体投与群では、コントロール群（PBS 投与群）と比べて変化はなかった（図 5）。

5. 経口投与用のキャリアーの創製と経口投与による評価

パン酵母である β 1,3-D-glucan の中で PEI と siRNA が複合体を形成していることが顕微鏡観察で確認できた（図 6）。また、マウス経口投与実験では、わずかながら肝臓と腎臓で蛍光強度が上昇していることが確認できた（図 7）。

D. 考察

PEI は siRNA デリバリーに有効な材料として期待が寄せられているが、毒性の問題は克服すべき課題である。我々はこの問題を PEI へプルランを導入することで解決した。プルランは急性毒性、慢性毒性など各毒性実験においてその安全性が確認され、我が国では添加物として承認されている。プルラン修飾 PEI ポリマーは PEI に比べてマウスの死亡率を顕著に軽減した。マウス実験では PEI/siRNA 複合体の C/A 比増加に伴い、マウスの死亡率の増加や肺組織での出血性赤斑点の拡大が確認できた。しかし、プルラン修飾 PEI ポリマー/siRNA 複合体によるマウスの死亡、臓器損傷を示すような結果は得られなかった。

一方、プルランを PEI へ導入することは、プルラン本来の肝臓指向能に影響を与える可能性があるため、マウスを用いた体内動態実験によりその影響を調べた。プルラン修飾 PEI ポリマー/siRNA 複合体を尾静脈によりマウスに投与したところ、肝臓で集積することを確認した。この結果から、プルランを PEI へ導入することは、肝臓指向能に影響を与えないことが明らかとなった。この結果から、開発したプルラン修飾 PEI ポリマーは肝臓選択的送達に有効な材料として期待される。本研究で、プルランの PEI への導入により、組織に対する毒性の軽減と同時に、肝臓への選択的送達に成功したことは画期的なものと言える。

さらに、ApoB siRNA は体内で非常に不安定であり、分解されやすいことが体内動態実験により確認した。この問題は、ポリマーと複合体を形成することで解決した。プルラン修飾 PEI ポリマー/siRNA 複合体は siRNA の血中安定性を著しく向上し、肝臓ターゲティングおよびイメージングを実現させた。

異なる分子量（4,900 と 107,000）のプルランを修飾したポリマーの評価では、低分子量のプルラン修飾 PEI ポリマー/siRNA 複合体（PEI-pullulan(MW, 5,900)/siRNA）よりも、高分子量のプルラン修飾 PEI ポリマー/siRNA 複合体（PEI-pullulan(MW, 107,000)/siRNA）のほうがより優れた血中安定性と肝臓集積を示した。これは、siRNA と複合体を形成する際、高分子量のプルランのほうが低分子量のものよりも、より広い範囲の親水性層を作り、血中タンパク質や分解酵素と siRNA との結合を阻害することで、siRNA を安定的に送達することができたと考えられる。

また、高コレステロールモデルマウスに 3 日間連続投与を行った治療実験でも、高分子量のプルラン修飾 PEI ポリマー/siRNA 複合体（PEI-pullulan(MW, 107,000)/siRNA）の投与群で、TC、LDL、および VLDL 値の著しい低下と肝臓 ApoB mRNA 発現の抑制も確認できた。特に、治療に使用された

siRNA の量（25 μg）は、以前報告された論文(100 μg)よりも低い濃度でありながら、コレステロール値の有意な低下と肝臓 ApoB mRNA 発現量の抑制効果を示した。

これらの結果は、低分子量よりも高分子量プルラン修飾 PEI ポリマー/siRNA 複合体が、高い siRNA の血中安定性、肝臓集積能、肝細胞への送達能を有していることを示唆する。また、治療用遺伝子を肝臓に選択的に送達することで、副作用のリスクを軽減することもできると考えられる。

さらに、siRNA の経口投与による肝臓へのデリバリーを実現するためにパン酵母であるβ1,3-D-glucan を経口投与用キャリアーとして使用した。β1,3-D-glucan の中で PEI と siRNA が複合体を形成していることを顕微鏡観察で確認できた。また、マウスへの経口投与 6 時間後、肝臓と腎臓での蛍光強度が上昇していることが確認できた。これは、複合体が封入されたβ1,3-D-glucan が、腸膜に存在する M 細胞を経由して血中に運ばれたことを示唆する。また、siRNA 単独投与群に比べ、1,3-D-glucan は胃腸内環境でより安定的に siRNA を送達することができた。これらの結果から、1,3-D-glucan をキャリアーとして使用することで、経口投与による siRNA の肝臓デリバリーは実現可能であることが示唆される。

E. 結論

肝臓指向能を有するプルランを導入することで肝臓選択的送達システムを構築した。プルラン修飾 PEI ポリマーは、肝臓選択的送達の実現だけではなく、毒性軽減や ApoB siRNA の血中安定性の向上にも効果的であった。高コレステロールモデルマウスを用いた投与実験からは、血中コレステロール値の有意な低下と肝臓での ApoB mRNA 発現量の抑制が確認できた。

また、β1,3-D-glucan を経口投与用キャリアーとして使用し、肝臓への siRNA デリバリーにも成功した。今後、複合体の最適化、より安定性の向上のためのコーティング剤などの検討などにより、ApoB siRNA の経口投与による治療は十分可能であると考えられる。

F. 健康危険情報

本研究では現在のところ健康に危険を及ぼす可能性はない。

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厚生労働科学研究費補助金（創薬基盤推進研究事業）
分担研究報告

創薬ターゲットとしての mPGES-1

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研究要旨　マクロファージに発現している誘導型プロスタグランジン E 合成酵素 microsomal-prostaglandin E synthase-1（mPGES-1）が、粥状硬化巣の炎症を抑制して粥腫を安定化させる新たな創薬ターゲット分子となりうるか否かを検討した。ヒト骨髄性白血病細胞株 THP-1 をリポ多糖（LPS）で刺激すると、mPGE-1 の発現が誘導されるが、細胞性粘菌由来分化誘導因子 differentiation-inducing factor（DIF）は、この mPGES-1 の誘導を強力に抑制することを見出した。臨床応用も視野に入れ、そのメカニズムの検討を現在行っている。

A．研究目的

血管壁の炎症は、粥状硬化巣の病変形成において、中心的な役割を果たすと考えられている。なかでも、マクロファージの活性化は粥腫を不安定化し、粥腫の破裂や血栓の形成を誘導することにより、急性冠症候群の発症に寄与すると考えられている。

プロスタグランジン E_2（PGE_2）は炎症反応の重要なメディエーターであり、マクロファージは炎症性反応の一つとして PGE_2 を産生する。PGE_2 の産生酵素としては、microsomal prostaglandin E synthase（mPGES-1、mPGES-2）と cytosolic prostaglandin E synthase（cPGES）の 3 種類が知られている。このうち mPGES-1 は炎症性刺激によりその発現が上昇し、炎症の進展に関与することが示唆されており、創薬のターゲットになる可能性がある。

そこで我々は、炎症性刺激に対するマクロファージの反応過程における mPGES 発現と PGE_2 産生への関与について、ヒト骨髄性白血病細胞株 U937 および THP-1 を用いて検討した。さらに、細胞性粘菌由来分化誘導因子 differentiation-inducing factor（DIF）の新規抗炎症薬としての可能性について検討した。また、恒常的に mPGES-1 を発現している 2 種類のヒト由来がん細胞 HCT-116（大腸がん）および HeLa（子宮頚がん）を用いて mPGES-1 の発現制御に及ぼす DIF の影響についての検討も併せて行った。

B．研究方法

1．細胞培養

THP-1 の培養には 10%ウシ胎仔血清を加えた RPMI1640 培地を用いた。また、phorbol 12-myristate 13-acetate（PMA、100nM）にて分化誘導した後、リポ多糖（LPS）および DIF-1 を用いて刺激した。ヒト由来大腸がん HCT-116 細胞および HeLa ヒト子宮頚がん由来 Hela 細胞の培養には 10%ウシ胎仔血清を加えた DMEM 培地を用いた。

2．siRNA 導入

THP-1 に mPGES 特異的 siRNA（Invitrogen）を RNAiMAX（Invitrogen）を用いて導入した。24 時間培養したのちに、PMA による分化誘導を行った。

3．ウェスタンブロット

刺激した細胞を回収し、SDS-PAGE にてタンパク質を分離した。タンパク質を転写したメンブレンを一次抗体（COX-2、mPGES、GAPDH）と反応させ、抗体と結合したタンパク質を検出した。

4．PGE_2 濃度測定

培養上清中に含まれる PGE_2 の濃度は、Prostaglandin E_2 Expression EIA Kit

(Cayman Chemical)にて測定した。

5．ヒト mPGES-1 プロモーター活性測定

24 穴培養用プレートに 1×10^5 個のがん由 HCT-116 細胞または HeLa 細胞を撒き、24 時間後、ヒト mPGES-1 プロモーター（-35/-1068 bp）組み込んだホタルルシフェラーゼレポーターベクター（pGL3-Basic）と導入効率の適正化のためのウミシイタケルシフェラーゼレポーターベクター(pRL-SV40)を細胞に導入した。24 時間後、DIF-1（10 または 30 μ M）で 6、12、及び 24 時間処理した。処理後の細胞におけるルシフェラーゼ活性を Promega 社のキット Dual Luciferase Assay System を用いて測定した。

6．リアルタイム PCR 解析

刺激した細胞を回収し、Torizol 試薬（GIBCO）を用いてトータル RNA を抽出した。抽出した RNA は High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) を用いて cDNA とし、リアルタイム PCR 装置 ABI-7500 を用いて TaqMan Gene Expression Assay Kit (Applied Biosystems) にて解析を行った。プライマーは GAPDH、mPGES-1 それぞれ Hs99999905_m1、Hs01115610_m1（Applied Biosystems）を使用した。

C．研究結果

1． mPGES に対する siRNA の効果

THP-1 に mPGES-1 に対する siRNA を導入した後、PMA で分化誘導を行い、LPS で刺激した。図 1 に示すように、用いた 2 種類の siRNA（#1、#2）は両方とも、LPS による mPGES－1 の発言誘導を抑制した。

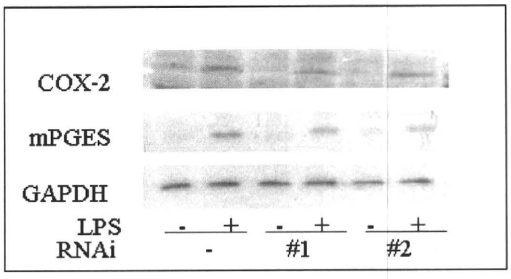

図 1　mPGES-1 発現に対する siRNA の効果

また、培養上清に含まれる PGE_2 の濃度を測定したところ、LPS により明らかな PGE_2 の産生上昇が認められたが、siRNA を導入した細胞では、PGE_2 の産生が有意に抑えられた（図 2）。

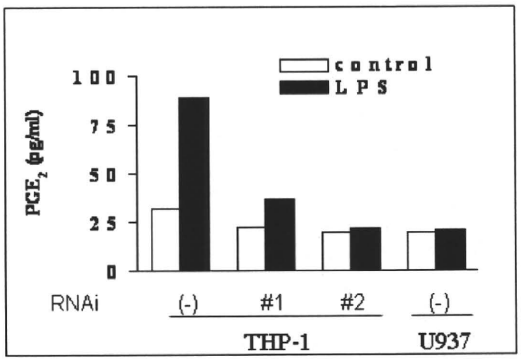

図 2　PGE_2 産生に対する siRNA の効果

2. DIF ファミリーによる mPGES-1 のタンパク質発現抑制

PMA を 24 時間作用させて THP-1 細胞に分化を誘導し、DIF-1（30 μ M）にて 3 時間刺激した後、LPS（1 または 10 μ g/ml）で 24 時間刺激した。回収したサンプルの COX-2 および mPGES-1 のタンパク質発現について検討したところ、DIF-1 での前処置によりこれらの酵素の発現が抑制されていた（図 3A）。

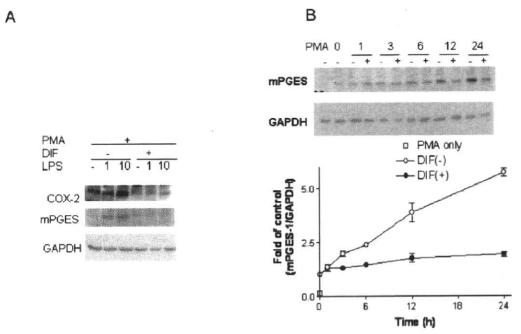

図 3 DIF の炎症関連酵素に及ぼす影響

さらに、DIF-1 による mPGES-1 発現抑制の時間依存性の検討を行った。PMA で分化誘導を行った THP-1 細胞を LPS（10 μ g/ml）で 24 時間刺激した後 DIF-1（30

μM）を添加し 1、3、6、12、24 時間後にサンプルを回収した。図 3B に示すように、LPS で 24 時間刺激することにより mPGES-1 の発現は明らかに上昇し、時間経過とともにさらに発現が上昇していく。一方 LPS で 24 時間刺激後に DIF-1 を添加した群では、時間経過にしたがって若干の発現上昇が認められるものの、コントロール群と比較して明らかな発現抑制効果が認められた。

次に DIF-1 の濃度を変化させて検討を行ったところ、図 4A に示すような濃度依存性が認められた。また、図 4B は DIF-1 のアナログの 1 つである DIF-3 の効果について検討したものであるが、DIF-3 にも DIF-1 と同様に mPGES-1 発現抑制作用が認められた。この結果から、mPGES-1 の発現抑制は DIF ファミリーに共通した作用である可能性が示唆された。

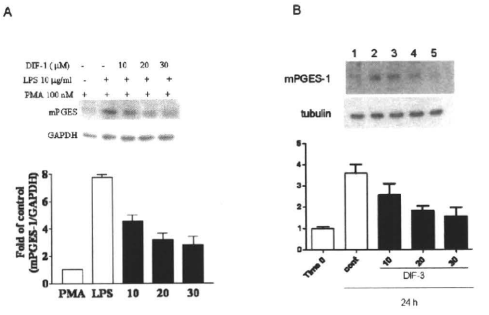

図 4 DIF ファミリーによる mPGES-1 のタンパク質発現抑制

3. DIF-1 による mPGES-1 mRNA 発現抑制

PMA で分化誘導を行った THP-1 細胞を LPS（10 μg/ml）で 24 時間刺激した後 DIF-1（30 μM）を添加し 24 時間後にサンプルを回収し総 RNA を抽出した。これを用いてリアルタイム PCR 法にて mRNA の発現量の変化を検討した。図 5 に示すように DIF-1 の添加により明らかな mPGES-1 の mRNA の発現量の低下が認められた。この結果から、DIF-1 が mRNA の発現量を低下させることにより、mPGES-1 のタンパク質発現量を減少させていることが示唆された。

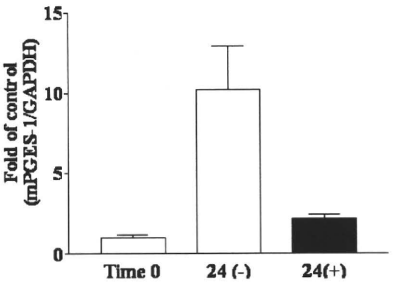

図 5 mPGES-1 の mRNA 発現に及ぼす DIF-1 の効果

4. DIF-1 の他 PGES への影響

PGES には、mPGES-1 の他にも数種類存在するので、他の PGES に及ぼす DIF-1 の効果を検討したが、特に変化はみられなかった（図 6）。

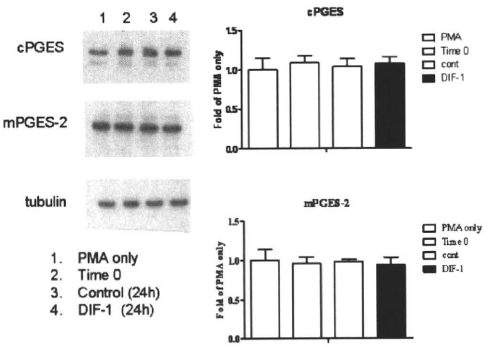

図 6　DIF-1 による他の PGES への影響

5.　がん細胞における非誘導性 mPGES-1 の DIF-1 によるタンパク質発現抑制

子宮けいがん細胞および大腸がん細胞では、炎症性の刺激が存在しなくても mPGES-1 が発現していることが知られている。そこで。それらの細胞 mPGES-1 の発現の及ぼす DIF の影響について検討した。図 7 に示したように、両細胞において時間依存性に DIF により mPGES-1 の発現が抑制されたが、その効果は刺激後 6 時間以降で明らかに認められた。

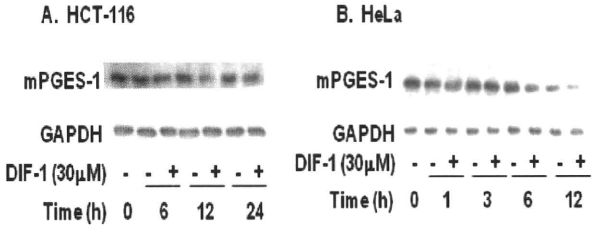

図 7 DIF の mPGES-1 の発現に及ぼす影響

6. DIF-1 の mPGES-1 プロモーター活性への影響

次に DIF-1 の mPGES-1 プロモーター活性に及ぼす影響について検討を行った。図 8 に示すように両がん細胞において、DIF-1 は mPGES-1 のプロモーター活性を抑制し、その効果はタンパク質の発現抑制よりも早い時間経過を示した。特に HeLa 細胞では刺激後 1 時間から、明らかな活性の低下が認められた。

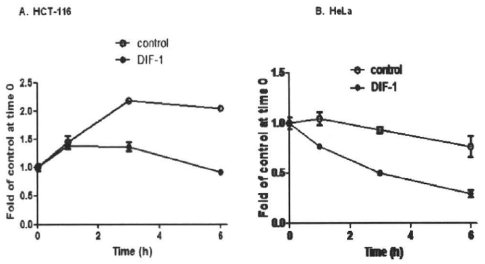

図 8 mPGES-1 プロモーター活性に及ぼす DIF-1 の効果

7. DIF-1 による mPGES-1 mRNA 発現抑制

HeLa 細胞に DIF-1 (30 μ M) を添加し 1、3、6 時間後にサンプルを回収し総 RNA を抽出した。これを用いてリアルタイム PCR 法にて mRNA の発現量の変化を検討した。図 9 に示すように DIF-1 の添加により明らかな mPGES-1 の mRNA の発現量の低下が認められた。この結果から、DIF-1 が mRNA の発現量を低下させることにより、mPGES-1 のタンパク質発現量を減少させていることが示唆された。

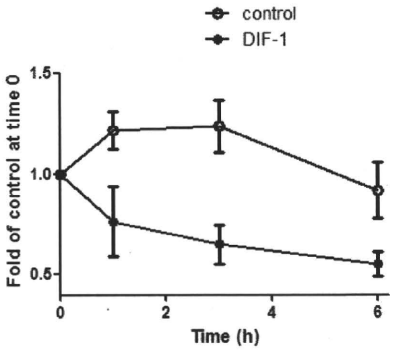

図 9 mPGES-1 の mRA 発現に及ぼす DIF-1 の効果

8. DIF-1 の炎症関連転写因子 Egr-1 への影響

mPGES-1 のプロモーター活性を調節する転写因子は Egr-1、NFkB、AP-1 などが知られているが、その中でも特に Egr-1 の発現上昇により mPGES-1 の発現が強力に誘導されることが知られている。そこで、Egr-1 の発現に及ぼす DIF-1 の影響について検討した。図 4 に示すように、予想に反して、DIF-1 により一過性に Egr-1 の発現が上昇することが示された。他にも数種類存在するので、他の PGES に及ぼす DIF-1 の効果を検討したが、特に変化はみられなかった（図 10）。

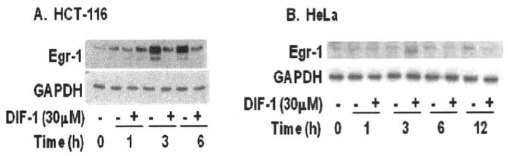

図 10　DIF-1 による Egr-1 の影響

D. 考察

粥腫の炎症を抑制する治療標的分子を

探索する目的で、マクロファージの活性化と炎症反応のメディエーターであるPGE_2の産生系に着目して検討を行った。

既存の抗炎症薬である非ステロイド性抗炎症薬（NSAIDs）は、シクロオキシゲナーゼ-2（COX-2）を抑制することにより下流のPGE_2の産生を抑制するが、NSAIDsはCOX-1をも抑制するため、生理機能の維持に必要なエイコサノイドの産生も低下させてしまう。この問題を解決するため、炎症時に誘導されるCOX-2に特異的な阻害薬（セレコキシブなど）が開発され、すでに臨床使用されているが、これらの薬剤は、プロスタサイクリンの産生減少によると思われる心血管イベントの増加を引き起こすことが報告され、その使用が制限されている。

そこで、炎症を増悪させるPGE_2の産生を特異的に抑制する方法を開発するため、炎症性刺激下における直接的なPGE_2産生酵素であるmPGES-1に着目した。

ヒト骨髄性白血病細胞株THP-1を、PMAで処置することによりマクロファージ様細胞に分化させ、これに炎症性刺激を加えると、mPGES-1タンパク質の誘導が認められるが、細胞性粘菌が産生する分化誘導因DIF-1が、炎症性刺激により誘導されるmPGES-1の発現を抑制した。さらに、DIFはmPGES-1のプロモーター活性を抑制することにより、mPGES-1の発現を抑制していることが示唆された。

さらに、炎症刺激を加えなくてもmPGES-1を発現しており、それにより産生されるpGE2ががんの進展に影響しているとされる２種類のヒト由来がん細胞HCT-116細胞とHeLa細胞を用いて検討を行った。両細胞においてタンパク質の減少に先行してプロモーター活性の低下並びにmRNA発現低下が認められたことから、DIF-1がmPGES-1の転写活性を阻害することにより発現を低下させていることが明らかとなった。

そこで、mPGES-1の代表的な転写因子であるEgr-1の発現におよぼすDIF-1の効果について検討したところ、予想に反してDIF-1によりこの転写因子の発現が上昇することが示された。この結果は、DIFが新しい機序によってmPGES-1の転写活性を阻害している可能性を示すものである。

E．結論

mPGES-1が有望な抗炎症薬の創薬ターゲットであり、DIFが抗炎症薬として機能する可能性が示唆された。

F．健康危険情報

本研究では現在のところ健康に危険を及ぼす可能性はない。

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出願中特許

・出願番号 特願 2009-238935、出願日 2009-10-16

・発明の名称 分化誘導因子又はその類縁体を含有する抗炎症剤

Bioorganic & Medicinal Chemistry 18 (2010) 3946–3950

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Liver-targeted siRNA delivery by polyethylenimine (PEI)-pullulan carrier

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ARTICLE INFO

Article history:
Received 5 March 2010
Revised 12 April 2010
Accepted 13 April 2010
Available online 18 April 2010

Keywords:
Polyethylenimine
Gene delivery
Cationic polymer
Cytotoxicity

ABSTRACT

Recently, small interfering RNA (siRNA)-based therapeutics have been used to treat diseases. Efficient and stable siRNA delivery into disease cells is important in the use of this agent for treatment. In the present study, pullulan was introduced into polyethylenimine (PEI) for liver targeting. PEI/siRNA or pullulan-containing PEI/siRNA complexes were delivered into mice through the tail vein either by a hydrodynamics- or non-hydrodynamics-based injection. The incidence of mortality was found to increase with an increase in the nitrogen/phosphorus (N/P) ratio of PEI/siRNA complexes. Moreover, the hydrodynamics-based injection increased mice mortality. Introduction of pullulan into PEI dramatically reduced mouse death after systemic injection. After systemic injection, the PEI/fluorescein-labeled siRNA complex increased the level of fluorescence in the lung and the PEI-pullulan/siRNA complex led to an increased fluorescence level in the liver. These results suggest that the PEI-pullulan polymer may be a useful, low toxic means for efficient delivery of siRNA into the liver.

1. Introduction

Small interfering RNA (siRNA)-based therapeutics, which are now recognized as a medical approach for the treatment of difficult-to-cure diseases such as viral infections and tumors, are attracting considerable attention in recent times.[1,2] However, naked siRNA is unstable in the bloodstream and is rapidly eliminated through the urinary system. Moreover, its negative charge inhibits efficient cellular uptake due to the negative charge of the cell surface. Thus, efficient and stable siRNA delivery into diseased cells is critical in this treatment modality. Many researchers have attempted to induce various chemical modifications into siRNA or to form complexes with several cationic carriers such as cationic polymers, liposomes, peptides, or proteins.[3–5]

Among cationic polymers, polyethylenimine (PEI) is the most popular synthetic polymer and has a high cationic charge density. It has been widely used to deliver siRNAs into cell lines or tissues. Naked siRNAs are unstable and are rapidly degraded, but PEI is able to form stable complexes with siRNAs, leading to the protection of genes from enzymatic degradation. Moreover, PEI shows a strong buffer capacity over a wide range of pH values; this plays an important role in the escape of genes from the endosome after endocytosis. On the other hand, the high cationic density of PEI allows for the formation of highly condensed complex with siRNAs, but complex formation with PEI can lead to cytotoxicity.[6–10] Information on the safety and biodistribution of PEI or PEI/siRNA complexes both in vitro and in vivo would contribute to improving the safety and efficiency of siRNA delivery using PEI.

In the present study, we introduced pullulan into PEI. Pullulan is a water-soluble polysaccharide consisting of three α-1,4-linked glucose polymers with different α-1,6-glucosidic linkages. It is used for liver targeting because of its high affinity for the asialoglycoprotein receptor in the liver.[11–13] We delivered PEI/siRNA or pullulan-containing PEI/siRNA complexes into mice through the tail vein by a hydrodynamics- or non-hydrodynamics-based injection. The incidence of mortality was found to increase with increasing the nitrogen/phosphorus (N/P) ratio of PEI/siRNA complexes. On the other hand, the introduction of pullulan into PEI reduced mouse mortality and increased liver-targeting efficiency.

2. Results and discussion

2.1. Polymers

A linear 22-kDa PEI was used for the synthesis of the siRNA and PEI-pullulan polymer complex (Fig. 1). The amount of pullulan in

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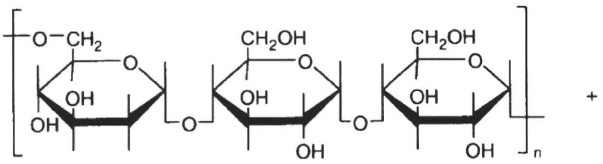
doi:10.1016/j.bmc.2010.04.031



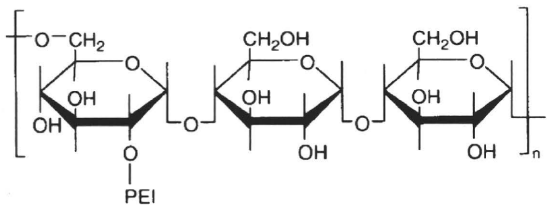


Figure 1. Chemical structure of pullulan and PEI-pullulan. To synthesize the PEI-pullulan polymer, 48.6 mg of pullulan (M_w, 107,000; 0.3 unit mmol) and 24.3 mg of carbonyldiimidazole (CDI; 0.15 mmol) were stirred in 30 mL of anhydrous dimethylsulfoxide (DMSO) at room temperature and then 13.2 mg of linear PEI (M_w, 22 kDa; 0.3 mmol) was added to the mixture.

the polymer was estimated to be 39 mol % and molecular weight of polymer was 2.6×10^5 (see Supplementary data). The zeta potentials of polymer/siRNA complex increased with increasing N/P ratio and showed nearly neutral at N/P ratios of 48 and 96 (see Supplementary data).

2.2. Measurements of complex diameters

The complexes of polymer and siRNA were prepared at several N/P ratios (1.5, 3, 6, 12, 24, and 48) and were determined using a Zetasizer. The particle size decreased with increasing N/P ratio. PEI/siRNA complexes showed <200 nm for all N/P ratios, whereas PEI-pullulan/siRNA complexes with ratios of 12 to 48 were <200 nm (Fig. 2).

2.3. Electrophoresis of the polymer/siRNA complex

Polymers were mixed with siRNA at several N/P ratios. The complexes were analyzed by electrophoresis. Bands corresponding

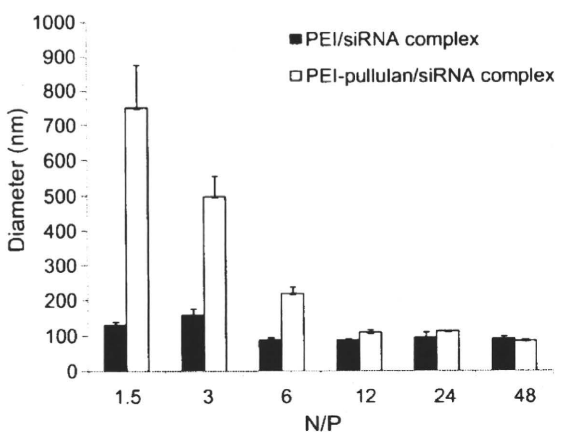


Figure 2. Diameter of the PEI/siRNA or PEI-pullulan/siRNA complexes. Polymer and siRNA complexes were simply prepared by incubating siRNA and polymer in water. The diameters of the complexes were determined using a Zetasizer.

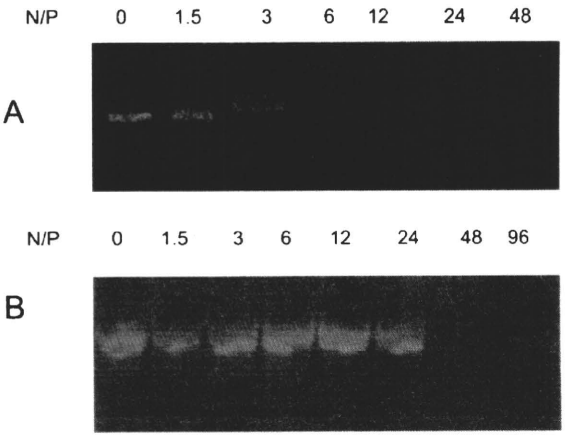


Figure 3. Electrophoresis of (A) PEI/siRNA and (B) PEI-pullulan/siRNA complexes. Various concentrations of the polymer were mixed with the siRNA and analyzed by 19% polyacrylamide gel electrophoresis. A N/P ratio of 0 implies siRNA alone.

to free siRNA in the PEI/siRNA complex were not observed when the polymer was present at N/P ratios of above 3, whereas when the N/P ratios were 1.5 and 3, bands corresponding to free siRNA were observed. In the case of the PEI-pullulan/siRNA complex, no suppression of siRNA was indentified in those complexes with N/P ratios of 1.5 to 24, while siRNA migration in complexes with N/P ratios of $\geqslant 48$ was suppressed (Fig. 3). These results show that introduction of pullulan into PEI weakens the polymer and siRNA complex.

2.4. Safety of polymer/siRNA complexes in vivo

PEI alone, the PEI/siRNA complex, and the PEI-pullulan/siRNA complex were injected into mice using a hydrodynamics-based or a non-hydrodynamics-based procedure. PEI alone or the PEI/siRNA complex with high N/P ratios ($\geqslant 6.0$) increased mice mortality after systemic injection using the non-hydrodynamics-based procedure (Fig. 4); note that all mice died when complexes with N/P ratios of $\geqslant 12$ were injected (data not shown). Similarly, previous studies reported that the PEI/DNA complex with a N/P ratio of 6 resulted in the death of 50% of the injected mice.[14,15] However, all mice died when PEI alone or the PEI/siRNA complex with a N/P ratio of 3 was injected using the hydrodynamics-based procedure. Hydrodynamics-based transfection was developed to deliver naked DNA or RNA into the liver by intravenous injection of a large volume of DNA or RNA solution at high velocity. This is an efficient method for liver-specific in vivo gene delivery.[16,17] However, in our study, high mouse mortality was observed when the hydrodynamics-based procedure was used for the in vivo delivery of PEI/siRNA complexes.

All dead mice lapsed into dyspnea less than 30 min after injection and showed hemorrhage-like dark red regions in the lung. There was no difference in mortality between mice injected with PEI alone and those injected with the PEI/siRNA complex, but more severe hemorrhage-like dark red regions were observed in the former (Fig. 4A and B).

Concerning the death of mice after systemic injection, Fahrmeir's group suggested that free PEIs after complex formation with DNA correlate with mouse mortality.[18] Several studies showed that increased gene expression in the lung is associated with lung damage and mouse mortality after intravenous injection of PEI/DNA or modified PEI/DNA.[15,19,20] In the present study, PEI/siRNA showed a similar in vivo toxicity to PEI/DNA.

On the other hand, no mortality was observed in mice injected with PEI-pullulan/siRNA complexes with N/P ratios of 6 to 48 by the hydrodynamics-based procedure mice (Fig. 4B) and the non-hydrodynamics-based procedure (data not shown). These

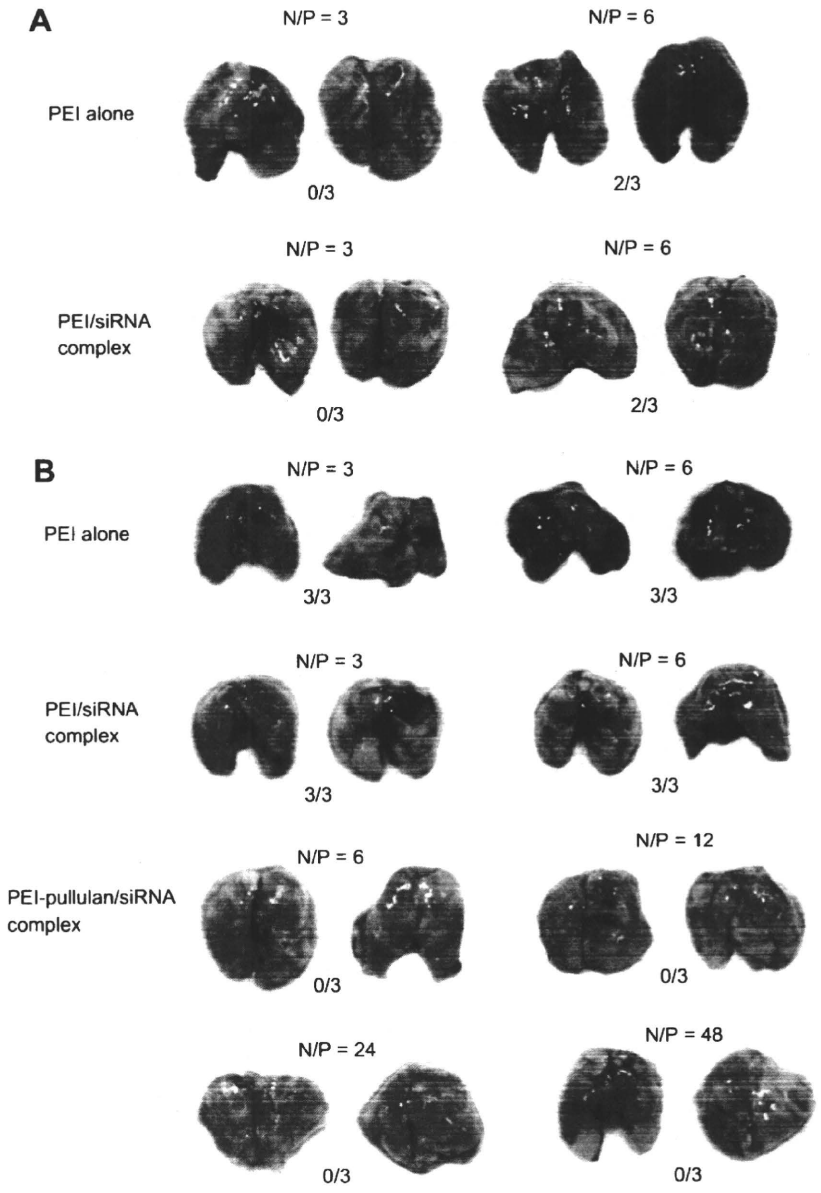


Figure 4. Delivery of PEI alone or polymer/siRNA complexes into mice by using the (A) non-hydrodynamics- or (B) hydrodynamics-based procedure. Numbers of dead mice per total mice are described below.

results suggest that intravenous injection with PEI alone or the PEI/siRNA complex at high N/P ratios can increase mortality, but introduction of pullulan into PEI results in low mortality. Moreover, hydrodynamics-based injection can increase the mouse mortality rate, compared to non-hydrodynamics-based injection. High in vivo toxicity or mortality caused by systemic injection of the PEI-based complex is an obstacle to be overcome. Many research efforts such as the introduction of poly(ethylene glycol) (PEG)[15] and removal of free PEIs after complex formation[18] were reported to efficiently reduce in vivo toxicity or mortality. In the present study, introduction of pullulan to PEI dramatically reduced in vivo toxicity and mortality.

2.5. Biodistribution after injection of the polymer/siRNA complex into mice

siRNA formed a complex with PEI at a N/P ratio of 3 and with PEI-pullulan at a N/P ratio of 48. Complexes were injected into the mice via the tail vein using the non-hydrodynamics-based procedure. The fluorescence in each tissue (heart, lung, liver, spleen, and kidney) was detected at 1 or 3 h after the injection. At 1 h after the injection of the PEI/siRNA or PEI-pullulan/siRNA complex, fluorescence was identified mainly in the lung and kidney. At 3 h, fluorescence increased in the livers of the PEI-pullulan/siRNA complex-injected mice, but was barely found in the livers of the PEI/siRNA-injected mice (Fig. 5).

Several studies have reported that linear and branched PEI/gene complexes show different biodistribution and transfection efficiency.[6–9] The linear PEI/gene complex exhibits more efficient transgene expression in the lung when injected intravenously, as compared to the branched PEI/gene complex;[6,7,9,14,21] however the transgene expression of the branched PEI/gene complex may be more efficient in other tissues (e.g., kidney).[9,22] Further, although PEI cytotoxicity depends on molecular weight and N/P ratios, the branched PEI/gene complex is found to have higher toxicity or cause more tissue damage as compared to the linear PEI/gene complex.[8,9,23]

In the present study, we used a linear 22-kDa PEI for complex formation with siRNA and for synthesizing the PEI-pullulan

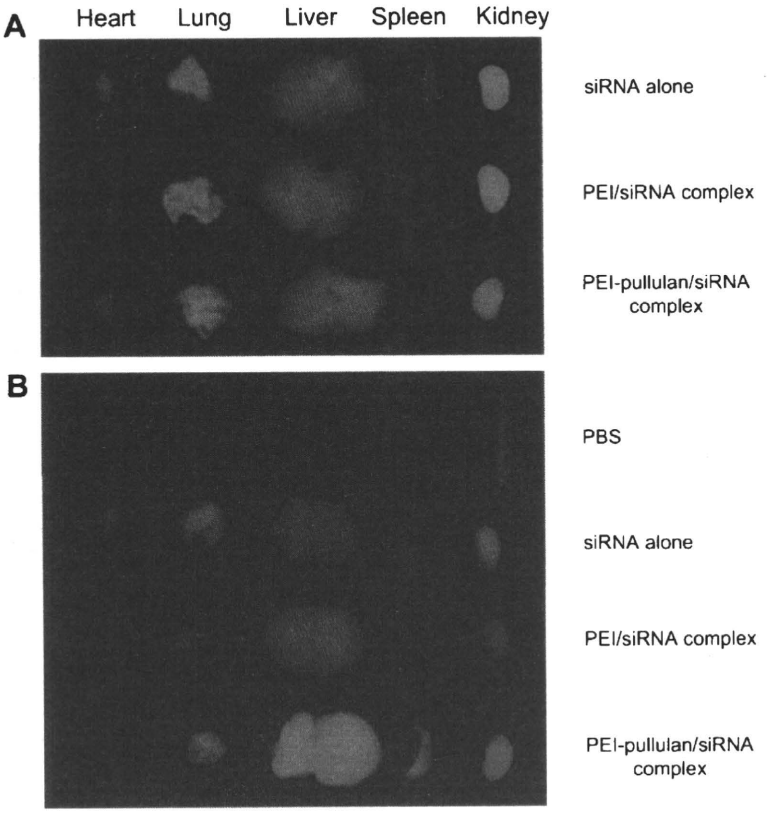


Figure 5. Biodistribution after injection of PBS, siRNA alone, or polymer/fluorescein-labeled siRNA complexes. The siRNA was bound with PEI at a N/P ratio of 3 and with PEI-pullulan at a N/P ratio of 48. The fluorescence in each tissue (heart, lung, liver, spleen, and kidney) was detected (A) 1 or (B) 3 h after the injection.

polymer. When the 22-kDa linear PEI/gene complexes were transfected via systemic administration, the main target was the lung and lower levels of transfection were found in the brain, heart, liver, spleen, and kidney.[14] High transgene expression in the lungs may relate to rapid crossing of the pulmonary endothelial barrier by the PEI/gene complexes.[21] Similarly, we found the highest level of fluorescence in the lung compared to other tissues (heart, liver, and spleen) at 1 h after intravenous injection of the PEI/siRNA complex at a N/P ratio of 3 (Fig. 5). Fluorescence in the kidney may be caused by elimination of biodegraded free fluorescein from the system.

siRNA-based therapeutics are recognized as a useful approach for liver (hepatic) diseases such as hepatitis B and C, but development of liver-targeted siRNA delivery system is an important problem to solve.[1] In the present study, pullulan, a water-soluble polysaccharide, was introduced into PEI to increase liver-targeting efficiency. At 3 h after the injection, we found highest level of fluorescence in the livers of the PEI-pullulan/siRNA complex-injected mice (Fig. 5). Thus, our system may be a useful means for efficient delivery of siRNA into the liver.

3. Conclusions

We found that introduction of pullulan to PEI increased the level of fluorescence in the liver. This finding may be explained by the fact that pullulan has a high affinity for asialoglycoprotein receptors in the liver.[11–13] Moreover, systemic delivery of PEI-pullulan polymer dramatically reduced mouse death. These results suggest that the PEI-pullulan polymer may be an efficient and low toxic means for siRNA delivery into the liver.

4. Materials and methods

4.1. Fluorescein-labeled siRNA

The gene (*apoB* siRNA) used in this study was amidated and its sequence was as follows: 5′-GUCAUCACACUGAAUACCAAUdTdT-3′ (sense) and 5′-dTdTCACAGUAGUGUGACUUAUGGUUA-3′ (antisense). Alexa Fluor 750 (Invitrogen, Tokyo, Japan) was used as an amine-reactive dye. The fluorescein-labeled siRNA was dialyzed against water containing 0.1% diethylpyrocarbonate (DEPC) for 2 days in a dialysis membrane bag with a molecular weight (MW) cut-off of 3500, followed by lyophilization.

4.2. Synthesis of PEI-pullulan polymer

A mixture of 48.6 mg of pullulan (M_w, 107,000; 0.3 unit mmol) and 24.3 mg of carbonyldiimidazole (CDI; 0.15 mmol) was stirred in 30 mL of anhydrous dimethylsulfoxide (DMSO) at room temperature. After 4 h, 13.2 mg of linear polyethyleneimine (PEI; M_w, 22 kDa; 0.3 mmol) was added to the mixture and further stirred at room temperature under a nitrogen-rich atmosphere for 1 day. The mixture was dialyzed against water for 3 days in a dialysis Spectra Pore membrane bag with a molecular weight cut-off of 10,000 (Spectrum Laboratories, Inc., Rancho Dominguez, CA), followed by lyophilization to obtain a PEI-pullulan polymer powder.

The buffering capacity of the PEI-pullulan polymer from pH 12 to 3 was determined by acid-base titration. Briefly, the polymer (4.8 mg) was dissolved in 8 mL of 150 mM NaCl to a final concentration of 0.6 mg/mL and the pH of the polymer solution was set to 12 with NaOH. The solution was subsequently titrated with 0.1 M HCl.

4.3. Measurements of the diameter of complexes

Polymer and siRNA complexes were prepared by incubating both the siRNA and the polymer in water for 30 min. The final concentration of the siRNA was adjusted to 1 μg/mL using water (pH 7.3). The diameters of the complexes were determined using a Zetasizer (Malvern Instruments, Malvern, UK) with the He/Ne laser at a detection angle of 173° and a temperature of 25 °C.

4.4. Electrophoresis of the polymer/siRNA complex

For the electrophoresis experiment, various concentrations of the polymer were mixed with the siRNA in ultrapure distilled water (Invitrogen) at room temperature for 30 min, and then analyzed by 19% polyacrylamide gel electrophoresis.

4.5. Delivery of polymer/siRNA complexes into mice by direct injection

All animal studies were performed in accordance with the Guidelines for Animal Experiments, established by the Ministry of Health, Labour and Welfare of Japan, and by the National Cardiovascular Center Research Institute. Male 6-week-old BALB/c mice (CLEA Japan Inc., Osaka, Japan) weighing approximately 22 g were used in this study. The mice were maintained in a temperature-controlled room (22 °C) with a 12-h light-dark cycle and were provided with a standard pellet diet (CE-2; CLEA Japan) and water ad libitum. One week after arrival, mice were divided into two groups, the hydrodynamics injection group and the non-hydrodynamics injection group. In the hydrodynamics injection group, 2 mL of 5% glucose solution containing each polymer/siRNA complex was injected, whereas in the non-hydrodynamics injection group, 0.2 mL was injected. For the hydrodynamics-based procedure, solutions were injected over 6–8 s into the tail vein using a 27-gauge needle. The mice were sacrificed 1 or 3 h after the injections, and thereafter each tissue type (lung, heart, liver, spleen, and kidney) was excised. Images were obtained with the Maestro In Vivo Imaging System (Cambridge Research & Instrumentation, Woburn, MA, USA).

Acknowledgment

This work was supported by a research grant from the Ministry of Health, Labour and Welfare (MHLW) and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), Japan.

Supplementary data

Supplementary data (Tables S1 and S2 describing molecular parameters of polymer and zeta potential of PEI-pullulan/siRNA complex, respectively) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.031.

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Clinical Chemistry 56:3
000–000 (2010)

Brief Communications

C-Reactive Protein Uptake by Macrophage Cell Line via Class-A Scavenger Receptor

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BACKGROUND: C-reactive protein (CRP) increases in response to inflammation and is purported to be a risk factor for atherogenesis. We recently demonstrated that a scavenger receptor, lectin-like oxidized LDL receptor (LOX-1), is a receptor for CRP. In light of the overlapping ligand spectrum of scavenger receptors such as modified LDL, bacteria, and advanced glycation end products, we examined whether other scavenger receptors recognize CRP.

METHODS: We analyzed the uptake of fluorescently labeled CRP in COS-7 cells expressing a series of scavenger receptors and in a monocytic cell line, THP-1, differentiated into macrophage with phorbol 12-myristate 13-acetate (PMA). We applied small interfering RNA (siRNA) against class-A scavenger receptor (SR-A) to THP-1 cells to suppress the expression of SR-A. We also analyzed the binding of nonlabeled CRP to immobilized recombinant LOX-1 and SR-A in vitro using anti-CRP antibody.

RESULTS: COS-7 cells expressing LOX-1 and SR-A internalized fluorescently labeled CRP in a dose-dependent manner, but cells expressing CD36, SR-BI, or CD68 did not. The recombinant LOX-1 and SR-A proteins recognized nonlabeled purified CRP and native CRP in serum in vitro. THP-1 cells differentiated into macrophage-like cells by treatment with PMA-internalized fluorescently labeled CRP. siRNA against SR-A significantly and concomitantly inhibited the expression of SR-A ($P < 0.01$) and CRP uptake ($P < 0.01$), whereas control siRNA did not.

CONCLUSIONS: CRP is recognized by SR-A as well as LOX-1 and taken up via SR-A in a macrophage-like cell line. This process might be of significance in the pathogenesis of atherosclerotic disease.

C-reactive protein (CRP),[1] which is synthesized by hepatocytes in response to inflammation and tissue damage *(1)*, binds to various ligands exposed on damaged tissues or bacteria promoting phagocytosis and complement activation with C1q *(1, 2)*. Plasma CRP concentrations may rise as much as 1000-fold during infection or inflammation *(3)*. In addition, CRP concentrations, within the reference range, can predict cardiovascular diseases *(4, 5)*, and there is a good correlation between plasma CRP concentrations and the degree of atherosclerosis in hypercholesterolemic rabbits *(6)*.

Fcγ receptors CD16, CD32, and CD64 have been reported as the receptors for CRP *(7–9)*. In addition, we recently demonstrated that CRP increases vascular permeability through a direct binding to lectin-like oxidized LDL receptor (LOX-1), which is expressed in endothelial cells *(10)*. Members of the scavenger receptor family, such as class A scavenger receptor (SR-A), CD36, LOX-1, and scavenger receptor B-I (SR-BI), recognize common ligands such as modified LDL, bacteria, and advanced glycation end products, and they are thought to affect the progression of atherosclerosis *(11, 12)*. In this study, to further elucidate the atherogenic properties of CRP, we addressed whether other scavenger receptors are involved in the recognition of CRP.

Human sera with high and normal concentrations of CRP were obtained from Dako. Human CRP purified from pleural fluid was purchased from Chemicon (AG723). Sodium azide in the solution was extensively removed by dialyzing 3 times against a 3000-fold volume of Dulbecco's PBS (Wako). Gram-negative bacterial endotoxins were undetectable by limulus amebocyte lysate (Associates of Cape Cod), which can detect as little as 0.03 endotoxin units per mL endotoxins. CRP was fluorescently labeled with CypHer5E (GE Healthcare) and dialyzed 3 times against a 3000-fold volume of PBS.

COS-7 cells maintained with Dulbecco's modified Eagle's medium (DMEM; Invitrogen)/10% fetal bovine serum (FBS) were seeded 1 day before transfection. After reaching 80%–90% confluency, we transfected the cells with the plasmid using Lipofectamin 2000 transfection reagent (Invitrogen). We used the following cDNAs: human LOX-1 (GenBank NM002543), SR-A (GenBank NM002445), CD36 (GenBank NM000072), SR-BI (GenBank NM005505), CD68 (GenBank NM001251), and

[1] Nonstandard abbreviations: CRP, C-reactive protein; LOX-1, lectin-like oxidized LDL receptor; SR, scavenger receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; siRNA, small interfering RNA; PMA, phorbol 12-myristate 13-acetate; SRA-C6, anti–SR-A antibody; DAPI, 4′,6-diamidino-2-phenylindole.

dectin-1 (GenBank NM197947), which were subcloned into pcDNA6.2/V5/GW/D-TOPO expression vector (Invitrogen). We used pcDNA3.1/V5-His/lacZ (Invitrogen) as a control. After 48 h, we washed the cells with DMEM:1% antibiotics and antimycotic (AbAm; Invitrogen). We replaced the medium with CypHer5E-CRP-containing DMEM/1% AbAm and incubated the cells for 2 h at 37 °C. After washing with PBS, the cells were fixed with phosphate-buffered formalin (Wako) and permeabilized with 0.1% Triton X-100/PBS. We detected the expression of each receptor by immunostaining with anti-V5 antibody (Nacalai Tesque) combined with Alexa 488 antimouse IgG (Invitrogen). The nuclei of the cells were counterstained with 0.5 mg/L 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). We divided the fluorescence intensities of CypHer5E and Alexa 488 by the cell number in a field, then divided the CypHer5E-CRP fluorescence intensity in the field by the Alexa 488 fluorescence value. These quantitative analyses were performed with an IN Cell Analyzer 1000 system (GE Healthcare).

We prepared recombinant human SR-A (amino acids 76–358) as described for LOX-1 *(10)*. Recombinant human SR-A (0.1 μg) or BSA (0.1 μg, Sigma) was immobilized to each well of 384-well plates (High Bind; Corning) by incubating at 4 °C in PBS overnight. After 2 washes with PBS, the plates were blocked with 80 μL of 20% ImmunoBlock (DS Pharma)/PBS at 4 °C for 8 h. After washing twice with PBS, we added CRP in the reaction buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L $CaCl_2$, 1% BSA, pH 7.0) to each well and incubated them at 4 °C overnight. We detected the binding of CRP with a TMB Peroxidase EIA Substrate kit (Bio-Rad) as described for LOX-1 *(10)*. We obtained small interfering RNA (siRNA) duplex oligoribonucleotides targeting the SR-A coding region (GenBank NM002445) from Invitrogen and used stealth RNAi duplex (Invitrogen) as a negative control. The siRNA sequences were as follows: 5′-GAUAUAACUCAAAGUCUCACGGGAA-3′, 5′-UUCCCGUGAGACUUUGAGUUAUAUC-3′ and 5′-CAGACCUUGAGAAAUAUCACUUUAA-3′, 5′-UUAAAGUGAUAUUUCUCAAGGUCUG-3′.

THP-1 cells were maintained with 10% FBS/1% AbAm/20 μmol/L mercaptoethanol:RPMI 1640 and differentiated with 100 nmol/L phorbol 12-myristate 13-acetate (PMA) (Sigma) for 48 h. We transfected the cells with siRNA oligos or control siRNA using Lipofectamin 2000 transfection reagent (Invitrogen) according to the manufacturer's instruction. After incubation at 37 °C for 24 h, we washed the cells with RPMI 1640/1% AbAm and replaced the medium with CypHer5E-CRP–containing RPMI 1640/1% AbAm, and the cells were incubated for 2 h. After washing with PBS, the cells were fixed with phosphate-buffered formalin (Wako) and permeabilized with 0.1% Triton X-100/PBS. We detected the effects of downregulation of SR-A gene expression by immunostaining with anti–SR-A antibody (SRA-C6; Trans Genic Inc) combined with Alexa 488 antimouse IgG. For detection of Fcγ receptors, we used anti-CD32 antibody (AT10; Santa Cruz) and anti-CD64 antibody (10.1; Santa Cruz). For CRP detection, we used anti-CRP antibody (Bethyl). The nuclei of the cells were counterstained with 0.5 mg/L DAPI. We divided the fluorescence intensities of CypHer5E and Alexa 488 by the cell number in a field. Quantitative analysis was performed with an IN Cell Analyzer 1000 system. All transfections were performed in triplicate.

All data are presented as mean (SE). Statistical analysis was performed with Student *t*-test. A P value <0.05 was considered statistically significant.

We examined whether CRP binds to scavenger receptors: LOX-1, SR-A, CD36, SR-BI, CD68, and dectin-1. Dectin-1 has the closest structural similarity to LOX-1 and belongs to C-type lectin-like molecule, although it is not a member of scavenger receptors.

Alexa546-labeled CRP at the concentration of 1 mg/L at 4 °C bound significantly to LOX-1–expressing cells ($P < 0.01$) but bound poorly to the cells expressing the other receptors (Supplemental Fig. 1, which accompanies the online version of this article at www.clinchem.org/content/vol56/issue3). Cellular uptake of CypHer5E-labeled CRP, which shows fluorescence after endocytosis, was significantly higher in SR-A–expressing cells, in a dose-dependent manner (1–30 mg/L), as well as in LOX-1–expressing cells, compared with cells expressing the other receptors (Fig. 1). Immunostaining with anti-V5 antibody revealed that all the receptors were expressed at a similar level in the respective cells.

Using anti-CRP antibody, we confirmed that nonlabeled CRP was also taken up by SR-A–expressing COS-7 cells. We further observed a significant binding of nonlabeled CRP (0.1–1 mg/L) to immobilized recombinant SR-A ($P < 0.01$) (online Supplemental Fig. 2). The binding was not affected by polymyxin B (5 mg/L), suggesting that it did not depend on the presence of endotoxin. Importantly, native CRP contained in human serum showed significant binding to SR-A, as well as to LOX-1 ($P < 0.01$) (online Supplemental Fig. 3). The binding was dependent on the concentration of CRP in the serum, suggesting that SR-A and LOX-1 have a capacity to bind to a native form of CRP in serum in the presence of other plasma proteins. These results indicate that SR-A and LOX-1 are the receptors for CRP among the examined receptors.

Because SR-A works in the monocyte-macrophage system, we assessed whether CRP is taken up by macrophages via SR-A. We used a human monocytic cell line,

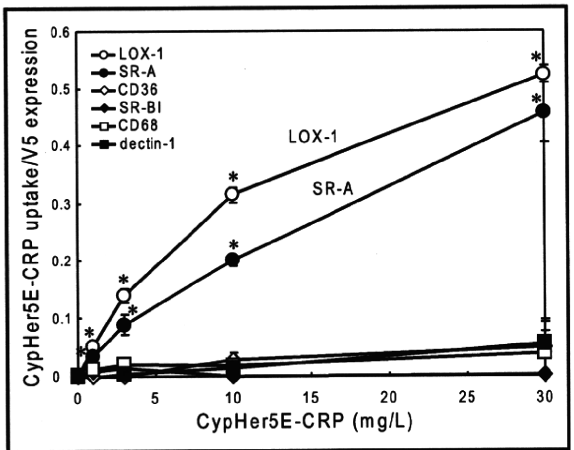

Fig. 1. Quantitative analyses of CypHer5E-CRP taken up by COS-7 cells expressing LOX-1, SR-A, CD36, SR-BI, and dectin-1.

Signals observed in the cells transfected with pcDNA3.1/V5-His/lacZ were considered as non-specific background. *Significant difference vs. negative control ($P < 0.01$).

THP-1, after inducing differentiation into macrophage by the treatment of PMA *(13)*. In PMA-treated THP-1 cells, CypHer5E-CRP was taken up in a dose-dependent manner (0.3–30 mg/L). SR-A expression and CRP uptake were concomitantly suppressed by 2 different siRNAs targeting SR-A, but not by control siRNA (Table 1). The siRNA targeting SR-A did not affect the expression of Fcγ receptors (data not shown), indicating that CRP is taken up mainly via SR-A in a macrophage cell line, at least under these conditions.

The ligand specificity of the scavenger receptor family overlaps considerably *(11, 12)*, and while all can bind to oxidized LDL, only SR-A or LOX-1 bound to CRP. Interestingly, dectin-1, the most structurally similar molecule to LOX-1, did not bind to CRP.

Using a monoclonal antibody, a previous report suggested the presence of an unknown receptor other than Fcγ receptors in macrophages *(14)*. It has been reported that fucoidin, a ligand for SR-A, inhibits the in vivo CRP-promoted uptake of oxidized LDL *(15)*. SR-A might be the unidentified CRP receptor. Fcγ receptors and SR-A are under different regulation of gene expression. In fact, in response to differentiation stimulus of PMA, the expression of SR-A is strongly induced, whereas the expression of Fcγ receptors is suppressed *(16, 17)*. Conversely, stimulation by interferon-γ enhances the expression of Fcγ receptors but suppresses the expression of SR-A *(18)*. These results suggest that Fcγ receptors and SR-A would work in the cells stimulated by different molecules.

Table 1. Suppression of the uptake of fluorescently labeled CRP by siRNA against SR-A in differentiated THP-1 cells.[a]

siRNA	SR-A expression, %	CypHer5E-CRP, %
None	103 (3.2)	108 (9.1)
Control siRNA	100 (2.7)	100 (9.8)
siRNA1 for SR-A	17 (1.6)[b]	31 (3.9)[b]
siRNA2 for SR-A	13 (0.0)[b]	32 (1.1)[b]

[a] Data are as mean (SE).
[b] Significant difference vs control siRNA groups ($P < 0.01$).

Interestingly, the activity of SR-A as CRP receptor was more pronounced in the uptake of CRP, whereas LOX-1 showed strong activity in both binding and uptake. Because SR-A works in phagocytes, the CRP uptake activity of SR-A is reasonable. CRP was originally identified as a binding protein for bacterial component C-polysaccharide *(3)*. SR-A may function to engulf bacteria, viruses, and harmful substances opsonized by CRP in a context of innate immunity.

Related to epidemiological risk factors for cardiovascular disease, the presence of CRP in atheroma has been reported in both rabbits and humans *(6)*. Furthermore, the colocalization of CRP and SR-A in macrophages in atheromas has been reported *(19)*. Taking these reports together with the present results, SR-A-mediated CRP uptake by macrophages in atheromas might affect the foam cell formation and progression of atherosclerotic disease.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures of Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Ministry of Health, Labour and Welfare of Japan; the National Institute of Biomedical Innovation; and Japan Science and Technology Agency.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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Previously published online at
DOI: 10.1373/clinchem.2009.140202