1月に承認されたアリピプラゾールの効能追加（追加適応症：双極性障害における躁症状の改善）で実施された国際共同治験の結果と審査時の論点について紹介します．

アリピプラゾールでは，双極Ⅰ型障害の躁病又は混合性エピソードと診断された患者に対するアリピプラゾールの有効性及び安全性を検討するため，プラセボ対照，ランダム化，二重盲検，並行群間比較試験が，日本，中国，インドネシア，マレーシア，フィリピン及び台湾の6ヵ国で実施されました．主要評価項目は躁病のスケールであるYoung Mania Rating Scale（以下，YMRS）の合計点のベースラインからの変化量です．主要評価項目の結果でプラセボ群との差をみると，日本人部分集団における改善効果は全集団と比べて若干高い，中国，台湾では若干低いことが示されました．点推定値でみるとインドネシアは全集団と比べて改善効果は高く，マレーシアは低いという結果ですが，症例数が少ないためばらつきが大きく地域間の比較は困難です．このように，どのような切り口で臨床試験の結果を見るかと，症例数が少ない場合の偶然性が審査時の議論となります．

例2）大規模国際共同治験：ダビガトランエテキシラートメタンスルホン酸塩

大規模国際共同治験の事例として，2011年1月に承認されたダビガトランエテキシラートメタンスルホン酸塩（以下，ダビガトラン）（適応症：非弁膜症性心房細動患者における虚血性脳卒中及び全身性塞栓症の発症抑制）で実施された国際共同治験の結果と審査時の論点について紹介します．

表1　国際共同治験を主要な臨床試験として構築した臨床データパッケージにより日本で承認された医薬品一覧
(2012年6月現在)

Name of Drug	Indication	Approval	Multiregional/ Asian
Tolterodine	Overactive bladder with symptoms of urge urinary incontinence, urgency, and frequency	Apr. 2006	Asian
Losartan	Diabetic nephropathy with proteinuria and hypertension in patients with type 2 diabetes	Apr. 2006	Multiregional
Trastuzumab	Adjuvant therapy for metastatic HER2-overexpressing breast cancer	Feb. 2008	Multiregional
Insulin glulisine	Diabetes mellitus	Apr. 2009	Asian
Tadalafil	Pulmonary arterial hypertension	Oct. 2009	Multiregional
Peramivir*	Type A and Type B Influenza virus infection	Jan. 2010	Asian
Everolimus	Metastatic renal cell carcinoma	Jan. 2010	Multiregional
Panitumumab	Metastatic colorectal carcinoma with wild-type KRAS tumors	Apr. 2010	Multiregional
Travoprost/Timolol*	Glaucoma	Apr. 2010	Multiregional
Temsirolimus	Advanced renal cell carcinoma	Jul. 2010	Asian
Laninamivir*	Type A and Type B Influenza virus infection	Sep. 2010	Asian
Nilotinib	Newly diagnosed chronic myeloid leukemia in chronic phase	Nov. 2010	Multiregional
Dabigatran	Stroke and systemic embolism in patients with non-valvular atrial fibrillation	Jan. 2011	Multiregional
Trastuzumab	Metastatic HER2-overexpressing gastric cancer	Mar. 2011	Multiregional
Pramipexole	Parkinson's disease	Apr. 2011	Multiregional
Edoxaban*	Prevention of venous thromboembolism after major orthopedic surgery	Apr. 2011	Asian
Dasatinib	Chronic myeloid leukemia (CML)	Jun. 2011	Multiregional
Indacaterol	Chronic obstructive pulmonary disease (COPD), including chronic bronchitis and/or emphysema.	Jul. 2011	Asian
Linagliptin	Type 2 diabetes mellitus (adjunctive to diet and exercise)	Jul. 2011	Multiregional
Gefitinib	EGFR-Positive unresectable or metastatic non-small cell lung cancer (NSCLC)	Nov. 2011	Asian
Everolimus	Progressive neuroendocrine tumors of pancreatic origin (PNET)	Dec. 2011	Multiregional
Denosumab	Bone complications in patients with multiple myeloma or solid tumour that has spread to the bone.	Jan. 2012	Multiregional
Aripiprazole	Manic episodes associated with bipolar disorder	Jan. 2012	Asian
Olanzapine	Depression episodes associated with bipolar disorder	Feb. 2012	Multiregional
Exenatide	Type II diabetes mellitus (adjunctive to diet, exercise and treatment with SU)	Mar. 2012	Asian
Crizotinib	Anaplastic lymphoma kinase (ALK)-positive, advanced or metastatic non-small cell lung cancer (NSCLC)	Mar. 2012	Multiregional
Budesonide/ Formoterol	Asthma (where use of a combination of inhaled corticosteroid and long-acting $\beta 2$ adrenoceptor agonist is appropriate)	Jun. 2012	Multiregional
Formoterol	Chronic obstructive pulmonary disease (COPD)	Jun. 2012	Multiregional

*：日本が世界最初の承認国である医薬品

ダビガトランの日本での開発は，Phase I実施後，Phase II試験として探索的な非盲検の用量設定試験が日本で実施され，その後合計18,000例を超える大規模な国際共同治験が実施されました．国際共同試験は，非弁膜症性心房細動患者における脳卒中及び全身性塞栓症の予防におけるダビガトラン２用量（110 mg１日２回及び150 mg１日２回）の有効性と安全性が用量調整ワルファリンに対して非劣性であることを検証するため，ランダム化，非盲検，盲検下エンドポイント評価（Prospective Randomized Open Blinded-Endpoint Method; PROBE法），並行群間比較試験が，日本を含む世界44ヵ国で実施されました．主要評価項目は全脳卒中（出血性を含む）又は全身性塞栓症の発生です．

有効性の結果について，全集団では，ダビガトランのHazard Ratio（以下，HR）はワルファリンと同等又は若干低い傾向にあります．日本人の組み入れは，全集団1群6,000例中100例程度ではありますが，日本人部分集団でのダビガトランのHRについても，全集団の結果と同様にワルファリンと同等又は若干低い傾向にあります．しかしながら，日本人症例数が少ないために，信頼区間は当然ながら非常に大きくなっています．そのため，日本人部分集団の結果から，日本人でのリスクが低いとは結論づけられませんが，少なくとも全集団と日本人部分集団の結果を比較して大きな違いはないと考えられます．

安全性の結果については，実際には色々な角度から検討されていますが，大出血の発現割合に着目しますと，全集団では，ダビガトランの大出血率はワルファリンと同程度又は若干低いという結果です．一方，日本人部分集団では，ワルファリンと同程度又は若干高いという結果です．当然，この結果から，日本人の大出血率が高いとは結論できませんが，傾向としてはそのような結果でした．この結果について，審査時の論点について紹介します．日本人を含む大規模臨床試験を実施し，その結果に基づき評価することは，外因性及び内因性の民族的な要因に国内外で大きな違いはないとの検討結果に基づき，問題がないと企業と総合機構で合意されています．また，日本人の組み入れは全集団1群6000例中100例と少なく，このような実施可能性に基づき設定された日本人症例数から一貫性を評価することは非常に困難であるが，得られた結果の範囲内では全集団と日本人部分集団の成績に大きな齟齬は認められないと評価されています．大出血率については，前述のとおり，全集団と比べて日本人で大出血率が若干高い傾向が認められましたが，これは目標とするINRが国内外で若干異なったことの影響ではないかといったことが議論されています．最終的な総合機構の審査チームの判断として，臨床的に許容可能な成績が得られており，日本人での有効性及び安全性が期待できることから，承認可能であるが，製造販売後の情報収集が非常に重要であると審査報告書中で記載されています．

製造販売後調査については，国際共同治験では日本人症例数が非常に少なかったことから，5000例規模の特定使用成績調査が計画され，タビガトランの安全性及び有効性に関して十分な情報収集が実施されています．当然ながら，出血を重点調査項目としています．

抗凝固薬ということで，出血のリスクは予想されていましたが，死亡症例等の報告を踏まえ，出血に関して，ダビガトランの発売３ヵ月後（2011年６月）に企業から最初の「適正使用のお願い」が出され，その１ヵ月後（７月）に２回目の情報提供による適正使用の推進策がとられています．さらに１ヵ月後（８月）には学会から緊急ステートメントが出され，それとは別にブルーレターも出されました．日本人患者のみを対象として臨床試験が実施された場合でも，承認前の安全性評価には限界がありますが，国際共同治験では，日本人症例数が少なくなり，日本人でのリスクの評価は更に限定的なものとなります．このような医薬品については，製造販売後のリスクマネジメントプランの早期計画等，効率的な安全性の情報収集

及び評価の実施を考慮しないと，適切な安全対策を早期に実施することが困難となります．従って国際共同開発時代では，製造販売後の安全性の情報収集及び評価がさらに重要になると考えます．

5．より適切な国際共同治験の実施を目指して

国際共同治験の計画及び実施

2007年の「基本的考え方」の通知後，国際共同治験の実施計画への助言，国際共同治験による承認事例が増加する中で，独立行政法人医薬品医療機器総合機構（以下，「PMDA」）でも事例を集積し，より適切な国際共同試験の実施について検討しており，現在，新たなガイダンスを作成しています．ここでは，これまでのPMDA内での議論の内容について，個人的な見解を含めて紹介します．【最終版は平成24年９月５日に公表された．http://www.pmda.go.jp/regulatory/file/guideline/new_drug/GCT_jirei.pdf を参照】

• 東アジア地域を主とする国際共同治験

日中韓での実施であれば，１集団としてよいのではないかという相談をよく受けますが，それを判断するためにも科学的なデータを集積していく必要があり，事前に民族的要因の影響を検討することが重要と考えます．むしろ東アジア地域での開発を積極的に行うことで，東アジアでのデータがより評価において信頼できるものとなり，積極的活用ができるようになると考えます．

• 日常的な国際連携が重要

日本が開発初期の段階から国際共同治験の実施国として参加しない場合であっても，日常的に国際連携を取り開発状況を把握し，いつでも日本が参加できるよう準備することが重要と考えます．早期の段階からPMDAの対面助言を実施し，継続的に相談しておくことも重要です．

• 東アジア地域での積極的な開発

欧米と比べて東アジアで罹患率が高い疾患，例えば，胃癌や肝炎などは，日本又は東アジアが主導権をとって全世界の１つの開発地域として育っていかなければならないと考えます．このような疾患については，日本が積極的にPOC試験を実施する，又は東アジア地域間のお互いの役割分担を検討する必要があると考えます．

• PKデータの比較

PKデータについては，海外在住の日本人での結果も評価可能と考えます．なぜなら，PKデータは比較的医療環境よりも遺伝的要因等の内因性要因の影響を受けること，また通常は患者対象ではなく健康成人を対象としているからです．なお，PKデータの比較については，同一の治験実施計画書での結果を比較するほうが望ましく，試験が実施された時期や測定方法が大きく異なるような試験を比較しても正しい評価ができない可能性があると考えます．

• 有効性及び安全性を評価する試験

有効性及び安全性を評価する試験については，内因性民族的要因のみならず，診断方法や標準治療等の医療環境，教育，文化等の社会的要因等の外因性民族的要因を考慮する必要があります．そのため，日本の医療環境下で実施すること，日本在住の日本人が適切に組入れられた臨床試験（国際共同治験又は国内単独の臨床試験）の結果に基づき評価する必要があると考えます．

次に，PKプロファイルを考慮した開発戦略の分類を示します（図３）．この分類は，日本人と白人のPKプロファイルを比較後に東アジア人間の比較試験を実施することを意図しているわけではなく，PKの人種間での差異に着目すると主に４つに分類することが可能になることを示しています．日本人と白人のPKプロファイルを比較して差がなく，東アジア人の間でも差がないという場合は，欧米，アジアを含む多地域による共同開発が実施可能と考えます．一方で，日本人と白人のPKプロファイルには差があるが，東アジア人間で差がない場合は，東アジア地域での共同開発を念頭に置いた開発戦略を考えることができます．このように，パターンに応じて，開発戦略を検討することが重要と考えます．

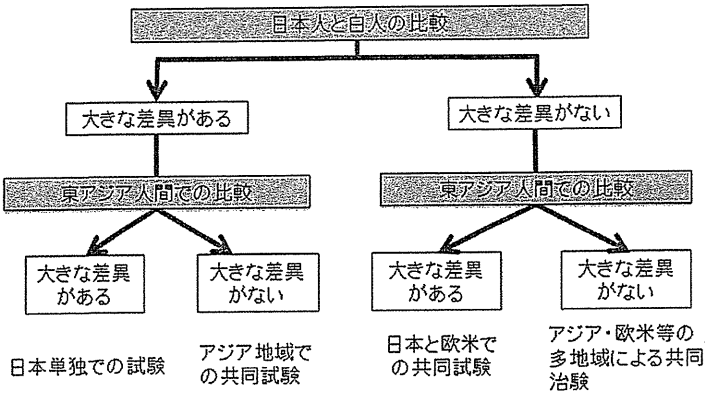


図3 PKプロファイルを考慮した開発戦略の分類

国際共同治験結果の評価

PMDAで国際共同治験の結果を評価した事例の集積から，全集団と日本人部分集団間での結果の一貫性を評価することは，最低でも我々が説明する責任を果たす上で必要な情報であると考えます．国際共同治験での日本人の集団は，部分集団であり，試験目的を達成するのに十分な症例数ではなく，全集団との間に差異が生じる可能性があります．PMDAでも，当然症例数が少なければ，偶然の結果として差異が出る可能性も考慮し，点推定値だけでなく，その精度，例えば標準偏差，推定値のばらつき等も考慮したうえで評価しています．また，主要評価項目の結果だけでなく，副次評価項目の結果も確認し，偶然の要素をできるだけ排除して評価していくことが重要と考えます．安全性についても有効性と同様に，全集団と日本人部分集団との間で著しく異なった傾向が認められていないかを確認すべきであると考えます．全集団と日本人部分集団の間で結果に差異が認められた場合には，要因毎の部分集団解析等も参考に差異が認められた原因について十分に考察し，当該国際共同試験の結果を日本人の有効性及び安全性の根拠とすることが可能であるか慎重に評価すべきと考えます．また，これらの評価結果及び考察については，申請時にCTDに適切に記載すべきと考えます．

大規模国際共同治験

事例として紹介したタビガトランのような大規模国際共同治験は，真の評価指標に関するエビデンスを構築するため，日本の積極的な参加が推奨されます．一方で，一貫性の検討を十分に行える日本人症例数が確保できない可能性があるという問題があります．したがって，既知情報や代替と真の評価指標との関係，国／地域間差異の影響等を精査し，日本を含む全集団を1つの集団としてみなすことができるかを十分に検討する必要があると考えます．2007年の「基本的考え方」の質問6に例示されている方法は，数百例規模での国際共同治験を想定しており，大規模国際共同治験に適用することは困難な場合があります．その場合に，主要評価項目（例えば生存率等の真の評価指標）との関係性が合理的に類推可能で，かつより少数例で評価可能な代替の評価指標に基づき，結果の一貫性が検討可能な症例数を必要最小例数とし，可能な限り多くの日本人症例を組み入れることも一案と考えます．また，副次評価項目等の結果についても検討することが重要であると考えます．これらは，あくまで一例であり，個別の状況に応じて，事前にPMDAと協議することが必要と考えます．

国際共同開発における長期安全性評価

国際共同開発では，承認申請までに収集できる日本人症例数が，国内単独開発に比べて減少し，特に安全性を評価する上で問題となる可能性があります．致死的でない疾患に対して長期投与が想定される医薬品については，十分に長期投与時の安全性を確認する必要があり，基本的には日本人で１年間投与された症例として100例以上の安全性データが収集できるように計画すべきと考えます．ただし，症例集積が困難な場合で以下に該当する場合には，上述の症例数を満たさなくても評価が可能な場合もあると考えます．

- 開発早期の探索的な段階から日本が継続的に国際開発に参加しており，複数の試験結果から，日本人と他の外国人との間で安全性に大きな差異がない
- 他の類似する効能･効果で既に承認されており，外国人と大きな差異がないことが，製造販売後での日本人における十分な安全性データから明らか

6．最後に

今後も個々の試験結果及び研究を集積しながら，できるだけ海外の開発と同調して国際共同開発を進めることが必要であり，また，アジア地域を主とする開発も１つの戦略だと思いますので，今後も，国際共同治験の適切な実施について積極的に検討していきたいと考えています．

質疑応答

(質問)

大きなテーマを示してくださって，ありがとうございました．途中で双極性障害のアジア地区の例を出されましたが，あの例を見ると，たしかインドネシアがかなり有効性で際立った効果を示していて，そのデータが全体を引っぱるという印象を少しもったのですが，国際共同治験の場合には日本以外の，つまり他国のデータも信頼して扱わなければいけない．そういう場合，一つの国がかなり有効性で際立った結果を示してきたときに，その国の民族性ということを，どのように評価するのか．あるいは，それぞれの国，質の違う臨床試験の有意の事象を，どの程度考慮して評価するのでしょうか？

(宇山)

インドネシアの件に関しては，あれは症例数が少ないので，あの結果に引っぱられて全体がよくなったということではなく，症例数が少ないために見かけ上良い成績にみえるということでだと思います．実際に推定値のばらつきが大きくなっていますので，症例を増やせばおそらくもっと収束すると考えます．そういう意味ではああいう症例数が少ないような結果については，むしろ慎重に評価する必要があると考えます．

こういう国際共同治験はこれから増加すると思いますが，１カ国なり１地域で極端に例数が少ないということになると，その結果の解釈は非常に難しく，そこから何か結論を導き出すことは困難だと思います．

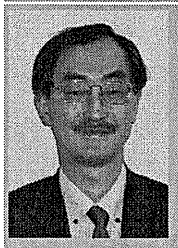
少なくとも，我々は，日本人の患者さんにきちんと使える医薬品であるのかどうかを最低限判断する必要がありますので，そういう意味では日本人の患者さんで一貫性が確認できるだけの症例数を組み入れていただきたいと思います．また，複数の試験に日本人を組み入れ偶然の要素をできるだけ排除するということも重要だと思います．

Pharmacogenomics of severe cutaneous adverse reactions

"Through pharmacogenomics, severe cutaneous adverse reactions have become avoidable adverse reactions. Efforts should continue to find more associations and develop diagnostic methods for prediction of high-risk patients to severe cutaneous adverse reactions, resulting in more patients being saved worldwide during the coming decade."

Naholo Kaniwa

Author for correspondence:
Division of Medicinal Safety Science, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
Tel.: +81 3 3700 1141 ext. 362
Fax: +81 3 3700 9768
nkaniwa@nihs.go.jp



Yoshiro Saito

Division of Medicinal Safety Science, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

KEYWORDS: abacavir ■ allopurinol ■ carbamazepine ■ *HLA-A*31:01* ■ *HLA-B*57:01* ■ *HLA-B*58:01* ■ *HLA-B75* ■ hypersensitivity syndrome ■ Stevens–Johnson syndrome ■ toxic epidermal necrolysis

Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are life-threatening severe adverse drug reactions with characteristic mucosal and cutaneous disorders, and are often accompanied by high fever and systemic complications. SJS/TEN are considered the same disease with different severity. Incidence is estimated to be two to three cases per million per year, depending on the ethnic groups. Among various causative drugs of SJS/TEN, aromatic antiepileptics, such as carbamazepine, phenytoin and lamotrigine, allopurinols and NSAIDs are the most common. SJS/TEN is considered to be a T-cell-involved delayed allergic reaction. Recently, reports on the involvement of some types of HLA class I molecules in the development of particular drug-induced SJS/TEN or other severe cutaneous adverse reactions (SCARs) have been accumulating.

Recent progress in pharmacogenomics in SCARs

A strong association between carbamazepine-induced SJS/TEN and *HLA-B*15:02* was initially detected in Han Chinese patients in Taiwan by Chung *et al.* [1]. The carrier frequency of *HLA-B*15:02* in these cases (n = 44) was 100%, which was significantly higher than that in carbamazepine-tolerant patients (3%; n = 101) ($p = 3.13 \times 10^{-27}$), and an extremely high odds ratio of 2504 was observed. This strong association was also observed in Han Chinese patients in Hong Kong [2], Asian originating patients living in Europe [3], Indian patients [4], Thai patients [5] and patients in mainland China [6]. However, no carriers of *HLA-B*15:02* have been detected in Caucasian [3] and Japanese [7] patients with carbamazepine-induced SJS/TEN. SJS/TEN incidence in some southeast Asian countries including Taiwan, Thailand, Malaysia and the Philippines is tenfold higher than in Europe, the USA and Japan [8]. The higher population allele frequencies of *HLA-B*15:02* (2–12%) in southeast Asian countries compared with those in Caucasians and in the Japanese (extremely low percentage) may explain the higher incidence of adverse reactions and observed strong associations.

Although association with *HLA-B*15:02* has not been detected, significant associations between *HLA-B*15:11* and carbamazepine-induced SJS/TEN have been found in Japanese and Korean patients (odds ratio: 16.3 and 18.0, respectively) [7,9]. Interestingly, *HLA-B*15:11* and *HLA-B*15:02* belong to the same serotype, HLA-B75. Other major members of HLA-B75 are *HLA-B*15:08* and *HLA-B*15:21*. Although statistical assessment has not been performed, carbamzepine-induced SJS/TEN patients in Asia who carry *HLA-B*15:11*, *HLA-B*15:08* or *HLA-B*15:21* have been reported [4,5]. Recently, Wei *et al.* estimated the molecular basis of these associations: carbamazepine noncovalently binds to HLA-B75 proteins including HLA-B*15:02 [10]. The 63rd amino acid in HLA-B75 members, which is the next amino acid of the carbamazepine putative binding site, is asparagine, while that of non-HLA-B75 members of *HLA-B*15*, which have no association with carbamazepine-induced SJS/TEN, is glutamic acid. A cell line transfected with *HLA-B*15* has shown that other HLA-B75 members including HLA-B*15:11 protein can undergo cell lysis by cytotoxic T cells activated by carbamazepine.

Future Medicine part of fsg

In addition to HLA-B75, *HLA-A*31:01* has been reported to be a biomarker for various carbamazepine-induced cutaneous adverse reactions ranging from mild skin rash such as maculopapular erythroderma to SCARs including SJS/TEN, in Europeans [11], Japanese [12] and Koreans [9].

> "*A strong association between carbamazepine-induced SJS/TEN and* HLA-B*15:02 *was initially detected in Han Chinese patients in Taiwan...*"

Allopurinol is used for hyperuricemia treatment and is known to often cause SCARs including SJS/TEN. Hung *et al.* reported for the first time a strong association between allopurinol-induced SCARs and *HLA-B*58:01* in Han Chinese patients living in Taiwan [13]. This association has been confirmed by studies with patients in Asia [14,15] and Europe [3], although the strength of the association depends on the ethnic groups. It is notable that *HLA-B*58:01* is a risk factor for various cutaneous reactions including SJS/TEN, hypersensitivity syndrome and maculopapular erythroderma, unlike *HLA-B*15:02*, which is a predictor specific for SJS/TEN induced by carbamazepine. A genome-wide association study with Japanese patients detected SNPs on chromosome 6 that absolutely linked with *HLA-B*58:01* [15], and we developed a screening test for *HLA-B*58:01* using one of the SNPs [16].

Usefulness of prescreening genetic biomarkers for preventing SCARs

Although biomarkers for SCARs have largely been found by retrospective studies, prospective randomized clinical studies may be required to evaluate the utility of biomarker screening to reduce drug-induced SCARs.

A large-scale, prospective, randomized, multicenter, double-blind study called PREDICT-I was conducted to evaluate screening for *HLA-B*57:01*, which has a strong association with abacavir-induced hypersensitivity [17]. In this study, nearly 2000 abacavir-naive HIV-infected patients in Europe and Australia were randomly divided into two groups. In one group, prospective screening for *HLA*57:01* was applied to exclude *HLA-B*57:01*-positive patients from abacavir treatment, while in the second group (control group), abacavir was given to all patients. Six weeks after the initiation of abacavir treatment, hypersensitivity incidence in both groups was compared. The incidence in the group receiving prospective screening was significantly lower (3.4%) than in the control group (7.8%). Thus, screening for *HLA-B*57:01* prior to treatment initiation is useful in reducing the risk of hypersensitive reactions to abacavir.

In Taiwan, a warning for *HLA-B*15:02* as a risk factor for carbamazepine-induced SJS/TEN was introduced to the package inserts of carbamazepine products in December 2007. Carbamazepine had been newly prescribed to approximately 50,000 patients each year, and the average incidence of carbamazepine-induced SJS/TEN between 2002 and 2004 was estimated to be 0.22% (~115 patients a year) [18]. In a prospective study performed in Taiwan using 4877 carbamazepine-naive patients from 23 hospitals, carbamazepine was administered to only *HLA-B*15:02*-negative patients. Within 2 months, none of the 4120 patients who took carbamazepine developed SJS/TEN. According to historical incidence, prescreening for *HLA-B*15:02* could prevent 9–10 out of 4120 patients from developing SJS/TEN.

> "*...screening for* HLA-B*57:01 *prior to treatment initiation is useful in reducing the risk of hypersensitive reactions to abacavir.*"

Insight into development of SCARs

Most patients who carry a risk allele for SCARs have been tolerant to a corresponding causative drug. Ko *et al.* examined T-cell receptor (TCR) repertoire usage in carbamazepine-induced SJS/TEN patients and carbamazepine-tolerant patients who carried *HLA-B*15:02* [19]. A specific third complementarity-determining region of the TCR, VB-11-ISGSY, was shared among 84% of the carbamazepine-induced SJS/TEN patients (n = 19), while it was not found in 17 tolerant patients. The study also suggested that carbamazepine-specific cytotoxicity could be primed *in vitro* in the peripheral blood mononuclear cells obtained even from healthy subjects carrying *HLA-B*15:02* and VB-11-ISGSY, which was blocked by an anti-VB-11 antibody. Thus, usage of a specific TCR repertoire may be critical for pathogenic responses to carbamazepine in patients.

> "*According to historical incidence, prescreening for* HLA-B*15:02 *could prevent 9–10 out of 4120 patients from developing SJS/TEN.*"

Carbamazepine is considered to bind noncovalently and directly to HLA [12], while abacavir

hypersensitivity may need a hapten/prohapten-related mechanism including antigen processing [20]. Norcross *et al.* showed differences in amino acid sequences between endogenous peptides isolated from abacavir-treated *HLA-B*57:01* B cells and from untreated cells. Novel drug-induced peptides contained predominantly isoleucine or leucine residues in the carboxyl terminal, which is characteristically different from the residues of the usual *HLA-B*57:01* peptides. The study suggested that abacavir induced loading of novel self-peptides into the HLA-B*57:01 molecule, resulting in presentation of neoantigenic peptides that drive polyclonal T-cell autoimmune responses and multiorgan systemic toxicity.

Strategy in drug safety issues

The pathogenesis of SCARs has not been completely elucidated. Only a limited number of genetic biomarkers for SCARs, including SJS/TEN, have been found, and further prospective/retrospective clinical studies are necessary to explore biomarkers for SCARs. One problem with association studies is that each study group cannot get sufficiently large sample sizes owing to extremely low SJS/TEN incidence. In addition, as shown above, most biomarkers are causative drug specific, which makes it more difficult to perform exploratory studies. Therefore, research groups in neighboring countries with similar ethnic backgrounds should collaborate to collect cases. In addition, synergy with various research areas such as immunobiology, allergy, structural biology, pharmacology and bioinformatics is necessary to reveal the mechanistic basis for SCARs development. These multidisciplinary efforts could generate supportive knowledge for epidemiological data in which only weak associations are detected owing to insufficient sample sizes.

SCARs such as SJS/TEN are generally not identified during the drug-development process owing to their extremely low frequencies but are initially recognized after broad use of a drug during the postapproval period. Therefore, the establishment of animal models and *in vitro* screening methods should be accelerated to identify high risk drug candidates for SCARs that are in the drug-development pipeline.

Conclusion

Through pharmacogenomics, SCARs have become avoidable adverse reactions. Efforts should continue to find more associations and develop diagnostic methods for prediction of high-risk patients to SCARs, resulting in more patients being saved worldwide during the coming decade.

Financial & competing interests disclosure

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References

1 Chung WH, Hung SI, Hong HS *et al.* A marker for Stevens–Johnson syndrome. *Nature* 428(6982), 486 (2004).

2 Man CB, Kwan P, Baum L *et al.* Association between *HLA-B*1502* allele and antiepileptic drug-induced cutaneous reactions in Han Chinese. *Epilepsia* 48, 1015–1018 (2007).

3 Lonjou C, Thomas L, Borot N *et al.* A marker for Stevens–Johnson syndrome...: ethnicity matters. *Pharmacogenomics J.* 6, 265–268 (2006).

4 Mehta TY, Prajapati LM, Mittal B *et al.* Association of *HLA-B*1502* allele and carbamazepine-induced Stevens–Johnson syndrome among Indians. *Indian J. Dermatol. Venereol. Leprol.* 75, 579–582 (2009).

5 Tassaneeyakul W, Tiamkao S, Jantararoungtong T *et al.* Association between *HLA-B*1502* and carbamazepine-induced severe cutaneous adverse drug reactions in a Thai population. *Epilepsia* 51, 926–930 (2010).

6 Wang Q, Zhou JQ, Zhou LM *et al.* Association between *HLA-B*1502* allele and carbamazepine-induced severe cutaneous adverse reactions in Han people of southern China mainland. *Seizure* 20, 446–448 (2011).

7 Kaniwa N, Saito Y, Aihara M *et al.* *HLA-B*1511* is a risk factor for carbamazepine-induced Stevens–Johnson syndrome and toxic epidermal necrolysis in Japanese patients. *Epilepsia* 51, 2461–2465 (2010).

8 Lim KS, Kwan P, Tan CT. Association of *HLA-B*1502* allele and carbamazepine-induced severe adverse cutaneous drug reaction among Asians, a review. *Neurology Asia* 13, 15–21 (2008).

9 Kim SH, Lee KW, Song WJ *et al.* Carbamazepine-induced severe cutaneous adverse reactions and HLA genotypes in Koreans. *Epilepsy Res.* 97(1–2), 190–197 (2011).

10 Wei CY, Chung WH, Huang HW *et al.* Direct interaction between HLA-B and carbamazepine activates T cells in patients with Stevens–Johnson syndrome. *J. Allergy Clin. Immunol.* 129, 1562–1569 (2012).

11 McCormack M, Alfirevic A, Bourgeois S *et al.* *HLA-A*3101* and carbamazepine-induced hypersensitivity reactions in Europeans. *N. Engl. J. Med.* 364, 1134–1143 (2011).

12 Ozeki T, Mushiroda T, Yowang A *et al.* Genome-wide association study identifies *HLA-A*3101* allele as a genetic risk factor for carbamazepine-induced cutaneous adverse drug reactions in Japanese population. *Hum. Mol. Genet.* 20, 1034–1041 (2011).

13 Hung SI, Chung WH, Liou LB *et al.* *HLA-B*5801* allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol. *Proc. Natl Acad. Sci. USA* 102, 4134–4139 (2005).

14 Tassaneeyakul W, Jantararoungtong T, Chen P *et al.* Strong association between *HLA-B*5801* and allopurinol-induced Stevens–Johnson syndrome and toxic epidermal necrolysis in a Thai population. *Pharmacogenet. Genomics* 19, 704–709 (2009).

15 Tohkin M, Kaniwa N, Saito Y *et al.* A whole-genome association study of major determinants for allopurinol-related Stevens–Johnson syndrome and toxic epidermal necrolysis in Japanese patients. *Pharmacogenomics J.* 13, 60–69 (2011).

16 Maekawa K, Nishikawa J, Kaniwa N *et al.* Development of a rapid and inexpensive assay for detecting a surrogate genetic polymorphism of *HLA-B*58:01*: a partially predictive but useful biomarker for allopurinol-related Stevens–Johnson syndrome/toxic epidermal necrolysis in Japanese. *Drug Metabol. Pharmacokinet.* 27(4), 447–450 (2012).

17 Mallal S, Phillips E, Carosi G *et al.* *HLA-B*5701* screening for hypersensitivity to abacavir. *N. Engl. J. Med.* 358, 568–579 (2008).

18 Chen P, Lin JJ, Lu CS *et al.* Taiwan SJS Consortium. Carbamazepine-induced toxic effects and *HLA-B*1502* screening in Taiwan. *N. Engl. J. Med.* 364, 1126–1133 (2011).

19 Ko TM, Chung WH, Wei CY *et al.* Shared and restricted T-cell receptor use is crucial for carbamazepine-induced Stevens–Johnson syndrome. *J. Allergy Clin. Immunol.* 128, 1266–1276 (2011).

20 Norcross MA, Luo S, Lu L *et al.* Abacavir induces loading of novel self-peptides into *HLA-B*57: 01*: an autoimmune model for HLA-associated drug hypersensitivity. *AIDS* 26, F21–F29 (2012).

Highlighted Paper selected by Editor-in-Chief

Development of Pyrrole–Imidazole Polyamide Targeting Fc Receptor Common Gamma Chain for the Treatment of Immune-Complex Related Renal Disease

Mamiko Kajiwara,[a] Takahiro Ueno,*[,a,b] Noboru Fukuda,[a] Hiroyuki Matsuda,[e] Toshibumi Shimokawa,[c] Maki Kitai,[a] Akiko Tsunemi,[a] Yoshinobu Fuke,[a] Takayuki Fujita,[a] Koichi Matsumoto,[a] Yoshiaki Matsumoto,[d] Chisei Ra,[c] and Masayoshi Soma[a,b,e]

[a] *Division of Nephrology, Hypertension and Endocrinology, Department of Medicine, School of Medicine, Nihon University;* [b] *Innovative Therapy Research Group, Nihon University Research Institute of Medical Science, School of Medicine, Nihon University;* [c] *Division of Molecular Cell Immunology and Allergology, Department of Biomedical Sciences, School of Medicine, Nihon University;* [e] *Division of General Medicine, Department of Medicine, School of Medicine, Nihon University; 30–1 Oyaguchi-kami, Itabashi-ku, Tokyo 173–8610, Japan: and* [d] *Department of Clinical Pharmacokinetics, Collage of Pharmacy, Nihon University; 7–7–1 Narashinodai, Funabashi, Chiba 274–8555, Japan.*

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Fcγ receptors I and III are thought to be involved in the development of lupus nephritis. Expression of Fc receptor common gamma chain (FcRγ) is necessary for the stable expression of Fcγ receptors I and III. The aim of this study was to develop a novel agent for the treatment of immune complex related renal disease using a gene regulator, pyrrole(Py)–imidazole(Im) (PI) polyamide, targeting the mouse FcRγ gene promoter. Two PI polyamides targeting FcRγ promoters were designed and synthesized. The effect of the PI polyamides on FcRγ mRNA expression was evaluated in J774.A cells by real-time polymerase chain reaction (PCR), and CD16/32 protein expression was determined by immunocytochemical analysis and flow cytometry. The effects of these polyamides on FcRγ gene expression and CD16/32 protein expression were evaluated in mouse peripheral blood mononuclear cells (PBMCs). One milligram per kilogram body weight of PI polyamide was injected *via* the tail vein every 2 d for 1 week and PBMCs were collected and analyzed. PI polyamide showed a specific binding to the target DNA in a gel mobility shift assay. Treatment of J774.A cells with 1.0 μM PI polyamide 1 significantly reduced FcRγ mRNA expression and CD16/32 surface protein expression in J774.A cells. Similarly, PI polyamide significantly decreased expression of FcRγ mRNA and CD16/32 in the PBMCs of C57B6 mice. PI polyamide designed to bind the FcRγ promoter decreased FcRγ gene and CD16/32 protein expression. PI polyamide targeting the FcRγ gene may be a novel gene regulator for the prevention of lupus nephritis or other immune complex-related disease.

Key words pyrrole imidazole polyamide; Fc receptor; immune complex; kidney disease

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of autoantibodies against wide arrays of self-antigens that induce tissue damage. Lupus nephritis is one of the most important clinical complications of SLE. Formation of immune complexes between these autoantibodies and the self antigens has been linked to lupus nephritis.[1] Immune complexes are formed in the circulation outside to the glomerulus and deposited on the renal capillaries. The deposition of immune complexes in the kidneys activates monocyte/macrophages by interacting with Fcγ receptor I and Fc receptor III, initiating an inflammatory cascade of cytokines and chemokines. Using mouse models lacking Fc receptors, the Fc receptor was found to play a pivotal role in disease development of various immune complex mediated diseases.[2–8] Mice with targeted disruption of Fc receptor common gamma chain (FcRγ) lacking Fcγ receptors I and III were completely protected from the development of lupus nephritis.[9] The gamma subunits of Fcγ receptors I and III are postulated to be the key determinant of maturation and surface expression of the receptors. Because of the critical role of gamma subunit in up-regulating Fcγ receptors I and III, there has been considerable interest in the development of chemical agent that regulate FcRγ genes.

Pyrrole(Py)–imidazole(Im) polyamides (PI polyamide) are synthetic DNA binding molecules designed to recognize and bind to specific sequences in the minor groove of the DNA double helix.[10,11] The pairing of an Im with a Py targets the G-C and C-G base pairs, and a Py-Py pairing targets the T-A and A-T base pairs.[12,13] The binding constant and sequence specificity of the PI polyamide is comparable to that of transcription factors. It has been demonstrated that PI polyamide competitively binds to regulatory sequences and inhibits the transcription-factor dependent gene regulation.[14] We have been developing PI polyamide compounds targeting the transforming growth factor beta 1 (TGFβ1) gene as potential therapy for renal fibrosis,[15,16] and targeting the lectin-like oxidative low density lipoprotein (LDL) receptor 1 gene for atherosclerotic disease.[17,18]

In this study, we employed a similar approach to develop a new agent for the treatment of immune complex-related renal disease. Accordingly, we designed PI polyamides targeting the mouse FcRγ gene promoter and determined their effects on Fcγ receptors expression.

MATERIALS AND METHODS

Luciferase Vector Construction Mouse FcRγ promoter was cloned into the pGL3-Basic luciferase vector. The promoter fragment was cleaved with *Nhe*I and *Mlu*I restriction endonucleases and self-ligated into the promoter site of the

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: ueno.takahiro@nihon-u.ac.jp

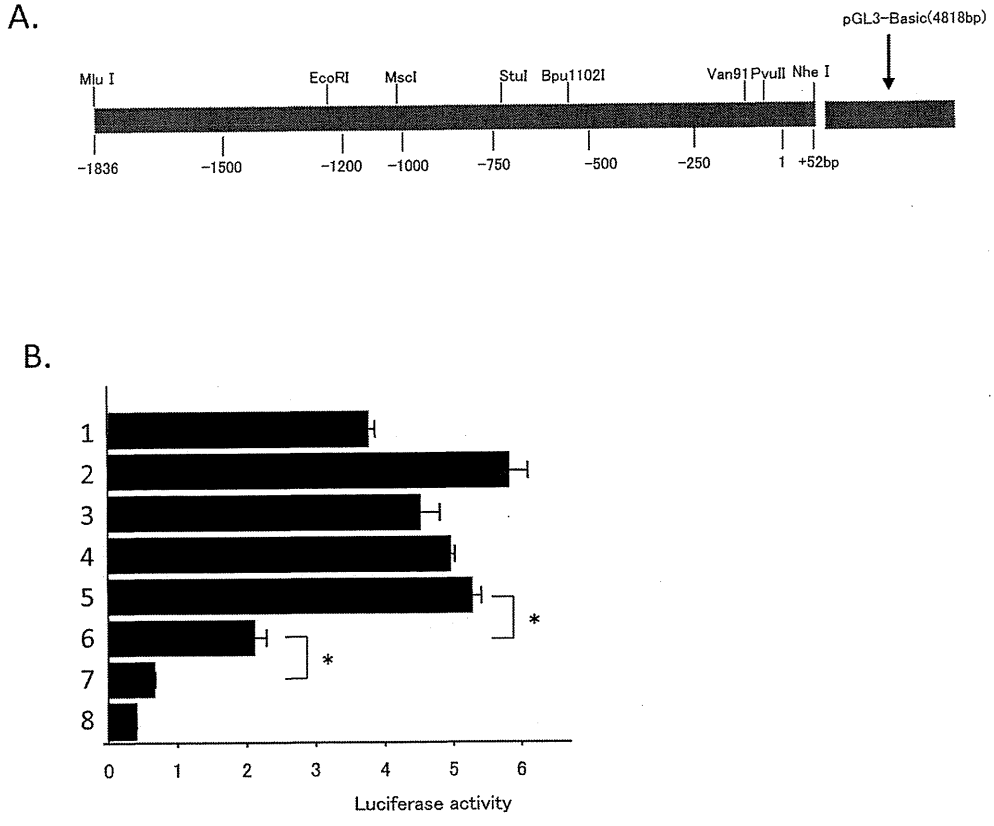

Fig. 1. FcR*y* Gene Promoter Analysis

(A) Restriction site map of mouse FcR*y* promoter used in the deletion mutant analysis. The promoter fragment was cleaved with *Nhe*I and *Mlu*1 restriction endonucleases and self-ligated to the promoter pGL3-basic vector. Other deletion mutants were generated by cleaving with *Nhe*I and *Pvu*II, *Van*91, *Bpu*I1021, *Stu*1, *Msc*I or *Eco*RI to obtain promoter fragment terminating at −50, −110, −595, −738, −1028, 1250, and 1836 bp, respectively. (B) Result of FcR*y* promoter assay using the deletion mutant luciferase assay. 1: *Nhe*I-*Mlu*1 (from +52 to −1836), 2: *Nhe*I-*Eco*RI (from +52 to −1250), 3: *Nhe*I-*Msc*I (from +52 to −1028), 4: *Nhe*I-*Stu*I (from +52 to −738), 5: *Nhe*I-*Bpu*1 (from +52 to −595), 6: *Nhe*I-*Van*91 (from +52 to −110), 7: *Nhe*I-*Pvu*II (from +52 to −50), 8: no insert. Data are shown as the mean±S.E. (n=5). *p<0.05 by Student t-test.

pGL3-basic vector using a DNA blunting kit (TaKaRa, Shiga, Japan). Other deletion mutants were generated by cleaving with *Nhe*I and *Pvu*II, *Van*91, *Bpu*I1021, *Stu*1, *Msc*I or *Eco*RI endonucleases to obtain promoter fragments terminating at positions −50, −110, −595, −738, −1028, 1250 and 1836 of the promoter sequence (Fig. 1A). HEK-293 cells were seeded onto 24-well plates and grown in Dulbecco's minimal essential medium (DMEM) (Sigma-Aldrich, St. Louis, MO, U.S.A.) containing 20% calf serum. When cells reached 70 to 90% confluence, a mixture of reporter plasmid (1 μg/well) and phRG-TK vector (0.01 μg/well; Promega, WI, U.S.A.) as an internal control, was used to transfect cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) as described previously[19] and then changed to fresh medium with or without 1.0 μmol/L phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich). Cells were incubated for an additional 24 h and then scraped into 100 μL of cold lysis buffer (phosphate buffered saline (PBS) (pH 7.4) containing 1 mmol/L phenylmethylsulfonyl fluoride). Luciferase activity was measured with a Dual-luciferase reporter assay system (Promega) and a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, U.S.A.).[20]

Synthesis of PI Polyamides PI polyamides were synthesized according to previously described methods[21,22] as described in patent number WO2007/060860. A mismatch polyamide which did not bind to the target transcription binding sites of FcR*y* promoter was also designed and synthesized.

DNA Binding Assay Fluorescein isothiocyanate (FITC)-labeled oligonucleotides including PI polyamide binding sequences were synthesized for use in a gel mobility shift assay. One micro mol per litter of the FITC-labeled oligonucleotides was incubated with 1.0 μmol/L of PI polyamide for 1 h at 37°C with or without 10 μmol/L unlabeled competitor dsDNA. The resulting complexes were separated by electrophoresis and visualized using a luminescent image analyzer LAS-4000 (FUJIFILM, Tokyo, Japan).

Determination of mRNA Expression For *in vitro* experiments, J774.A cells (Health Science Research Resource Bank, Han-nan, Japan) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Invitrogen) and 50 mg/mL streptomycin (Invitrogen). Cells were differentiated using phorbol-12-myristate-13-acetate (PMA) at a concentration of 0.1 μmol/L. To determine the effect of PI polyamide on FcR*y* mRNA expression, J774.A cells were incubated with 1.0, 0.1, or 0.01 μmol/L PI polyamide targeting FcR*y* or 1.0 μmol/L mismatch PI polyamide in DMEM containing 0.5% FCS for 8 h in the presence of 1.0 μmol/L PMA. Following incubation, total RNA was isolated and reverse-transcribed as described previously.[23] Real-time quantitative PCR was performed with 4× diluted cDNA using TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, U.S.A.) and an ABI 7500

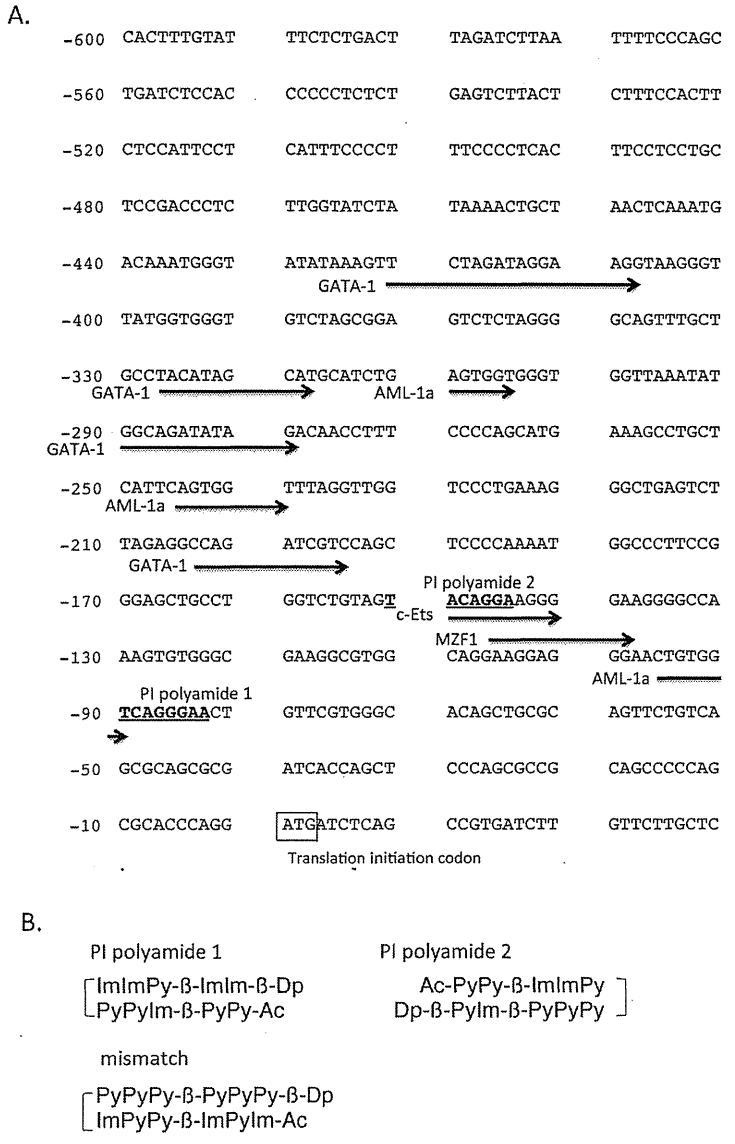

Fig. 2. Mouse FcRγ Promoter Sequence Analysis and the Structures of PI Polyamides Targeting FcRγ

(A) Mouse FcRγ sequence. Predicted transcription factor binding sites between −595 bp and the transcription start site using TFSEARCH. This region contained 9 possible transcription factors binding sites. PI polyamide targeting the upstream AML-1a site was designated as PI polyamide 1 and cEts-MZF1 site was designated as PI polyamide 2. PI polyamide binding sequences are indicated by underlines. Transcription factors binding sequences predicted by TFSEARCH are indicated by black arrows. (B) Structure of PI polyamides targeting FcRγ (PI polyamide 1 and PI polyamide 2) and mismatch PI polyamides.

sequence detector (Applied Biosystems) according to the manufacturer's instructions. Assay-on-Demand primers and probes were purchased from Applied Biosystems. Bands corresponding to 18S rRNA were quantified for sample normalization. Real-time PCR data were analyzed using a standard curve. In all cases, the correlation coefficients for the standard curves were >0.90.

Immunocytochemistry Cells were grown in chamber slides (NUNC, Rochester, NY, U.S.A.) and fixed in 4% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100, blocked with 1% normal goat serum and 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS), and incubated overnight at 4°C with mouse monoclonal anti-CD16/32 antibody (1:500; BD Bioscience, San Jose, CA, U.S.A.). The following day cells were washed 3 times with PBS then incubated with the secondary antibody (Alexa 594 goat anti-mouse immunoglobulin G (IgG), 1:500; Invitrogen) for 1 h at room temperature. After washing 3 times with PBS, the nuclei were counterstained with 5 μg/mL 4′,6-diamidino-2-phenylindole (Dojindo Molecular Technologies, Inc., ML, U.S.A.). The samples were air dried, mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL, U.S.A.), and photographed using a digital camera under a fluorescent microscope (Nikon Eclipse TE 2000-U).

Flow Cytometric Analysis J774.A cells were suspended in PBS containing 0.1% bovine serum albumin (Sigma-Aldrich) at a density of 5×10^5 cells per tube. After blocking, cells were stained for 30 min on ice with anti mouse CD16/32 antibody. Binding of first antibody was detected by secondary staining with phycoerythrin-conjugated anti-rabbit IgG antibody (BD Biosience, NJ, U.S.A.). Cells were analyzed with a FACSCalibur flow cytometer with the Cell Quest software

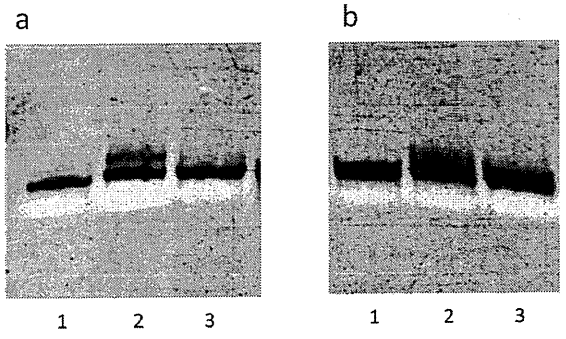

Fig. 3. Binding of PI Polyamide to the Target Sequences

A double-stranded DNA (dsDNA) fragment corresponding to bp −70 to −160 of the FcRγ promoter was labeled with FITC. One μmol/L labeled dsDNA was incubated with 1 μmol/L PI polyamides designed for FcRγ with or without 10 μmol/L unlabelled competitor dsDNA. a: PI polyamide 1, b: PI polyamide 2. Lane 1: FITC-conjugated synthetic dsDNA corresponding to the target sequence; Lane 2: FITC-conjugated target dsDNA+PI polyamide (1:1); Lane 3: FITC-conjugated target dsDNA+PI polyamide+unlabeled dsDNA (1:1:10).

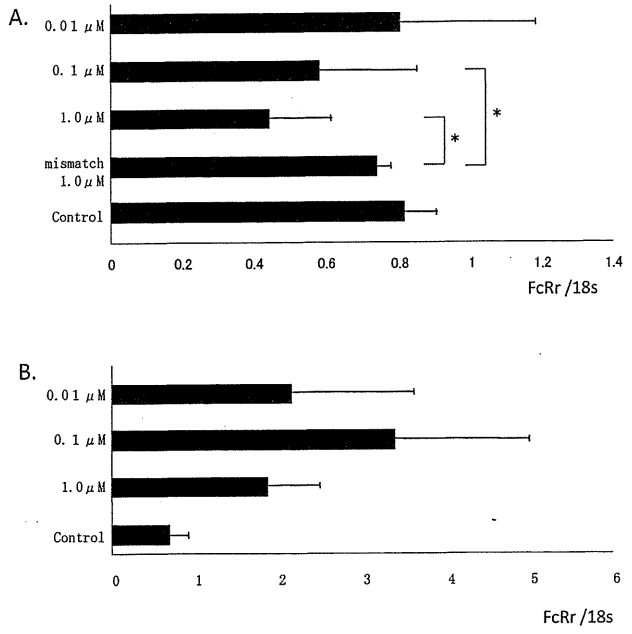

Fig. 4. Effect of PI Polyamide on FcRγ mRNA Expression in J774.A Cells

RT-PCR analysis of expression of FcRγ mRNA with 0.01, 0.1, and 1.0 μmol/L PI polyamide 1 (A) or PI polyamide 2 (B). The ratios of FcRγ mRNA to 18S rRNA were evaluated by analysis of the concentration of amplified PCR products. Data are shown as the mean±S.E. (n=6). *p<0.05 *vs.* control cells.

package (Becton Dickinson, Bedford, MA, U.S.A.). Positive cells were counted and compared with the signal of corresponding control cells.

***In Vivo* Experimental Design** This study conformed to the guidelines published in the 'Guide for the Care and Use of Laboratory Animals' of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by Nihon University Institutional Animal Care and Use Committee. Male C57BL/6 mice (Charles River Breeding Laboratories, Stone Ridge, NY, U.S.A.) were used in all of the experiments.

Mice were divided into 2 groups and were fed normal chow diet (Oriental Yeast, Tokyo, Japan) *ad libitum* for 2 weeks. One milligram per kilogram body weight of PI polyamide targeting FcRγ (or mismatch polyamide) was dissolved in 100 μL of DMSO plus 100 μL of H_2O and injected *via* the tail veins every 2 d for 1 week (total 7 mg of polyamide per rat). Mice were euthanized by a lethal injection of sodium pentobarbital (IP, 100 mg/kg of body weight) at 14 d after PI polyamide administration. Peripheral blood was collected in heparinized tubes, layered on a Ficoll-Paque density gradient (Sigma-Aldrich) and centrifuged at 400×***g*** for 30 min at room temperature to isolate peripheral blood mononuclear cells (PBMCs). PBMCs were fixed onto glass slides using Cyto-spin and immunocytochemical staining was performed with anti mouse CD16/CD32 antibodies. Subsequently, total RNA was isolated from the PBMCs, reverse-transcribed into cDNA, and real-time quantitative PCR was performed with the cDNA.

Statistical Analysis Results are given as the mean±standard error of the mean (S.E.M.). The significance of differences between means of 2 groups was evaluated by Student's t-test for unpaired data. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

Mouse FcRγ Promoter Analysis A mouse FcRγ promoter-pGL3-basic luciferase reporter chimeric plasmid was created as a tool for identification of the transcription factor elements responsible for activation of the mouse FcRγ gene. Deletion of the 110 bp sequence downstream of the transcription start site (−110 bp deletion construct) markedly reduced the promoter activity compared with deletion of −595 bp downstream sequence. Deletion of −50 bp downstream sequence resulted in a greater reduction of promoter activity compared with deletion of −110 bp sequence (Fig. 1B). These results suggest that the regions corresponding to sequence −595 to −110 bp downstream of the transcription initiation site and −110 to −50 bp downstream sequence contain distinct elements controlling the basal transcriptional regulation of the gene.

We searched for transcription factor binding sites between the-595 bp downstream location and the transcription start site with TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html). This region contained 9 possible transcription factors binding sites. At first, we focused on the upstream transcription factor binding sites and designed PI polyamids targeting the upstream AML-1a site (PI polyamide 1) and cEts-MZF1 site (PI polyamide 2) (Fig. 2A). Structures of the 2 PI polyamides targeting the FcRγ gene and the mismatch polyamides are shown in Fig. 2B.

Binding and Specificity of PI Polyamide to Double-Stranded DNA Gel mobility shift assays allow for the determination of the binding of PI polyamides to the target DNA sequences. Both PI polyamides bound the double stranded DNA. Bands that appeared by the binding of the PI polyamide to the target sequences disappeared by the addition of excess unlabeled double strand target DNA showing the specific binding (Fig. 3).

Effect of PI Polyamide on FcRγ mRNA Expression in J774.A Cells Addition of 1.0 or 0.1 mmol/L PI polyamide 1 significantly (p<0.05) reduced-the-abundance of FcRγ mRNA by 50%, compared with non-treated cells. Addition of 0.001 mmol/L PI polyamide 1 or mismatch polyamide, did not

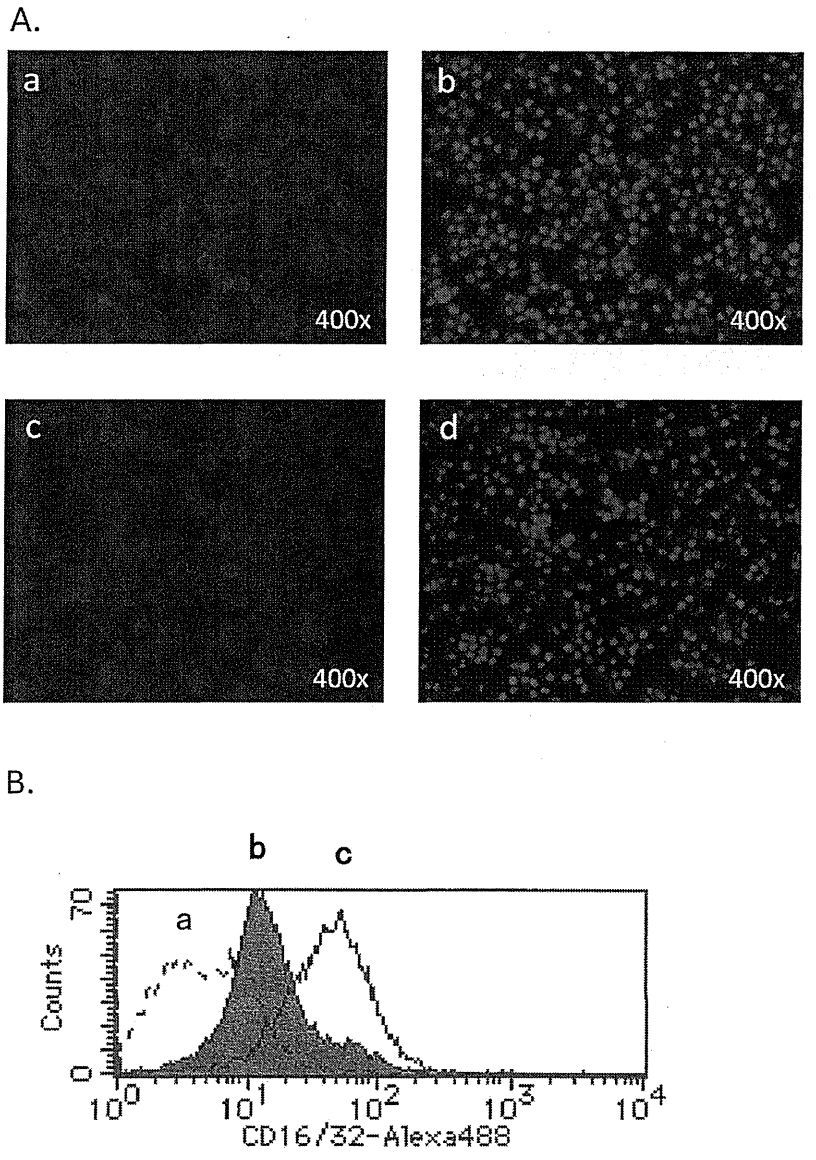

Fig. 5. Effect of PI Polyamide 1 on Cell Surface Expression of CD16/32 in J774.A Cells

J774. A cells were incubated with 1 mmol/L of mismatch PI polyamide (a: anti CD16/32 antibody (1:500; BD Bioscience), b: anti CD16/32 antibody and Hoechst staining) or 1 mmol/L of PI targeting FcRγ promoter (c: anti CD16/32 antibody, d: anti CD16/32 antibody and Hoechst staining), and further labeled with secondary antibody (Alexa 594 goat anti-mouse IgG, 1:500; Invitrogen) to anti-CD16/32 antibody by immunocytochemical staining (A). CD16/32 expression in PI polyamide treated cells was then evaluated by flow cytometric analysis (B). (a: Isotype control, b: PI polyamide 1, c: Mismatch PI polyamide).

affect the expression of FcRγ mRNA compared with the non-treated cells (Fig. 4A). Whereas PI polyamide 2, increased the abundance of FcRγ mRNA (the difference was not-statistically significant and the effect was not dose-dependent) (Fig. 4B). Therefore, only PI polyamide 1 was used in the subsequent experiments.

Effect of PI Polyamide 1 on CD16 Expression in J774.A Cells To confirm the inhibitory effect of PI polyamide 1 on expression of CD16 proteins in J774.A cells, we performed immunocytochemical analysis against CD16/CD32. Incubation with 1.0 μmol/L PI polyamide 1 for 8 h suppressed the expression of CD16/CD32 proteins in J774.A cells compared with mismatch PI polyamide (Fig. 5A). Flow cytometric analysis showed that treatment of J774.A cells with PI polyamide 1 targeting FcRγ decreased the number of CD16/CD32 positive cells (21.6%) compared with the cells treated with mismatch PI polyamide (75.1%) (Fig. 5B, Table 1).

Table 1. Effect of PI Polyamide on CD16/32 in J774.A Cells Evaluated by Flow Cytometric Analysis

%	Mean	S.D.
Isotype conrtol	1.5	0.8
FcRγ PI polyamide	21.2*	0.1
Mismatch PI polyamide	75.1	6.4

J774.A cells were incubated with 1 mmol/L of mismatch PI polyamide or PI targeting FcRγ, and CD16/32 expression was evaluated by flow cytometric analysis. Data are shown as the mean±S.E. (n=6). *p<0.05 *vs.* mismatch PI.

Effect of PI Polyamide *in Vivo* We examined *in vivo* effects of PI polyamide 1 on CD16 expression and FcRγ mRNA

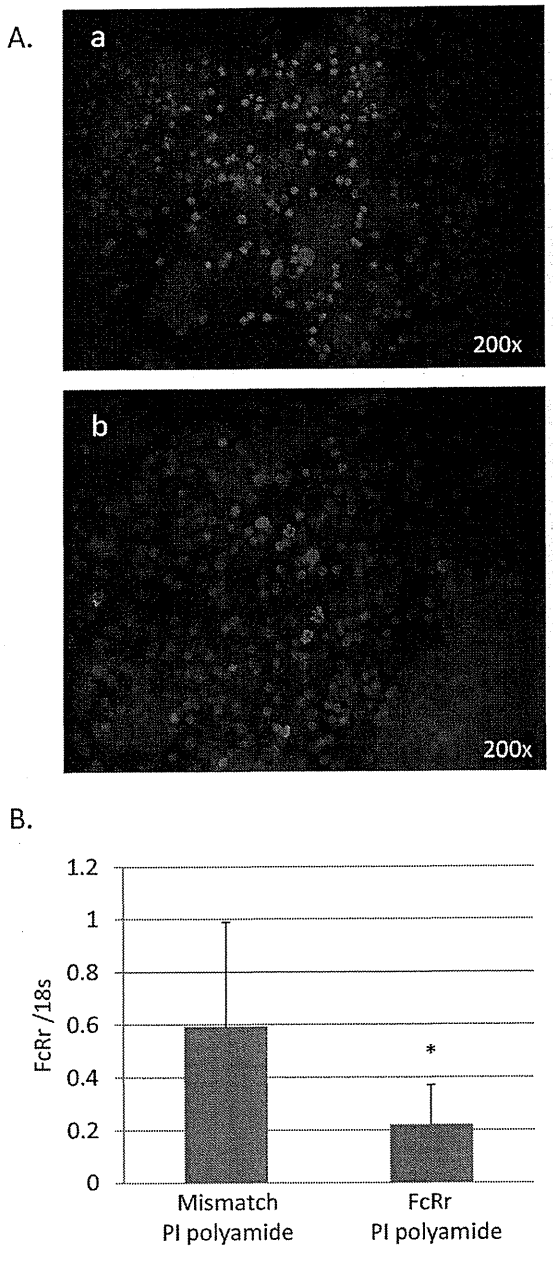

Fig. 6. Effect of PI Polyamide 1 on Mouse Peripheral Blood Mononuclear Cell (PBMC) CD16/32 Surface Expression and FcRγ mRNA Level

One milligram per kg body weight of PI polyamide targeting FcRγ or mismatch was injected to C57BL/6 mice *via* the tail veins every 2 d for 1 week. At 14 d after PI polyamide administration, peripheral blood was collected and PBMCs were isolated and fixed on the slides using Cyto-spin followed with immunocytochemical staining with anti mouse CD16/32 antibody (A). Subsequently, total RNA was isolated from PBMCs and reverse-transcribed to obtain cDNA and real-time quantitative PCR was performed with cDNA (B). Data are shown as the mean±S.E. (n=3). *$p<0.05$ *vs.* mismatch PI polyamide.

expression in peripheral blood mononuclear cells (PBMCs) from mice. *In vivo* treatments with PI polyamide 1 considerably decreased PBMC surface expression of CD16/32 by immunocytochemical analysis (Fig. 6A). Treatment with PI polyamide 1 also significantly ($p<0.05$) decreased the abundances of FcRγ mRNA in PBMCs (Fig. 6B).

DISCUSSION

Cell surface receptors that bind IgG-immune complexes, known collectively as Fcγ receptors, play essential roles in diseases mediated by antibodies.[24] In particular, mice deficient in the common r-chain required for expression of the murine activating Fcγ receptors are protected from development of acute and progressive glomerulonephritis.[5,9] Moreover, we have previously reported that PI polyamide was strongly localized to almost all nuclei in the renal tubules after intravenous administration, and persisted in the nuclei of renal tubules 14 d later. Based on these observations, we developed a novel gene silencer, PI polyamide, to suppress FcRγ gene expression as a potential therapeutic approach for immune-complex related renal diseases.[15]

We analyzed the regulatory elements of FcRγ that are critical for expression of the gene. Results of the luciferase assay revealed the existence of regulatory element that strongly affects FcRγ gene expression spanning between −50 to −110 bp and −110 to −595 bp region of the FcRγ promoter. Nine possible transcription factor binding sequences were predicted by TFSEARCH analysis, and we first focused on the 3 transcription factor binding sites (AML-1a, MZF-1 and c-Ets) located in closer region from the taranscription start site. We designed 2 PI polyamides for the sequences close to AML-1a binding site (PI polyamide 1) and close to c-Ets and MZF1 binding site (PI polyamide 2). Real-time PCR analysis showed a significant decrease of FcRγ mRNA expression in J774.A cells by PI polyamide 1, whereas PI polyamide 2 increased the expression (but not statistically significantly). This difference of the effect of PI polyamide on FcRγ mRNA expression might be associated with the difference in the function of the target transcription factors. Takahashi *et al.*[25] and Juang *et al.*[26] reported that the Ets-related transcription factor, Elf-1, binds to the GGAA element on the FcRγ promoter and represses its expression. The enhancing effect of PI polyamide 2 may result from the competition with Elf-1 transcription factor.

PI polyamide 1 binds to the sequence including the AML-1a (also called RUNT related transcription factor 1; RUNX1) transcription factor binding site. The regulating mechanisms of AML-1a on FcRγ gene expression have not been clarified. However, recent studies investigating the genetic susceptibility of SLE, rheumatoid arthritis, and psoriasis have revealed potential roles for RUNX1 protein in the development of autoimmune diseases.[27–29] These studies on the relationship of autoimmune disease and RUNX1 gene and AML-1a/RUNX1 binding sites suggest that these regulatory sequences are good targets to design PI polyamides as therapeutic agents for the prevention of the complication associated with autoimmune diseases.

Since Fcγ receptors I and III play significant roles in the development of lupus nephritis, the current study was undertaken to develop PI polyamides as novel agents that suppress the expression of Fcγ receptors I and III on the protein level. Because the gamma subunit of Fcγ receptors I and III is known to be a key determinant of maturation and surface expression level of Fcγ receptors I and III, we designed and synthesized PI polyamides targeting the FcRγ gene. In the present study, PI polyamides targeting the FcRγ reduced the expression of CD16/32 level on the macrophage cell surface. This is evidence for the suppression of FcRγ resulting from a

reduction in expression of Fcγ receptors I and III.

Nucleic acid medicines such as antisense DNA, ribozymes, and decoys have been developed as gene-silencing agents. Decoys, in particular, inhibit the binding of transcription factors to their targets in a manner similar to PI polyamides. However, because these agents are degraded easily by nucleases, they require drug-delivery systems for sufficient distribution into organs. By contrast, PI polyamides are completely resistant to the biological degradation by nucleases and are thus more stable *in vivo*.[10] Also, all nucleic acid-based medicine require virus vectors, non-virus vectors or other transfection reagents to incorporate into cells or distribute to target tissues. PI polyamides do not require any transfection agent, and are easily taken up into the nucleus of cells and remain in nucleus at least 7 d. Because of these desirable properties, PI polyamides could be used as novel gene silencing agents in the near future.

To develop PI polyamide as a novel agent to regulate lupus nephritis, we need further evaluation in animal models of the disease. Mice of the NZB/NZW F1 mouse strain spontaneously develop autoimmune lupus nephritis. Female mice from the NZB x NZWF1 cross develop proteinuria and only small number (<20%) survive to 52 weeks.[30] This animal model of lupus nephritis could be a suitable animal model to evaluate the effect of PI polyamide on the development of lupus nephritis.

In a previous study we demonstrated that PI polyamide targeting the TGFβ1 gene reduced urinary TGFβ excretion by 60%, resulting in marked inhibition of renal injury in an animal model of hypertension-induced renal disorder. Results of the previous study supports the findings relative to the *in vivo* effect of the PI polyamid targeting FcRγ that showed a reduction in peripheral blood CD16/32 cell count of about 60%.[15]

We have developed a PI polyamide targeting the TGFβ1 gene as a therapeutic tool for renal fibrosis in end-stage renal disease. Treatment with PI polyamide targeting TGFβ1 resulted in a significant reduction in renal fibrosis *in vivo*, suggesting PI polyamide is a biologically active drug in this animal model of chronic disease. There were no apparent side effects in animals treated with PI polyamide.[15,16] However, basic studies about the biological behavior of PI polyamide, such as pharmacokinetics or toxicity have not been completed. Because PI polyamide is a potentially novel medicine, and is commercially unavailable, we need perform further analysis of the basic features of this agent to develop this drug as a therapeutic agent.

In conclusion, the synthetic PI polyamide designed to bind the FcRγ promoter decreased FcRγ gene expression with a corresponding decrease in CD16/32 protein expression. PI polyamide targeting the FcRγ gene may be a novel gene regulator for the prevention of lupus nephritis or other immune complex-related disease.

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REFERENCES

1) Ebling F, Hahn BH. Restricted subpopulations of DNA antibodies in kidneys of mice with systemic lupus. Comparison of antibodies in serum and renal eluates. *Arthritis Rheum.*, **23**, 392–403 (1980).
2) Hazenbos WL, Gessner JE, Hofhuis FM, Kuipers H, Meyer D, Heijnen IA, Schmidt RE, Sandor M, Capel PJ, Daëron M, van de Winkel JG, Verbeek JS. Impaired IgG-dependent anaphylaxis and Arthus reaction in Fc gamma RIII (CD16) deficient mice. *Immunity*, **5**, 181–188 (1996).
3) Clynes R, Ravetch JV. Cytotoxic antibodies trigger inflammation through Fc receptors. *Immunity*, **3**, 21–26 (1995).
4) Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV. FcR gamma chain deletion results in pleiotrophic effector cell defects. *Cell*, **76**, 519–529 (1994).
5) Park SY, Ueda S, Ohno H, Hamano Y, Tanaka M, Shiratori T, Yamazaki T, Arase H, Arase N, Karasawa A, Sato S, Ledermann B, Kondo Y, Okumura K, Ra C, Saito T. Resistance of Fc receptor-deficient mice to fatal glomerulonephritis. *J. Clin. Invest.*, **102**, 1229–1238 (1998).
6) Meyer D, Schiller C, Westermann J, Izui S, Hazenbos WL, Verbeek JS, Schmidt RE, Gessner JE. FcgammaRIII (CD16)-deficient mice show IgG isotype-dependent protection to experimental autoimmune hemolytic anemia. *Blood*, **92**, 3997–4002 (1998).
7) Ra C, Jouvin MH, Blank U, Kinet JP. A macrophage Fc gamma receptor and the mast cell receptor for IgE share an identical subunit. *Nature*, **341**, 752–754 (1989).
8) Suzuki Y, Shirato I, Okumura K, Ravetch JV, Takai T, Tomino Y, Ra C. Distinct contribution of Fc receptors and angiotensin II-dependent pathways in anti-GBM glomerulonephritis. *Kidney Int.*, **54**, 1166–1174 (1998).
9) Clynes R, Dumitru C, Ravetch JV. Uncoupling of immune complex formation and kidney damage in autoimmune glomerulonephritis. *Science*, **279**, 1052–1054 (1998).
10) Dervan PB. Molecular recognition of DNA by small molecules. *Bioorg. Med. Chem.*, **9**, 2215–2235 (2001).
11) Gottesfeld JM, Turner JM, Dervan PB. Chemical approaches to control gene expression. *Gene Expr.*, **9**, 77–91 (2000).
12) Mrksich M, Wade WS, Dwyer TJ, Geierstanger BH, Wemmer DE, Dervan PB. Antiparallel side-by-side dimeric motif for sequence-specific recognition in the minor groove of DNA by the designed peptide 1-methylimidazole-2-carboxamide netropsin. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 7586–7590 (1992).
13) White S, Baird EE, Dervan PB. Effects of the A.T/T.A degeneracy of pyrrole—imidazole polyamide recognition in the minor groove of DNA. *Biochemistry*, **35**, 12532–12537 (1996).
14) Gottesfeld JM, Neely L, Trauger JW, Baird EE, Dervan PB. Regulation of gene expression by small molecules. *Nature*, **387**, 202–205 (1997).
15) Matsuda H, Fukuda N, Ueno T, Katakawa M, Wang X, Watanabe T, Matsui S, Aoyama T, Saito K, Bando T, Matsumoto Y, Nagase H, Matsumoto K, Sugiyama H. Transcriptional inhibition of progressive renal disease by gene silencing pyrrole-imidazole polyamide targeting of the transforming growth factor-β1 promoter. *Kidney Int.*, **79**, 46–56 (2011).
16) Matsuda H, Fukuda N, Ueno T, Tahira Y, Ayame H, Zhang W, Bando T, Sugiyama H, Saito S, Matsumoto K, Mugishima H, Serie K. Development of gene silencing pyrrole-imidazole polyamide targeting the TGF-betal promoter for treatment of progressive renal diseases. *J. Am. Soc. Nephrol.*, **17**, 422–432 (2006).
17) Ueno T, Fukuda N, Tsunemi A, Yao EH, Matsuda H, Tahira K, Matsumoto T, Matsumoto K, Matsumoto Y, Nagase H, Sugiyama H, Sawamura T. A novel gene silencer, pyrrole-imidazole polyamide targeting human lectin-like oxidized low-density lipoprotein receptor-1 gene improves endothelial cell function. *J. Hypertens.*, **27**, 508–516 (2009).
18) Yao EH, Fukuda N, Ueno T, Matsuda H, Matsumoto K, Nagase H, Matsumoto Y, Takasaka A, Serie K, Sugiyama H, Sawamura T. Novel gene silencer pyrrole-imidazole polyamide targeting lectin-like oxidized low-density lipoprotein receptor-1 attenuates

restenosis of the artery after injury. *Hypertension*, **52**, 86–92 (2008).
19) Magin-Lachmann C, Kotzamanis G, D'Aiuto L, Wagner E, Huxley C. Retrofitting BACs with G418 resistance, luciferase, and oriP and EBNA-1—new vectors for *in vitro* and *in vivo* delivery. *BMC Biotechnol.*, **3**, 2 (2003).
20) Harger JW, Dinman JD. An *in vivo* dual-luciferase assay system for studying translational recoding in the yeast *Saccharomyces cerevisiae*. *RNA*, **9**, 1019–1024 (2003).
21) Wurtz NR, Turner JM, Baird EE, Dervan PB. Fmoc solid phase synthesis of polyamides containing pyrrole and imidazole amino acids. *Org. Lett.*, **3**, 1201–1203 (2001).
22) Bando T, Sugiyama H. Synthesis and biological properties of sequence-specific DNA-alkylating pyrrole-imidazole polyamides. *Acc. Chem. Res.*, **39**, 935–944 (2006).
23) Tahira Y, Fukuda N, Endo M, Suzuki R, Ikeda Y, Takagi H, Matsumoto K, Kanmatsuse K. Transforming growth factor-beta expression in cardiovascular organs in stroke-prone spontaneously hypertensive rats with the development of hypertension. *Hypertens. Res.*, **25**, 911–918 (2002).
24) Schmidt RE, Gessner JE. Fc receptors and their interaction with complement in autoimmunity. *Immunol. Lett.*, **100**, 56–67 (2005).
25) Takahashi K, Hayashi N, Shimokawa T, Umehara N, Kaminogawa S, Ra C. Cooperative regulation of Fc receptor gamma-chain gene expression by multiple transcription factors, including Spl, GABP, and Elf-1. *J. Biol. Chem.*, **283**, 15134–15141 (2008).
26) Juang YT, Sumibcay L, Tolnay M, Wang Y, Kyttaris VC, Tsokos GC. Elf-1 binds to GGAA elements on the FcRgamma promoter and represses its expression. *J. Immunol.*, **179**, 4884–4889 (2007).
27) Prokunina L, Castillejo-López C, Oberg F, Gunnarsson I, Berg L, Magnusson V, Brookes AJ, Tentler D, Kristjansdóttir H, Gröndal G, Bolstad AI, Svenungsson E, Lundberg I, Sturfelt G, Jönssen A, Truedsson L, Lima G, Alcocer-Varela J, Jonsson R, Gyllensten UB, Harley JB, Alarcón-Segovia D, Steinsson K, Alarcón-Riquelme ME. A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. *Nat. Genet.*, **32**, 666–669 (2002).
28) Helms C, Cao L, Krueger JG, Wijsman EM, Chamian F, Gordon D, Heffernan M, Daw JA, Robarge J, Ott J, Kwok PY, Menter A, Bowcock AM. A putative RUNX1 binding site variant between SLC9A3R1 and NAT9 is associated with susceptibility to psoriasis. *Nat. Genet.*, **35**, 349–356 (2003).
29) Tokuhiro S, Yamada R, Chang X, Suzuki A, Kochi Y, Sawada T, Suzuki M, Nagasaki M, Ohtsuki M, Ono M, Furukawa H, Nagashima M, Yoshino S, Mabuchi A, Sekine A, Saito S, Takahashi A, Tsunoda T, Nakamura Y, Yamamoto K. An intronic SNP in a RUNX1 binding site of SLC22A4, encoding an organic cation transporter, is associated with rheumatoid arthritis. *Nat. Genet.*, **35**, 341–348 (2003).
30) Foster MH. Relevance of systemic lupus erythematosus nephritis animal models to human disease. *Semin. Nephrol.*, **19**, 12–24 (1999).

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Research Article

Quantitation of Pyrrole-Imidazole Polyamide in Rat Plasma by High-Performance Liquid Chromatography Coupled with UV Detection

Tomonori Kamei,[1] Takahiko Aoyama,[1] Chihiro Tanaka,[1] Takafumi Nagashima,[1] Yukio Aoyama,[1] Hiroyuki Hayashi,[2] Hiroki Nagase,[3] Takahiro Ueno,[4] Noboru Fukuda,[4] and Yoshiaki Matsumoto[1]

[1] *Department of Clinical Pharmacokinetics, School of Pharmacy, Nihon University, 7-7-1 Narashinodai, Chiba, Funabashi City 274-8555, Japan*
[2] *Department of Clinical Pharmacotherapy, School of Pharmacy, Nihon University, 7-7-1 Narashinodai, Chiba, Funabashi City 274-8555, Japan*
[3] *Chiba Cancer Center Research Institute, 666-2, Nitonacho, Chuo-ku, Chiba, Chiba City 260-8717, Japan*
[4] *Department of Medicine, School of Medicine, Nihon University, Tokyo 173-8610, Japan*

Correspondence should be addressed to Yoshiaki Matsumoto, matsumoto.yoshiaki@nihon-u.ac.jp

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A simple and robust method using high-performance liquid chromatography with UV detection was developed and validated for the determination of six pyrrole-imidazole (PI) polyamides (HN.49, TGF-β1f, TGF-β1t, HN.50f, HN.50t, and LOX-1) in rat plasma. After the plasma proteins were precipitated with methanol containing phenacetin as an internal standard, the analytes were separated on a Luna C18 (2) (5 μm, 4.6 × 150 mm). Calibration curves were linear over the range of 0.5 to 200 μg/mL for HN.49, 0.25 to 200 μg/mL for TGF-β1f, TGF-β1t, HN.50t, and LOX-1, 1 to 200 μg/mL for HN.50f in rat plasma. The inter- and intraday precision were below 15%, and the accuracy was within 15% at the quality controls. The validated method was successfully applied to sample analysis for the pharmacokinetic study.

1. Introduction

Pyrrole(Py)-imidazole(Im) (PI) polyamides are small synthetic molecules composed of aromatic rings of *N*-methylpyrrole and *N*-methylimidazole amino acids [1]. Synthetic polyamides recognize and bind to specific nucleotide sequences in the minor groove of double-helical DNA with high affinity [2]. Various sequence-specific DNA-binding PI polyamides have been developed to regulate gene expression by targeting the promoter regions of enhancer and transcription factor-binding elements in vitro [3]. PI polyamide targeting rat transforming growth factor (TGF)-β_1 has been reported to inhibit the expressions of TGF-β_1 mRNA and protein in the renal cortex of Dahl-S rats. The targeted PI polyamide also reduced glomerulosclerosis and interstitial fibrosis without side effects. These observations indicate that PI polyamides will be effective for TGF-β_1-related diseases, including progressive renal injury [4, 5]. PI polyamides targeting human aurora kinase A (AURKA) and B (AURKB) promoters significantly inhibited the promoter activities, and mRNA and protein expression levels of AURKA and AURKB. They also demonstrated a marked antiproliferative synergy in human tumor cell lines as a result of induction of apoptosis-mediated severe catastrophe of cell-cycle progression [6]. PI polyamides specifically inhibited lectin-like oxidized low-density lipoprotein receptor-1 mRNA expression and apoptosis induced by oxidized low-density lipoprotein and angiotensin II in human umbilical vein endothelial cells [7]. PI polyamide that targets the activator protein-1-binding site of the matrix metalloproteinase (MMP)-9