表1　アレルギー検査法

	CAP	MAST	DiaPack	イムファストチェック	ケンタウルス	ヒラソルチップ
1回での検査種類	1種類	26→33種類	1種類	3種類	1種類	50種類以上受託可*
検査時間	2時間40分	20→4時間	16→11分	20分	47分	40分 [→20]
血清量	40μl / 1種類	200μl / 26種類	50μl / 1種類	30μl / 3種類	25μl / 1種類	200μl / 50種類 [→100]
方式	ニトロセルロースプレート	セルロース糸	多孔性ガラスフィルター	免疫クロマト	鉄微粒子	マイクロアレイ
保険（価格）	10種類まで120点/1種	2000点	(1050円)	(3000円)		
装置価格	295万円	400万円	900万円	−		200万円

[ ] 内は開発中を示す。
*研究用、試験用に受託で様々なアレルゲン固定化マイクロアレイ・チップを作製可能

る。現在，遺伝子を解析するゲノミクスから，プロテオミクス，グリコミクス，セロミクス，メタボロミクスと解析対象範囲は拡張を続けており，バイオインフォマティクスの発展とともにますます重要になってきている[1, 2]。臨床診断の分野では，DNAマイクロアレイが欧州で認可を受けた。抗体マイクロアレイや特殊なタンパク質マイクロアレイは一部研究用に市販されるようになってはいるものの，一般的な生体分子をマイクロアレイしたバイオチップは開発途上である。

その原因は，DNAを固定化する場合に比べて，タンパク質などの生体分子を固定化するには，本質的に困難な点があることによる。DNAは多様な遺伝情報を担うものの，化学構造としてはほとんど類似の4塩基の配列が変わるだけで，ほぼ均質な高分子と考えてよく，末端修飾も容易で，原理的に多種類のDNAを同一の固定化法で固定化することが可能である。これに対し，タンパク質は，化学的に多様で，官能基の種類，量が異なる上に，分子上での存在場所もそれぞれ異なる。このため，同一の方法で一つの基板上に固定化するのは非常に困難である。

2. 光固定化法

そこで，筆者らは，このような問題点を解決するために光固定化法を考案した[3, 4]。この方法は，三つの特徴をもつ。

第一は，有機分子であれば「なんでも」マイクロアレイ固定化できることである。これは，ラジカル反応による架橋固定化反応であるため，可能となった。

第二は，固定化する際のマトリックスに非特異的な相互作用を弱める高分子を用いている点である。これにより高S/N比の特異的な相互作用解析が可能になった。

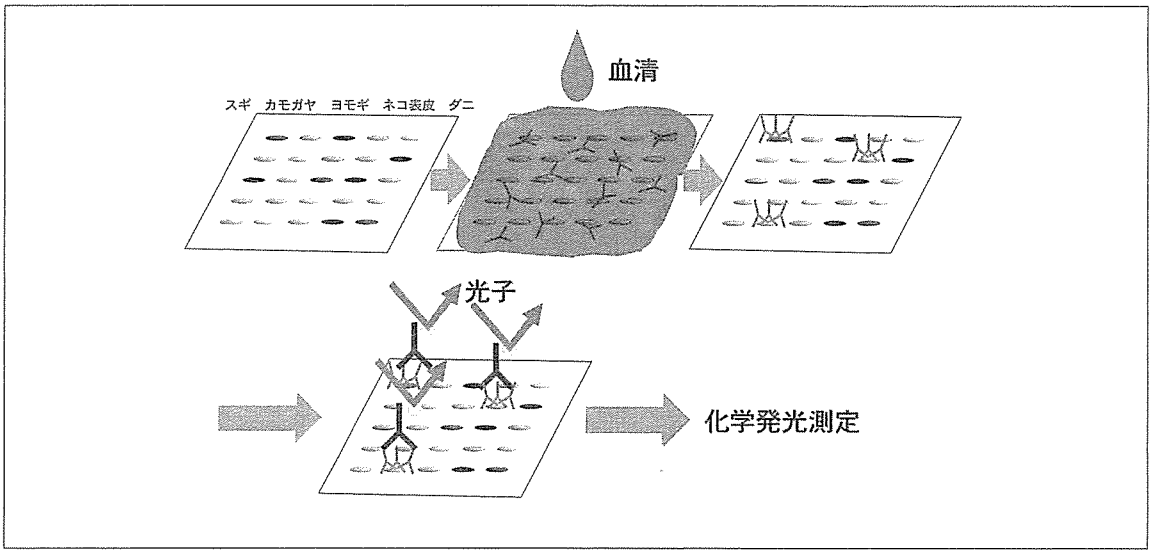

図1 アレルゲン・マイクロアレイを用いたIgEの検出法。マイクロアレイ・チップ上に血清を添加した後，洗浄し，抗ヒトIgEを反応させた後，化学発光のための試薬を加えて洗浄し，スポットの発光を測定する。

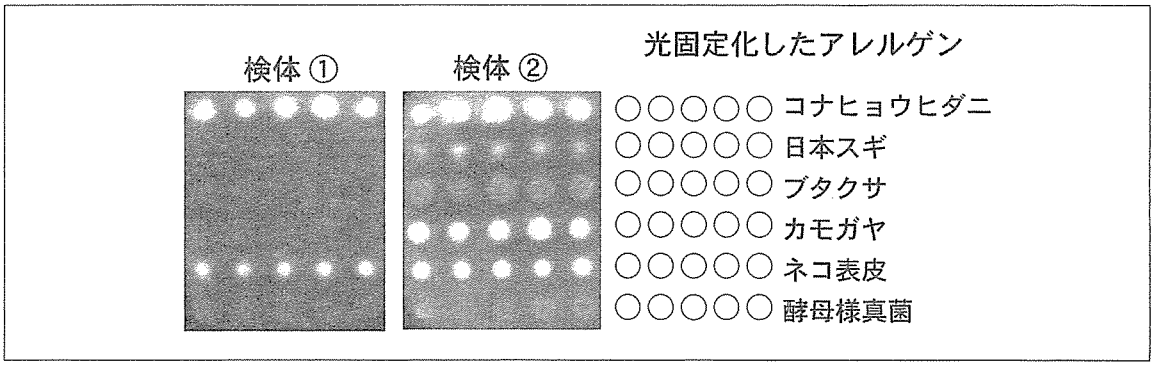

図2 アレルゲン・マイクロアレイを用いたIgEの検出例。発光量により検体中のIgE量を定量できる。

第三は，ランダムな配向性で固定化できることである。アレルゲンのように様々なエピトープ部位をもち，ポリクローナル抗体全体量を調べるためには，全エピトープ部位をランダムに露出させると非常に有利となる。

3. アレルゲン・マイクロアレイチップ

図1には，アレルゲンをマイクロアレイして，吸着するIgEを測定する方法を示す。マイクロアレイチップ上に血清を載せ，洗浄し，化学発光試薬を加えることで，どの固定化アレルゲンにIgEが吸着するかを特定することができる。図2には，測定結果の例を示す。

図3には，図1のプロセスを完全自動で行える装置を示す。チップを装填して，測定したい溶液（血清）を試験管に入れて装填するだけで，測定データを得ることができ，検体が，どのアレルゲンに対するIgEを含むかを一目で判定できる。

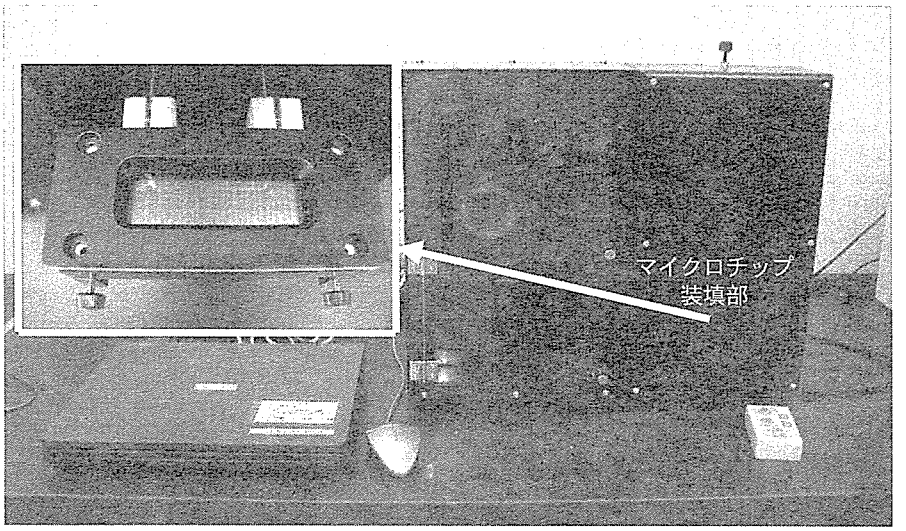

図3　光固定化マイクロチップ用自動測定装置。

おわりに

本研究は科学技術振興機構（JST）の大学発ベンチャー創出推進事業からの研究助成により行われた。現在，ヒラソルバイオ（株）がその成果を生かすために設立され，企業活動を行っている。多くの情報量を生み出すバイオチップは，これからの医療における重要性をますます増やすと考えられるが，まだチップ製造法には課題が多かった。

本稿で紹介した光固定化は，そのような問題を解決して，基盤技術としては完成できた。しかし，まだ搭載するコンテンツについては，充分でないのが現状である。臨床家，臨床研究者の方々のご協力をいただき，日本発の世界初バイオチップ検査システムの構築を目指したい。

文献

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4）Oyama, K., Omura, K., Ito, Y.: Allerg. Int. 54: 627-631, 2005

＜話題あれこれ＞

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なんでも固定化バイオチップ

Everything-Immobilizable Biochip

伊藤嘉浩[*1]　大村　馨[*2]

光架橋反応で有機分子なら「なんでも」固定化できる方法を，マイクロアレイ・チップ作成に応用した。この方法により各種細胞，環境・食物アレルゲン，自己免疫疾患関連抗原を固定化することができ，血液中に存在する各々に対応する抗体量を測定できた。さらに，測定を完全に自動化して行える装置も開発した。また，SPR や QCM 測定用のチップの固定化法にも利用できることを示した。

1. はじめに

最近のライフサイエンスの研究は，材料の微細加工やナノテクノロジーの発展により新たな展開が見られる。本特集でも主な部分を占める DNA マイクロアレイ（DNA チップ）の出現は，まさにバイオテクノロジーと微細加工技術の融合の賜物である。現在，遺伝子を解析するゲノミクスから，プロテオミクス，グリコミクス，セロミクス，メタボロミクスと解析対象範囲は拡張を続けており，バイオインフォマティクスの発展とともにますます重要になってきており，いよいよ臨床診断の分野でも DNA マイクロアレイは使用されるようになってきている。研究用には抗体マイクロアレイ，タンパク質マイクロアレイ，ペプチドマイクロアレイが市販され，多くの研究が行われているものの，まだ一般的なタンパク質や糖をマイクロアレイしたバイオチップは開発途上である[1]。

その原因は，DNA を固定化する場合に比べて，タンパク質などの生体分子を固定化するには，本質的に困難な点があることによる。DNA は多様な遺伝情報を担うものの，化学構造としてはほとんど類似の 4 塩基の配列が変わるだけで，ほぼ均質な高分子と考えてよく，末端修飾も容易で，原理的に多種類の DNA に同一の固定化法を利用することが可能である。これに対し，タンパク質は，化学的に多様で，官能基の種類，量が異なる上に，分子上での存在場所もそれぞれ異なる。このため，同一の方法で一つの基板上に固定化するのは非常に困難である（図 1）。

2. 光固定化法

そこで，筆者らは，このような問題点を解決するために光固定化法を考案した[2, 3]。この方法は，新たに合成した光反応性マトリックスを用いて生体分子を光架橋固定化するもので，次の 3 つの特

[*1]Yoshihiro Ito　㈱理化学研究所　伊藤ナノ医工学研究室　主任研究員；㈶神奈川科学技術アカデミー　伊藤「再生医療バイオリアクター」プロジェクト　プロジェクトリーダー
[*]Kaoru Omura　ヒラソルバイオ㈱　代表取締役

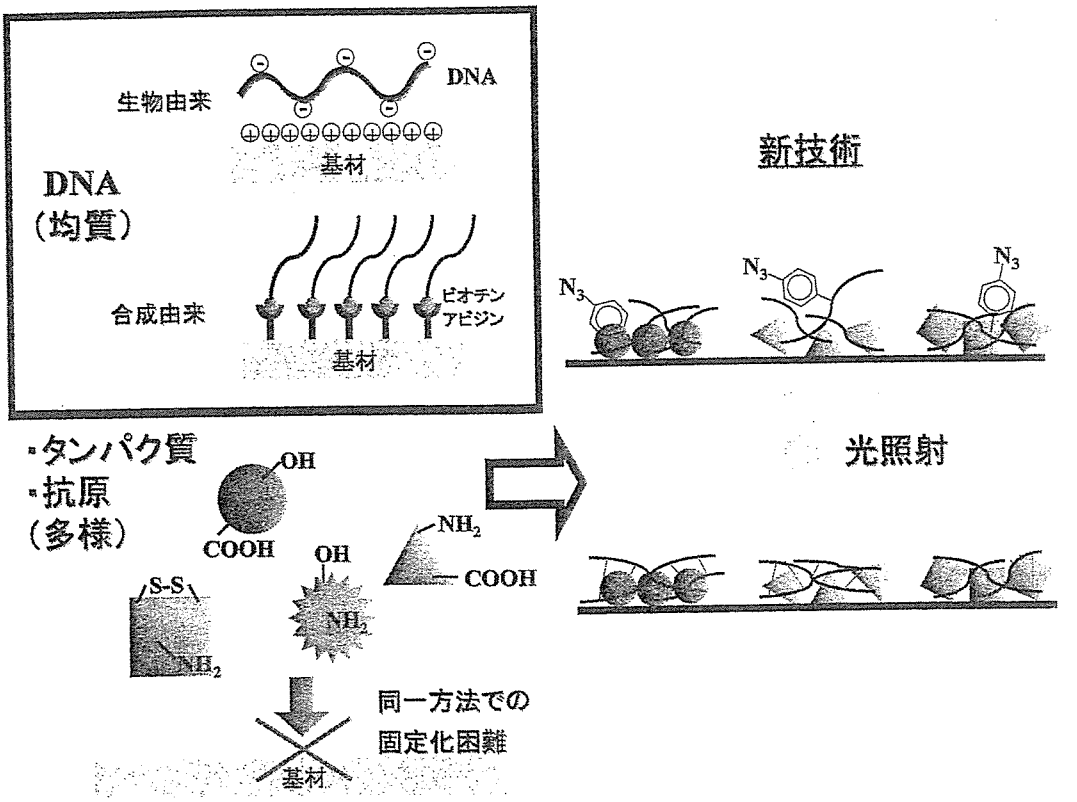


図1　DNA は異なる種類でも同じ方法でマイクロアレイ固定化できるが，タンパク質は種類が異なると同じ方法では困難なため，新しく光固定化法を開発した

徴をもつ。

①有機分子であれば「なんでも」マイクロアレイ固定化できること。ラジカル反応による架橋で固定化するため，可能となった。

②固定化する際のマトリックスにポリエチレングリコールのような両親媒性高分子を用い非特異的な相互作用を弱めている。これにより高 S/N 比の特異的な相互作用解析が可能になった。

③生体分子をランダムな配向性で固定化できることである（図 2)。従来の共有結合による固定化法では，基板側にカルボキシル基を用いると固定化される生体分子のアミノ基を介して固定化することになり，固定化される分子の配向性はアミノ基の分布に依存する。タンパク質工学によって，固定化用の配列を導入しても，一定の配向性となる。このような従来の方法と異なり，様々な生体分子相互作用を相互作用部位に関係なく網羅的に調べ上げることができる。この技術が広く応用されるようになると，従来の固定化技術を用いた生体分子相互作用研究が，果たして真の相互作用解析になっていたかさえ問題となる可能性がある。一方，応用的な側面からは，アレルゲンのように様々なエピトープ部位をもち，ポリクローナル抗体全体量を調べるためには，全エピトープ部位をランダムに露出させることから非常に有利となる。

3. 光固定化マイクロアレイの展開

このような特徴をもつ光固定化法は，特に抗体を検出することに有効と考えられる。ここでは，これまでに行ってきた細胞固定化と，アレルゲンや自己免疫疾患関連抗原の固定化の場合について述べる。

3.1　細胞固定化マイクロアレイチップ

輸血の際には血液型の一致が重要になる。A 型血液（赤血球）型のヒトは抗 A 抗体は持たな

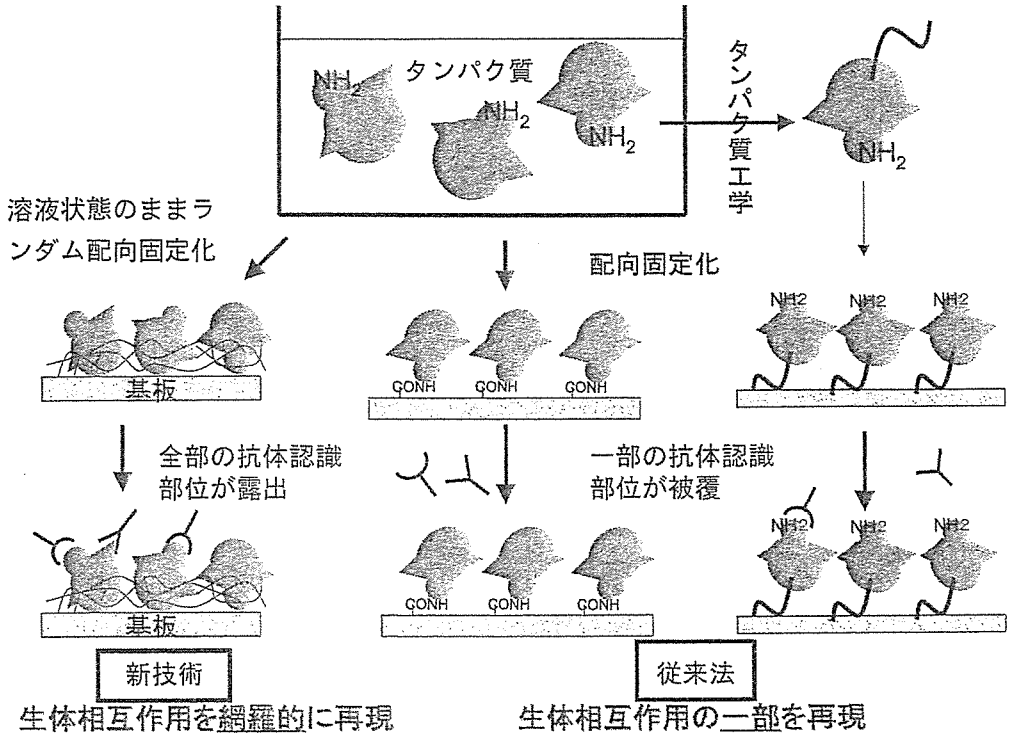


図2　光固定化法では，従来の化学固定化法や，組換えタンパク質を用いた方法と異なり，生体分子のランダム配向固定化が可能。通常の化学固定化法では，タンパク質中の官能基の分布によって配向性が決まる。タンパク質工学的にアンカー配列を導入した場合は，タンパク質のN端あるいはC端が基板に結合し，この周辺は表面には露出しないことになる

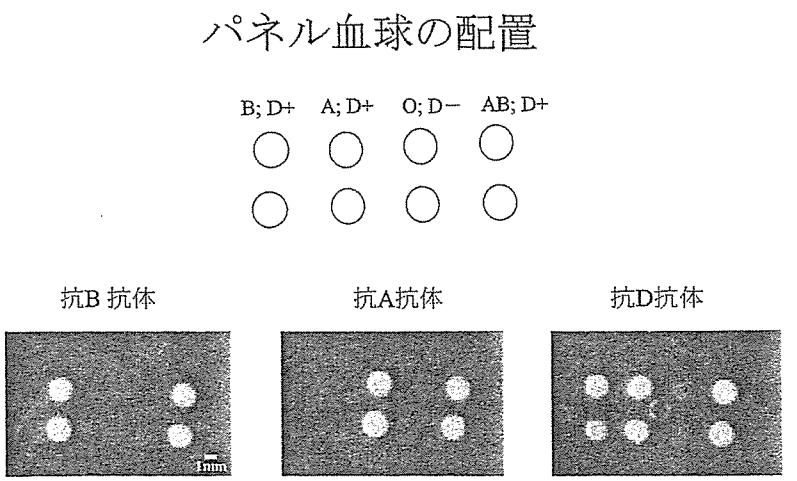


図3　パネル血球をマイクロアレイ固定化した基板への各種抗体の結合
上段にはマイクロスポットした異なる血液型のパネル血球のチップ上での配置を示す。下段には，各々の抗体との接触後のチップの化学発光の様子を示す。抗体結合スポットで発光が観察された。(協力：日本赤十字社)

表1　アレルギー検査法の比較

	CAP	MAST	DiaPack	イムファストチェック	ケンタウルス	ヒラソルチップ
1回での検査種類	1種類	26→33種類	1種類	3種類	1種類	50種類以上受託可*
検査時間	2時間40分	20→4時間	16→11分	20分	47分	40分(→20)
血清量	40μl/1種類	200μl/26種類	50μl/1種類	30μl/3種類	25μl/1種類	200μl/50種類(→100)
方式	ニトロセルローススプレート	セルロース糸	多孔性ガラスフィルター	免疫クロマト	鉄微粒子	マイクロアレイ
保険（価格）	10種類まで	2,000点	(1,050円)	(3,000円)		
装置価格	295万円	400万円	900万円	−		200万円

（ ）内は開発中を示す。
＊研究用，試験用に受託で様々なアレルゲン固定化マイクロアレイ・チップを作製可能。

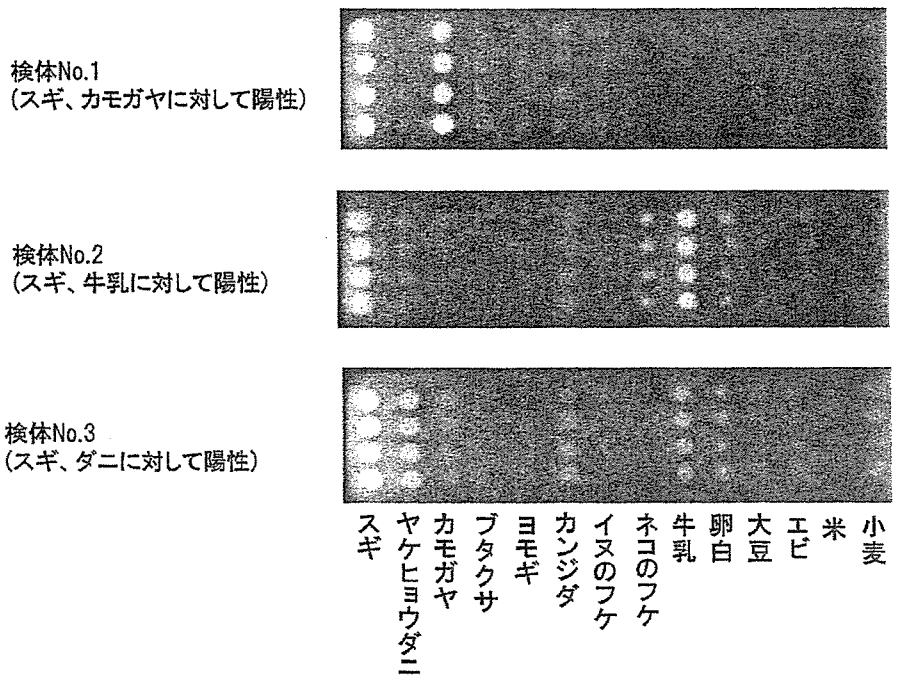


図4　アレルゲン・マイクロアレイを用いたIgEの検出例
発光量により検体中のIgE量を定量できる。(協力：相模原病院)

いものの，抗B抗体を持つため，B型血液（赤血球）と混ぜると抗原抗体反応して凝集が起こる。B型のヒトの場合，抗A抗体をもつのでA型血液と混合すると凝集が起こる。このような現象を血液型のわかった赤血球を光反応性マトリックスと混合し，マイクロアレイした後乾燥し，光を照射して固定化することにより調べることができた[4]。図3には，パネル血球を固定化しておいて抗体を反応させた例を示す。

妊娠や過去の輸血により変わることがある不規

則抗体が現れる場合があり，輸血の際に調べる必要がある。そのために，現在は，凝集法による確認が行われているが，これを簡便にするために，本研究のようなパネル血球を光固定マイクロアレイしたチップを利用できるようになると，医療の進歩につながると期待される。

3.2 アレルゲン固定化マイクロアレイチップ

アレルギー検査は，皮膚試験と試験管内試験に大別される。皮膚試験として，即時型アレルギーの検査にはプリックテスト，遅延型アレルギーの検査にはパッチテストが最もよく行われる。しかし，皮膚試験は患者への負担が大きいため，試験管内試験が現在のアレルギー検査の主流となっている。中でも，測定が簡便なうえ，結果が客観的な数値として表れることから，血中の特異 IgE 抗体の測定が最も多く用いられている。アレルギー治療の第一歩は起因アレルゲンの特定であるため，特異 IgE 抗体のスクリーニング検査は重要である。

表 1 には，現在主に市販あるいは開発されている検査法をまとめる。筆者らは，わずかな血液量で，様々な抗原に対するアレルギーを，一括して一度に検査できる安価なシステムを，新しいタイプの抗原マイクロアレイ・チップ（ヒラソルチップ）を用いて可能にしたので，これについて解説する[5, 6]。

この場合も上述の細胞マイクロアレイと同様，アレルゲンを光反応性マトリックスと混合した後，

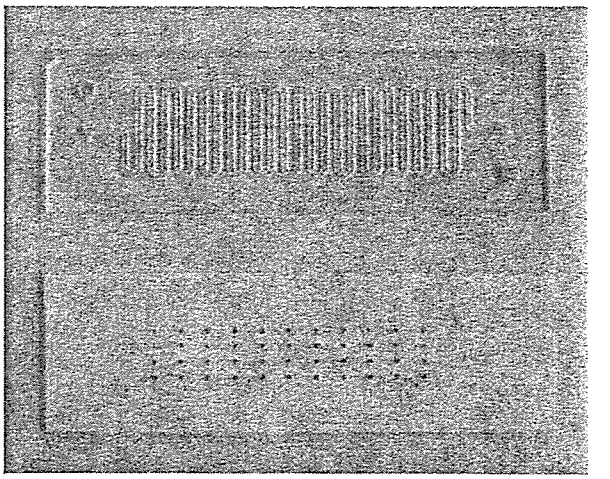


写真 1　マイクロアレイ固定化板（下）にマイクロ流路を彫ったポリジメチルシロキサン製板（上）を張り合わせ，自動化装置（図 6）に装填。

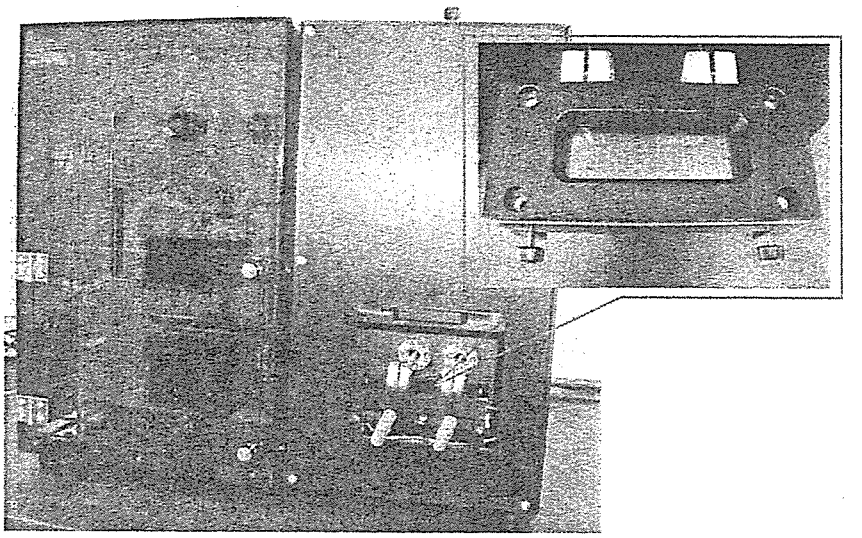
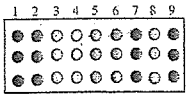


写真 2　光固定化マイクロチップ用自動測定装置
送液部：シリンジポンプ 1 台，6 方切り替えバルブ 2 台，使用サンプル量 90μl，速度 3.3μl/sec，検出部：高感度 CCD カメラ，反応：シリコン樹脂流路，重量：約 23kg，寸法：480(W)×360(D)×420(H)mm，電源：AC100V，制御用 PC：USB，RS232C，WindowsPC



1.SS-A/52kDa　4.U1snRNP68kDa　7.Jo-1
2.SS-A/60kDa　5.Sm　8.dsDNA
3.SS-B　6.Scl-70　9.CENP-B

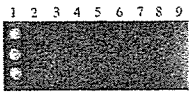
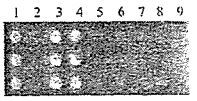
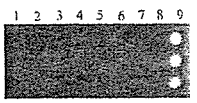
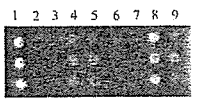
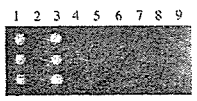
	病　　名	患者血清中に見出される主な自己抗体と出現頻度	従来法による評価結果	化学発光法
No.4	SjS （シェーグレン症候群）	抗 SS-A/Ro 抗体：50-70% 抗 SS-B/La 抗体：20-30%	抗 SS-A/Ro 抗体陽性	
No.13	MCTD （混合性結合組織病）	抗 U1RNP 抗体：100%	抗 SS-A/Ro 抗体陽性 抗 SS-B/La 抗体陽性 抗 U1RNP 抗体陽性	
No.42	SSc （全身性硬化症）	抗 Scl-70 抗体：15-30% 抗セントロメア抗体：20-30%	抗セントロメア抗体陽性	
No.46	SLE （全身性エリテマトーデス）	抗 Sm 抗体：15-30% 抗 dsDNA 抗体：40-70%	抗 SS-A/Ro 抗体陽性 抗 U1RNP 抗体陽性 抗 Sm 抗体陽性 抗 dsDNA 抗体陽性	
No.51	SjS （シェーグレン症候群）	抗 SS-A/Ro 抗体：50-70% 抗 SS-B/La 抗体：20-30%	抗 SS-A/Ro 抗体陽性 抗 SS-B/La 抗体陽性	

図5　自己免疫疾患での抗原マイクロアレイでの患者の抗体検出
（協力：慶應義塾大学病院）

アレイヤーを用いてマイクロアレイして，光照射によって固定化してアレルゲン・マイクロアレイを作成する。測定では，まずマイクロアレイ・プレートを血清と接触させる。しばらくしてから洗浄し，酵素標識した抗 IgE 抗体を反応させ，最後に化学発光試薬を加えることで，どの固定化アレルゲンに IgE が吸着するかを特定することができる。図4には，測定結果の例を示す。検体中に固定化アレルゲンに対する IgE が存在すると，そのスポットが化学発光して，何に対するアレルギーがあるかが一目瞭然となる。

このような測定を微量の血清で行えるようにするために，写真1に示すようなマイクロ流路プレートも作製した。このプレートはポリジメチルシロキサンからなり，これとマイクロアレイ固定化基板をはさんでから，装置に装填し測定を行う。送液を自動で行えるように製作した全自動測定装置

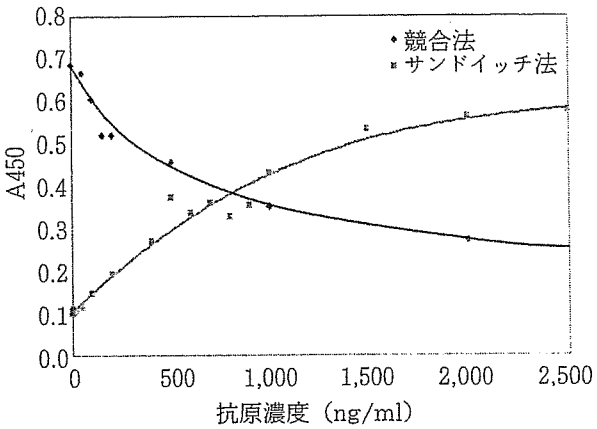


図6　ウシ血清アルブミン（BSA）を光固定化して競合法で BSA 量を定量した場合と，通常のサンドウィッチ法で BSA を定量した場合の比較

を写真2に示す。この装置では，チップを装填して，測定したい溶液（血液あるいは血清）を試験管に入れて装填するだけで自動的に測定データを

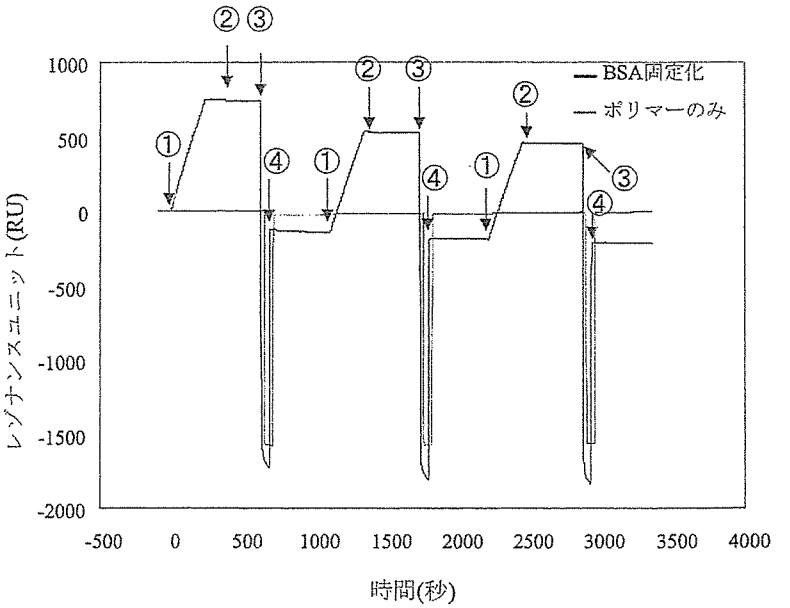


図7　ウシ血清アルブミンを光固定化して抗 BSA 抗体の吸着を表面プラズモン共鳴（SPR）法で調べた結果

BSA 固定化表面では，①抗体溶液を流し，②バッファーで洗浄した後，抗体の吸着が観測され，③塩酸処理をすると脱着し，④再度バッファーで洗浄し，新たな抗体溶液を流すとまた再現性よく吸着が始まる。ベースラインに若干変化があるものの，この一連の過程を繰り返すことができた。

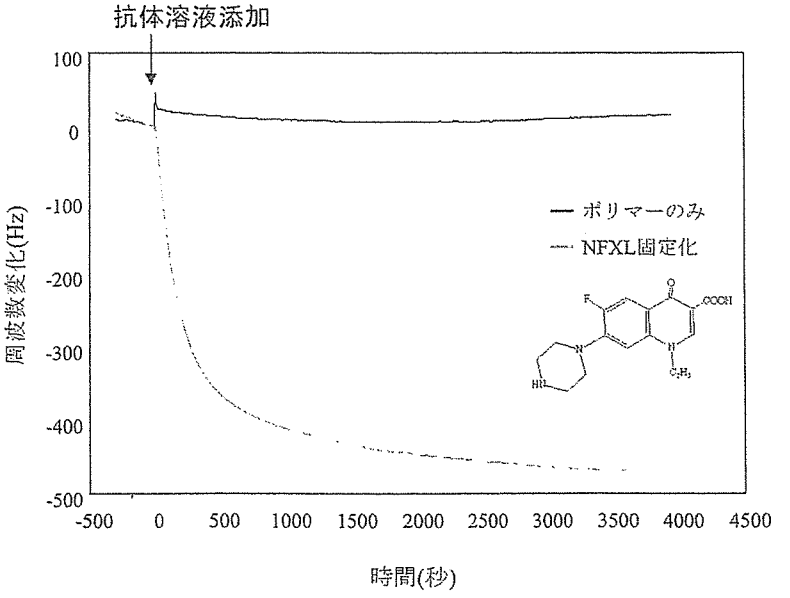


図8　ノルフルオロキサシン（NFXL）を金基板上に光固定化し，抗 NFXL 抗体の吸着挙動を水晶発振マイクロバランス（QCM）で測定した結果

光反応性マトリックスだけでは吸着（振動数の変化）がみられなかったものの，固定化表面には吸着が観測された。

得ることができる。

3.3 自己免疫疾患抗原マイクロアレイ

自己免疫疾患に関連する複数の抗原も同様にマイクロアレイ状に固定化し，患者血清中の自己抗体を検出することも可能となった（図5）。生体には，自分の体の中に侵入した有害な異物や細菌，ウイルスに対して，防衛網を何重にも張り巡らし守る免疫機能がある。しかし，なんらかの原因で免疫の機構の一部が狂ってしまうことがある。その結果，本来，味方である，自分自身の体に向かって，攻撃を仕掛けてしまう。それが，自己免疫疾患である。症状が多彩であり，また，表現しにくいため，伝えることが困難なことが多く，慢性関節リウマチ，全身性エリテマトーデス，混合結合組織病，　シェーグレン症候群，等が知られている。マイクロアレイ技術を用いれば，複数の抗体の種類を同時に知ることができる。

4. 研究用チップ

光固定化法は，以上のような診断応用への他に基礎研究用のチップへの利用も可能である。例えば，ELISA用プレートに定量したい物質を光反応性マトリックスを用いて光固定化することにより，ELISA測定への応用も可能となっている（図6）。

チオール化合物と組み合わせることにより，金基板上への光固定化も行うことができる。図7には，タンパク質を金基板上に光固定化してその抗体との相互作用を表面プラズモン共鳴（SPR）法で調べた結果を示す。あらかじめ金基板に有機チオールを反応させて有機化した後，光反応性マトリックスとタンパク質を混合して固定化することができ，その上でのタンパク質吸着を測定するものである。図8には，低分子化合物であるノルフロキサシン（NFXL）を固定化し，抗体との相互作用を水晶振動子マイクロバランス（QCM）法で調べた結果を示す。タンパク質のような高分子化合物ばかりでなく，低分子化合物も固定化できることから，より広い分野での利用が期待できる。

5. おわりに

筆者らの光固定化法を用いたバイオチップ開発は，科学技術振興機構の大学発ベンチャー創出促進事業からの研究助成により行われた。現在，ヒラソルバイオ㈱がその成果を生かすために設立され，企業活動を行っている。多くの情報量を生み出すバイオチップは，これからの医療における重要性をますます増すと考えられるが，チップ製造法にはまだ課題が多かった。本稿で紹介した光固定化は，そのような問題を解決して，基盤技術としては完成できた。しかし，まだ搭載するコンテンツについては，充分でない。臨床家，臨床研究者の方々のご協力をいただき，日本発の世界初バイオチップ検査システムの構築を目指したい。

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Direct Modulation of Rheumatoid Inflammatory Mediator Expression in Retinoblastoma Protein–Dependent and –Independent Pathways by Cyclin-Dependent Kinase 4/6

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Objective. **It is known that the cyclin-dependent kinase inhibitor (CDKI) gene $p21^{Cip1}$ suppresses rheumatoid inflammation by down-modulating type I interleukin-1 receptor (IL-1RI) expression and inhibiting JNK activity. The purpose of this study was to determine whether CDK activity directly modulates the production of inflammatory molecules in patients with rheumatoid arthritis (RA).**

Methods. **Genes for the CDKIs $p16^{INK4a}$ and $p18^{INK4c}$, a constitutively active form of retinoblastoma (RB) gene product, cyclin D1, and CDK-4, were transferred into RA synovial fibroblasts (RASFs). RASFs were also treated with a synthetic CDK-4/6 inhibitor (CDK4I). Levels of matrix metalloproteinase 3 (MMP-3), monocyte chemoattractant protein 1 (MCP-1), and IL-1RI expression were determined by Northern blotting, real-time polymerase chain reaction analysis, and enzyme-linked immunosorbent assay. CDKIs were immunoprecipitated to reveal their association with JNK.**

Results. **Transfer of the $p16^{INK4a}$ and $p18^{INK4c}$ genes and CDK4I suppressed the production of MMP-3 and MCP-1. Unlike $p21^{Cip1}$, neither CDKI gene inhibited IL-1RI or JNK. The expression of MMP-3 was up-regulated when CDK-4 activity was augmented. This regulation functioned at the messenger RNA (mRNA) level in MMP-3, but not in MCP-1. Transfer of active RB suppressed the production of MMP-3 and MCP-1 without changing their mRNA levels.**

Conclusion. **CDK-4/6 modulated the production of MMP-3 and MCP-1. MMP-3 production was regulated primarily at the mRNA level in an RB-independent manner, whereas MCP-1 production was controlled posttranscriptionally by RB. These results show that cell cycle proteins are associated with control of mediators of inflammation through multiple pathways.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation, hyperplasia, and destruction of the cartilage and bone. In the rheumatoid joint, inflammatory cells, such as lymphocytes and macrophages, infiltrate and produce a variety of cytokines. The inflammatory cells stimulate synovial fibroblasts to proliferate vigorously and to secrete inflammatory cytokines, and they also recruit more inflammatory cells into affected joints. Proliferating RA synovial fibroblasts (RASFs) and infiltrating cells shape a hyperplastic granulomatous synovial tissue called pannus. Pannus offers a platform where many mediators of inflammation, including tissue-degrading proteases, are produced and osteoclasts are activated to absorb the bone matrix. These processes eventually lead to destruction of the affected joints (1,2).

A goal of antirheumatic treatment is prevention of irreversible joint damage. Clinical experience with

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blockage of inflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and IL-6, has demonstrated that antiinflammatory cytokine treatment is an attractive therapeutic choice for RA (3). Nevertheless, the effects of such new antiinflammatory treatment as well as conventional treatment are never satisfactory for all RA patients. We have been exploring cell cycle regulation of RASFs as a new antirheumatic strategy, assuming that suppression of inflammation, together with synovial cell proliferation, should be the ultimate therapeutic combination. The efficacy of cell cycle regulation was substantiated previously by transfer of cyclin-dependent kinase inhibitor (CDKI) genes $p16^{INK4a}$ and $p21^{Cip1}$ into inflamed joints in animal models of RA (4–6).

In general, the cell cycle is driven by kinase activity of cyclin–CDK complexes. These kinases phosphorylate retinoblastoma (RB) gene products, which results in inactivation of the RB function that keeps E2F transcription factors from promoting cell cycle progression. CDKIs are intracellular proteins that inhibit the kinase activity of CDKs. They consist of 2 families, INK4 and Cip/Kip. The INK4 family proteins, including $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$, specifically inhibit the cyclin D–CDK-4/6 complex, which is important for the G_1/S transition of the cell cycle. The Cip/Kip family proteins, including $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, inhibit all cyclin–CDK complexes (7).

While CDKIs act as inhibitors of cell cycling, we have observed that CDKI gene delivery into arthritic joints suppresses not only the proliferation of synovial fibroblasts, but also the production of inflammatory cytokines, infiltration by inflammatory cells, and destruction of bone and cartilage (5). We have also found that expression of $p21^{Cip1}$ in RASFs in vitro down-regulates the messenger RNA (mRNA) expression of proteinases and mediators of inflammation involved in the pathology of RA (8). These observations are consistent with reports showing that $p21^{Cip1}$ binds to JNK to exert antiinflammatory effects (9,10). In addition, we have found that expression of IL-1 receptor type I (IL-1RI) is down-regulated and that transcription factor activities, including NF-κB and activator protein 1 (AP-1), are suppressed by $p21^{Cip1}$ (8).

In contrast, we identified no mechanistic interaction between $p16^{INK4a}$ and other molecules in RASFs (8). Nevertheless, some inflammatory molecules, including matrix metalloproteinase 3 (MMP-3) and monocyte chemoattractant protein 1 (MCP-1), were down-regulated, commonly by $p16^{INK4a}$ and $p21^{Cip1}$ (8). This led us to assume that CDK activity directly modulates the expression of inflammatory molecules. The findings of the present study have shown that this is indeed the case, at least in terms of MMP-3 and MCP-1 production. Their protein levels were regulated by RB-dependent as well as RB-independent pathways. We found that cell cycle progression and inflammatory processes in arthritic joints are closely related.

MATERIALS AND METHODS

Cell culture. RA synovial tissues were obtained from 5 patients who had undergone joint replacement surgery or synovectomy at Tokyo Medical and Dental University Hospital, Tokyo Metropolitan Bokuto Hospital, or National Shimoshizu Hospital in Chiba. All patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the classification of RA (11). The mean ± SD duration of disease was 10.6 ± 3.9 years. At the time samples were collected, the patients had been taking disease-modifying antirheumatic drugs (DMARDs) (methotrexate, gold sodium thiomalate, bucillamine, or sulfasalazine) with or without prednisolone. The RA was refractory to these medications. The mean ± SD erythrocyte sedimentation rate was 53 ± 27.0 mm/hour before surgery.

Synovial tissue was also obtained from a patient with osteoarthritis (OA). Adult normal human dermal fibroblasts (NHDF-Ad) derived from 1 subject were purchased from Cambrex (East Rutherford, NJ). RASFs and OA synovial fibroblasts (OASFs) were isolated and cultured as described elsewhere (4). All fibroblast samples were used at early passages (from passage 3 to 9).

Patients gave their consent to all procedures in the present study. The study protocol was approved by the ethics committees of Tokyo Medical and Dental University and of RIKEN.

Adenovirus infection. Recombinant adenoviruses containing a human $p16^{INK4a}$ gene (AxCAp16) (12), a human $p18^{INK4c}$ gene (Ad-RGD-p18) (13–15), a human $p21^{Cip1}$ gene (AxCAp21) (12), a human cyclin D1 in conjunction with a nuclear localization signal (Ad-D1-NLS) and a human CDK-4 gene (Ad-CDK-4) (16,17) that encodes a nonphosphorylatable, constitutively active form of a human RB gene (Ad-RB) or a β-galactosidase gene (Ad-LacZ) (18,19), control Ax1w1 adenovirus (RIKEN Gene Bank, Tsukuba, Japan), and control Ad5-RGD, which lacks insert genes (20), were either purchased, received as gifts, or constructed in our laboratory. RASFs, OASFs, and NHDF-Ad were infected with one of these recombinant adenoviruses at a minimal multiplicity of infection (MOI) that ensured 100% efficacy of infection (typically, 50–200 MOI).

Three days after infection, when expression of the transferred genes reached maximal levels, the fibroblasts were examined for proliferation or were stimulated for 5 hours with 5 ng/ml of TNFα (Genzyme, Cambridge, MA), 5 ng/ml of IL-1β (PeproTech, Rocky Hill, NJ), and 25 μM indomethacin (Sigma, St. Louis, MO) to examine the production of mediators of inflammation. Indomethacin was included to avoid possible suppression by prostaglandins released from the stimulated RASFs (8,21). Preliminary experiments had shown that

5 ng/ml of each cytokine was the optimal concentration for stimulating RASFs.

Cell proliferation assay. Cell growth was assessed by incorporation of ^{3}H-thymidine. Three days after the adenoviral infection, RASFs were stimulated for 36 hours with IL-1β, TNFα, or indomethacin. ^{3}H-thymidine (0.3 μCi; Amersham Biosciences, Buckinghamshire, UK) was added during the last 24 hours of culture, and the incorporated radioactivities were quantified. Numbers of live cells were determined using Cell Counting Kit 8 (Dojin, Kumamoto, Japan).

Flow cytometry for cell cycle analysis. Cells were fixed in phosphate buffered saline containing 0.15% Triton X-100 for 10 minutes, and then incubated with 50 μg/ml of propidium iodide (Sigma) and 5 μg/ml of RNase A. Cells were analyzed using a FACSCalibur (BD Biosciences, San Diego, CA), and data were collected.

Northern blot and real-time polymerase chain reaction (PCR) analyses. Northern blot analyses to detect MMP-3, MCP-1, and IL-1RI mRNA were performed as described elsewhere (8). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and sets of primers specific for MMP-3 (22) or MCP-1 (23) complementary DNA. Data were standardized against human GAPDH mRNA using the threshold cycle method (24).

Enzyme-linked immunosorbent assay (ELISA). The adenovirus-infected RASFs were cultured for 60 hours and transferred to microwells. Twelve hours after transfer, the culture supernatants were replaced with fresh Dulbecco's modified Eagle's medium containing 10% fetal bovine serum with IL-1β, TNFα, and indomethacin. Supernatants from this 24-hour culture were collected, and levels of MMP-3 (Fuji Chemical, Toyama, Japan), MCP-1 (BioSource International, Camarillo, CA), macrophage inflammatory protein 3α (MIP-3α; R&D Systems, Minneapolis, MN), and IL-6 (BioSource International) were determined by ELISA.

Analysis of the effects of a small-molecule CDK-4/6 inhibitor. A CDK-4/6 inhibitor, CDK4I (2-bromo-12,13-dihydro-5*H*-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6*H*)-dione; Merck, Whitehouse Station, NJ) (25), was dissolved in DMSO and added to the culture medium. RASFs were pretreated with CDK4I for 6 hours. The RASFs were then stimulated with IL-1β and TNFα for either 36 hours (for cell and culture supernatant analysis) or 12 hours (for RNA extraction and analysis).

Immunoprecipitation and Western blot analyses. For immunoprecipitation, cell lysates of RASFs were prepared on day 3 after adenovirus infection (4,8). JNKs 1–3 were immunoprecipitated using mouse anti-JNK monoclonal antibody (sc-7345; Santa Cruz Biotechnology, Santa Cruz, CA) (26). Rabbit anti-human p16^{INK4a}, mouse anti-human p18^{INK4c}, and p21^{Cip1} polyclonal antibodies (sc-468, sc-9965, and sc-387, respectively; Santa Cruz Biotechnology) were used as primary antibodies for Western blot analyses.

Multiwell colorimetric transcription factor assays. To assess transcription factors and JNK activities in RASFs, nuclear extracts were prepared using a nuclear extract kit (Active Motif, Carlsbad, CA). Trans AM AP-1/c-Jun, NF-κBp50, and NF-κBp65 transcription factor assay kits (Active Motif) were used to quantify DNA binding activities of AP-1 and NF-κB transcription factors.

Statistical analysis. ^{3}H-thymidine incorporation, signal intensity ratios from the real-time PCRs, and protein concentrations in the supernatants were compared by Student's paired *t*-test using StatView 5.0J software (SAS Institute, Cary, NC).

RESULTS

Suppression of RASF production of MMP-3 and MCP-1 production by p16^{INK4a}, but no down-regulation of IL-1RI expression or association with JNK. RASFs derived from joints with active rheumatoid inflammation were cultured in vitro. It has been shown that endogenous p16^{INK4a} is not expressed in cultured RASFs (4). Cells were infected with the AxCAp16 adenovirus containing the human p16^{INK4a} gene or with the control Ax1w1 blank adenovirus. When the transgene expression was at the highest level, the cells were examined for proliferation and cell cycle progression.

^{3}H-labeled thymidine incorporation by the p16^{INK4a}-expressing RASFs was profoundly suppressed as compared with incorporation by RASFs infected with control virus. This suppression was accompanied by an increase in the number of cells in the G_0/G_1 phase of the cell cycle (Figure 1A).

Preliminary DNA array analyses of gene expression in RASFs samples with and without gene transfer of p16^{INK4a} suggested that a set of genes related to RA pathology, including MMP-3 and MCP-1, was down-regulated by p16^{INK4a}. In RASFs in which the p21^{Cip1} gene had been transfected, the expression of those 2 molecules as well as MIP-3α and IL-6 was found to be down-regulated (8). Therefore, we next tested the expression of all 4 molecules in stimulated RASFs with and without p16^{INK4a} gene transfer, using a specific ELISA. When p16^{INK4a} was introduced into RASFs, the production of both MMP-3 and MCP-1 in culture supernatants was suppressed, whereas the production of MIP-3α and IL-6 was essentially unaffected (Figure 1B). In contrast, the overexpression of p16^{INK4a} in OASFs and in NHDF-Ad did not appreciably alter the production of MMP-3 and MCP-1 (Figure 1C).

Because of variations in basal and up-regulated levels of MMP-3, MCP-1, MIP-3α, and IL-6 in culture supernatants, their production by stimulated RASFs was assessed relative to that of control RASFs infected with control adenovirus. Typically, culture supernatants from stimulated RASFs contained approximately 500, 50, 3, and 300 ng/ml of MMP-3, MCP-1, MIP-3α, and IL-6, respectively. The relative production of MMP-3 and IL-6 protein by stimulated RASFs infected with the control Ax1w1 adenovirus as compared with uninfected

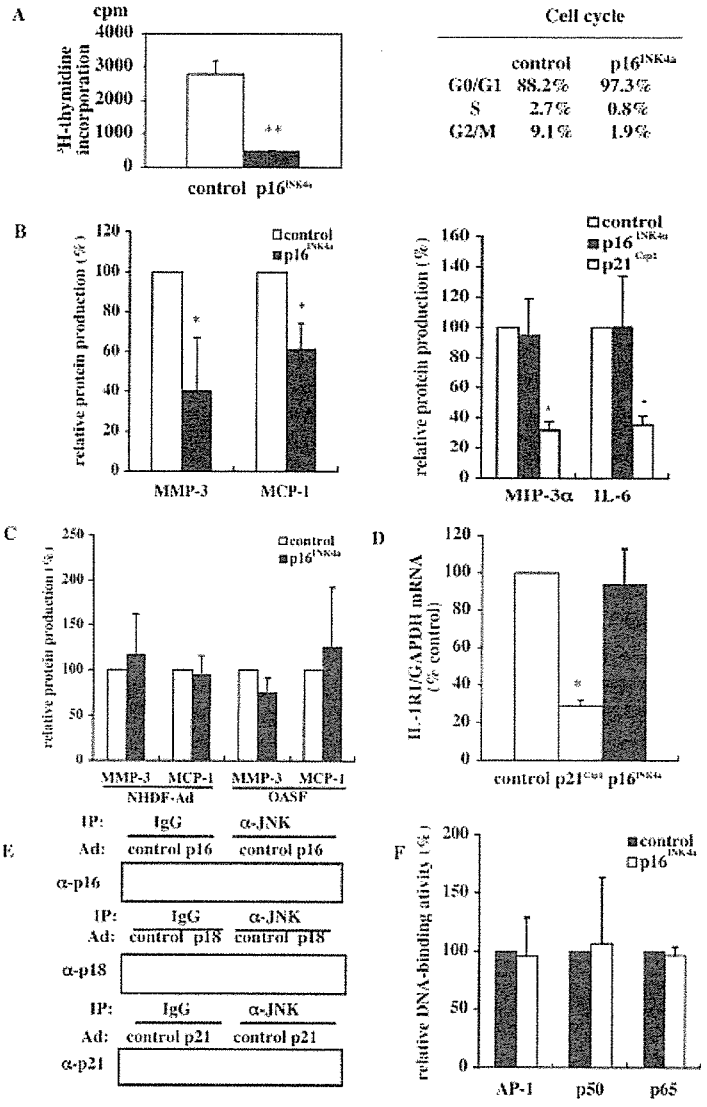

Figure 1. Suppression of fibroblast expression of matrix metalloproteinase 3 (MMP-3) and monocyte chemoattractant protein 1 (MCP-1) by $p16^{INK4a}$. **A,** Rheumatoid arthritis synovial fibroblasts (RASFs) infected with $p16^{INK4a}$ or control adenovirus were stimulated for 24 hours with interleukin-1β (IL-1β) plus tumor necrosis factor α (TNFα), and ^{3}H-thymidine incorporation was assessed 3 days later (left). Mean reduction in ^{3}H-thymidine incorporation induced by $p16^{INK4a}$ was 83% compared with controls. Flow cytometry showed an increase in cells at G_0/G_1 phase in RASFs expressing $p16^{INK4a}$ (right). Results are from 1 of 3 samples. **B,** RASFs infected with $p16^{INK4a}$ or control adenovirus were stimulated for 24 hours with IL-1β plus TNFα, and MMP-3, MCP-1, macrophage inflammatory protein 3α (MIP-3α), and IL-6 in culture supernatants were measured by enzyme-linked immunosorbent assay. Mean reduction in MMP-3 and MCP-1 induced by $p16^{INK4a}$ was 78% and 91%, respectively, and mean reduction in MIP-3α and IL-6 production induced by $p21^{Cip1}$ was 69% and 67%, respectively, compared with controls. **C,** Adult normal human dermal fibroblasts (NHDF-Ad) and osteoarthritis synovial fibroblasts (OASFs) infected with $p16^{INK4a}$ or control adenovirus were stimulated for 24 hours with IL-1β plus TNFα, and the production of MMP-3 and MCP-1 was determined as in **B. D,** RNA from RASFs infected with $p16^{INK4a}$ or control adenovirus was examined for IL-1 receptor type I (IL-1RI) and GAPDH mRNA expression by Northern blotting. Mean reduction in IL-1RI mRNA expression induced by $p21^{Cip1}$ was 71% compared with controls. **E,** Whole cell extracts from RASFs infected with $p16^{INK4a}$ or $p18^{INK4c}$ adenovirus (Ad) were immunoprecipitated (IP) with anti-JNK antibody (α-JNK) or control IgG and analyzed by Western blotting using antibodies specific for each cyclin-dependent kinase inhibitor: anti-$p16^{INK4a}$ (α-p16), anti-$p18^{INK4c}$ (α-p18), and anti-$p21^{Cip1}$ (α-p21). Results are from 1 of 2 samples. **F,** RASFs infected with $p16^{INK4a}$ or control adenovirus were stimulated for 24 hours with IL-1β plus TNFα, and the DNA binding activities of activator protein 1 (AP-1), NF-κBp50, and NF-κBp65 were determined by colorimetric assay. Values are the mean and SD of 5 wells in **A,** 3 samples in **B** and **D,** 3 experiments in **C,** and triplicate cultures in **F.** * = $P < 0.05$; ** = $P < 0.01$. Data from previous experiments (8) showing the effects of $p21^{Cip1}$ (AxCAp21 adenovirus) on the proliferation of MIP-3α and IL-6 (**B**) and on IL-1RI mRNA expression (**D**) are also shown.

RASFs was 74 ± 37% and 101 ± 32%, respectively (mean ± SD). Thus, the production of these mediators of inflammation was not significantly affected by simple infection with control adenovirus.

Suppression of the release of mediators of inflammation by $p21^{Cip1}$ should be at least partly attributable to a down-regulation of IL-1RI expression (8). However, IL-1RI mRNA expression was not appreciably reduced in RASFs expressing $p16^{INK4a}$ as compared with RASFs infected with control adenovirus (Figure 1D). In addition, we and other investigators (8–10) have shown that $p21^{Cip1}$ associates with JNK to reduce JNK enzymatic activity. We further demonstrated previously that the DNA binding activity of AP-1, which is downstream of the JNK pathway, was reduced in RASFs expressing $p21^{Cip1}$ (8). This prompted us to examine $p16^{INK4a}$ for binding with JNK. Cell lysates of RASFs infected with AxCAp16 or AxCAp21 adenoviruses containing the human $p21^{Cip1}$ gene were immunoprecipitated with anti-$p16^{INK4a}$ or anti-$p21^{Cip1}$ antibody. Subsequent immunoblotting revealed that $p21^{Cip1}$, but not $p16^{INK4a}$, was associated with JNK (Figure 1E). Unlike $p21^{Cip1}$, $p16^{INK4a}$ did not appreciably inhibit AP-1 or NF-κB DNA binding activities in RASFs stimulated with inflammatory cytokines (Figure 1F). These data show that $p16^{INK4a}$ does not depend upon the suppression of JNK pathways or the down-regulation of IL-1RI expression for inhibition of the production of mediators of inflammation.

Suppression of RASF expression of MMP-3 and MCP-1 by $p18^{INK4c}$. The molecule $p18^{INK4c}$ is another member of the INK4 family of CDKIs that specifically inhibits CDK-4/6. Like $p16^{INK4a}$, this molecule did not bind to JNK (Figure 1D). It was not expressed by cultured RASFs, which were subsequently infected with Ad-RGD-p18 adenovirus containing a human $p18^{INK4c}$ gene or with control adenovirus. RASFs that expressed $p18^{INK4c}$ incorporated less ^{3}H-labeled thymidine than did the controls (Figure 2A). Flow cytometric analyses revealed that cell cycle progression was inhibited at the G_0/G_1 phase in $p18^{INK4c}$-expressing RASFs (Figure 2A). ELISA of the culture supernatants showed that $p18^{INK4c}$ gene transfer down-regulated MMP-3 and MCP-1 production by RASFs (Figure 2B). These results show that $p16^{INK4a}$ and $p18^{INK4c}$ had the same effect and suggest that inhibition of CDK-4/6 activity should account for the suppression.

Suppression of RASF production of mediators of inflammation by a small-molecule CDK-4/6 inhibitor. A common function of $p16^{INK4a}$, $p18^{INK4c}$, and $p21^{Cip1}$ is to interact with cyclin D–CDK-4/6 complexes to suppress

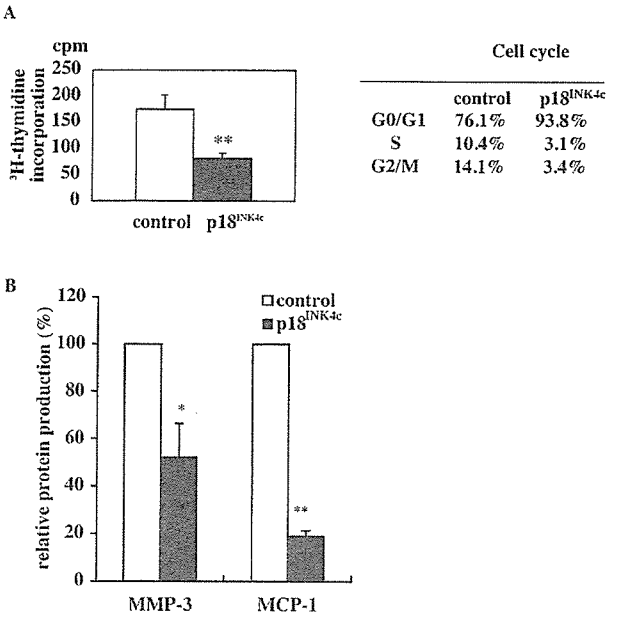

Figure 2. Suppression of RASF production of MMP-3 and MCP-1 by $p18^{INK4c}$. **A**, RASFs infected with $p18^{INK4c}$ or control adenovirus were stimulated with IL-1β plus TNFα, and ^{3}H-thymidine incorporation was assessed (left). Mean reduction in ^{3}H-thymidine incorporation induced by $p18^{INK4c}$ was 43% compared with controls. Flow cytometry showed an increase in cells at G_0/G_1 phase in RASFs expressing $p18^{INK4c}$ (right). Results are from 1 of 3 samples. **B**, RASFs infected with $p18^{INK4c}$ or control adenovirus were stimulated with IL-1β plus TNFα, and MMP-3 and MCP-1 in culture supernatants were measured by enzyme-linked immunosorbent assay. Mean reduction in MMP-3 and MCP-1 production induced by $p18^{INK4c}$ was 48% and 71%, respectively, compared with controls. Values are the mean and SD of 5 wells in **A** and 3 samples in **B**. * = $P < 0.05$; ** = $P < 0.01$. See Figure 1 for definitions.

CDK-4/6 activity. To examine whether inhibition of cyclin D–CDK-4/6 activity per se suppresses the production of mediators of inflammation by RASFs, we used CDK4I, a synthetic compound that specifically inhibits CDK-4/6. CDK4I inhibited ^{3}H-thymidine incorporation by cytokine-stimulated RASFs in a dose-dependent manner. Even at the highest concentration examined, the number of cells in the G_0/G_1 phase of the cell cycle was increased without losing cell viability (Figure 3A). The amounts of MMP-3 and MCP-1 protein produced by RASFs treated with CDK4I were significantly less than those produced by controls (Figure 3B).

Up-regulation of cell proliferation and RASF expression of MMP-3 by augmented CDK-4 activity. We next augmented the activity of CDK-4/6 to study its effects on the production of mediators of inflammation.

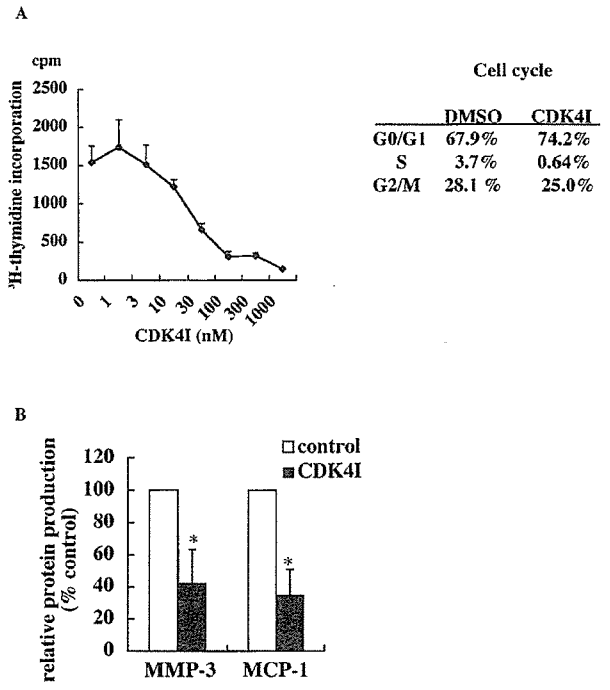

Figure 3. Suppression of RASF production of MMP-3 and MCP-1 by CDK4I, a small-molecule cyclin-dependent kinase 4/6 inhibitor. **A,** RASFs were treated for 12 hours with the indicated concentrations of CDK4I, stimulated with IL-1β plus TNFα, and ^{3}H-labeled thymidine incorporation was assessed (left). Flow cytometry showed an increase in cells at G_0/G_1 phase in RASFs treated with 1 μM CDK4I compared with control (DMSO) (right). Results are from 1 of 2 independent experiments. **B,** RASFs were treated with 1 μM CDK4I or 0.5% DMSO (controls), stimulated with IL-1β and TNFα, and MMP-3 and MCP-1 in culture supernatants were measured by enzyme-linked immunosorbent assay. Mean reduction in MMP-3 and MCP-1 production induced by CDK4I was 57% and 64%, respectively, compared with controls. Values in **A** and **B** are the mean and SD of 3 samples. * = $P < 0.05$. See Figure 1 for definitions.

Although CDK-4/6 activity in normal cells is controlled by the amount of cyclin D, gene transfer of cyclin D1 alone did not accelerate cell cycle progression (17). To promote the function of cyclin D that binds to intranuclear CDK-4, products of the cyclin D1 transgene were directed to nuclei by adding a minigene that encodes a nuclear localization signal (16). Cotransfer of the cyclin D1–NLS gene construct and the CDK-4 gene into RASFs by adenoviruses resulted in phosphorylation (i.e., inactivation) of RB. Cotransfer also up-regulated ^{3}H-thymidine incorporation into cultured RASFs and decreased the number of cells in the G_0/G_1 phase (Figure 4A). Because of limited titers of the prepared adenoviruses, the culture supernatants were subjected to ELISA only for MMP-3. When RASFs overexpressing cyclin D1–NLS and CDK-4 were stimulated, they produced more MMP-3 than did stimulated RASFs infected with the control virus (Figure 4B). Thus, the level of MMP-3 expression correlated directly with the activity of CDK-4.

Regulation of MMP-3 production, but not MCP-1 production, by CDK activity at the mRNA level. Levels of mRNA for MMP-3 and MCP-1 in RASFs were studied to discern underlying molecular events. Real-time PCR analysis showed that the MMP-3 mRNA level was reduced in $p16^{INK4a}$-expressing RASFs, whereas no significant change was observed in the MCP-1 mRNA level (Figure 4C). These results were confirmed by Northern blot analysis, which revealed significant reduction of MMP-3 mRNA levels, but not MCP-1 mRNA levels, in the $p16^{INK4a}$-expressing RASFs (Figure 4D). Treatment of RASFs with CDK4I also reduced the levels of mRNA for MMP-3, but not MCP-1, in the activated RASFs (Figure 4E). Thus, the levels of mRNA for MCP-1 did not account for the decrease in the amount of secreted protein.

No dependence of transcriptional control of MMP-3 on RB. It has been reported that introduction of active (i.e., unphosphorylated) RB suppresses the production of MMP-1, another tissue-degrading enzyme involved in rheumatoid inflammation, at the posttranscriptional level (27). Because mRNA levels of MMP-3 and MCP-1 were differentially controlled by CDK activity, we next investigated the regulation of these 2 molecules by RB. To manipulate the function of RB, which is the major substrate of CDK-4/6, we used a mutant RB gene that had replacement mutations at some of the phosphorylation sites. Adenoviral introduction of this gene increased the active, unphosphorylated form of RB, thus mimicking the suppression of the CDK-dependent phosphorylation of RB (18). We found that when RASFs overexpressed the active RB, they incorporated less ^{3}H-thymidine as compared with control RASFs (Figure 5A). Flow cytometric analysis of the cell cycle showed that the active RB stopped their cell cycle at the G_0/G_1 phase (Figure 5A).

ELISA analyses of the culture supernatants showed that the production of both MMP-3 and MCP-1 was reduced in RASFs expressing the active RB (Figure 5B). Real-time PCR analysis revealed that MMP-3 and MCP-1 mRNA expression in RASFs overexpressing the active RB was preserved in comparison with the control RASFs (Figure 5C).

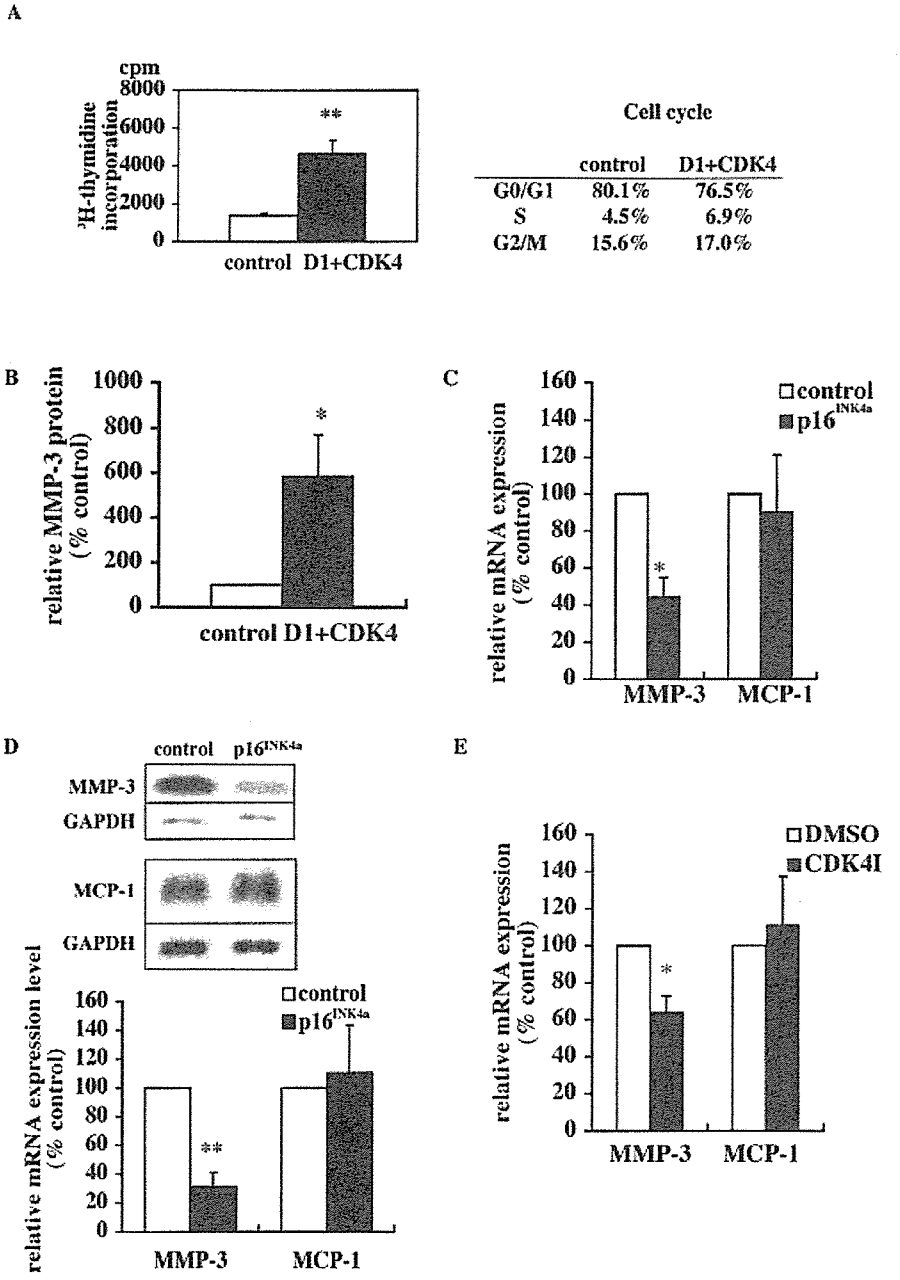

Figure 4. Effects of the combination of cyclin D1–nuclear localization signal (NLS) and cyclin-dependent kinase 4 (CDK-4) gene transfer on RASFs. **A,** RASFs were infected with cyclin D1–NLS plus CDK-4 (D1+CDK-4) or control adenovirus, and ^{3}H-thymidine incorporation was assessed 60 hours later (left). Mean increase in RASFs was 240% compared with controls. Flow cytometry showed a decrease in cells at G_0/G_1 phase in RASFs expressing cyclin D1–NLS plus CDK-4 compared with control (right). Results are from 1 of 2 independent samples. **B,** RASFs infected with cyclin D1–NLS plus CDK-4 or control adenovirus and MMP-3 in culture supernatants was measured by enzyme-linked immunosorbent assay. Mean increase in RASFs was 490% compared with controls. **C–E,** RASFs infected with $p16^{INK4a}$ or control adenovirus (**C** and **D**) or treated with 1 μM CDK4I or DMSO (control) (**E**) were stimulated with IL-1β plus TNFα, and MMP-3 and MCP-1 mRNA were analyzed by real-time polymerase chain reaction (**C** and **E**) or Northern blotting (**D**). Northern blotting results are from 1 of 3 samples. Levels of mRNA were standardized against GAPDH mRNA. Mean reduction in MMP-3 production induced by $p16^{INK4a}$ and by CDK4I was 56% (**C**), 70% (**D**), and 36% (**E**) compared with controls. Values are the mean and SD of 5 wells in **A** and 3 samples in **B–E**. * = $P < 0.05$; ** = $P < 0.01$. See Figure 1 for other definitions.

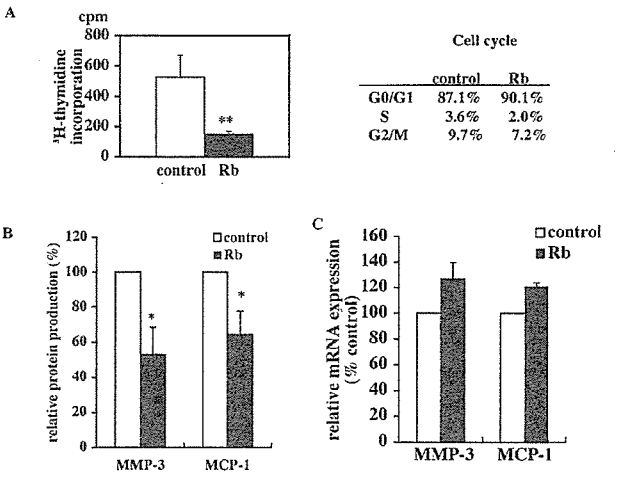

Figure 5. Suppression of RASF expression of MMP-3 and MCP-1 by constitutively active retinoblastoma (RB). **A,** RASFs were infected with nonphosphorylatable RB or control adenovirus, stimulated with IL-1β plus TNFα, and ^{3}H-thymidine incorporation was assessed (left). Results are from 1 of 2 samples. Flow cytometry showed an increase in cells at G_0/G_1 phase in RASFs expressing the constitutively active RB (right). Results are from 1 of 2 independent samples. **B,** RASFs infected with the constitutively active RB or control adenovirus were stimulated for 24 hours with IL-1β plus TNFα, and MMP-3 and MCP-1 in culture supernatants were measured by enzyme-linked immunosorbent assay. Mean reduction in MMP-3 and MCP-1 production induced by RB was 48% and 36%, respectively, compared with controls. **C,** RASFs infected with RB or control adenovirus were stimulated with IL-1β plus TNFα, and mRNA for MMP-3 (**C**) and MCP-1 (**D**) was analyzed by real-time polymerase chain reaction. The mRNA levels are standardized against those of GAPDH. Values are the mean and SD of 5 wells in **A** and 3 samples in **B** and **C**. * = $P < 0.05$; ** = $P < 0.01$. See Figure 1 for other definitions.

DISCUSSION

The present study revealed that CDK-4/6 activity controls the production of MMP-3 by RASFs in a RB-independent manner. The regulation occurs at the mRNA level. RB, which is a substrate of CDK-4/6, can also regulate the expression of MCP-1 as well as MMP-3 at the posttranscriptional level (Figure 6). These features were not seen in control synovial or dermal fibroblasts that were not derived from an inflammatory milieu. Although the RA patients had been treated with various DMARDs, the reactivity of their fibroblasts was similar. We thus assume that the observed regulation is the result of an aberrant activation of the synovial fibroblasts in the rheumatoid joint rather than an intrinsic character of the RASFs or modification by therapeutic agents. The only functional CDK-4/6 substrate known at present is RB, which regulates the functional availability of E2F transcription factors for cell cycle progression. Our results predict the presence of other associating molecules that modulate the production of MMP-3 mRNA.

Bradley et al (27) reported that the phosphorylation status of RB correlates with the production of MMP-1 and IL-6. They showed that unphosphorylated RB suppresses expression of MMP-1 at the posttranscriptional level, and they suggested that this suppression is mediated by inhibition of p38 kinase. We found that a similar regulation is operative during the translation of MMP-3 and MCP-1. However, RB-independent control seems to dominate the regulation of MMP-3 production because it works at the mRNA level. Also, it was noted that CDK-inhibiting molecules suppressed MMP-3 production no less effectively than did the active RB (Figures 3B and 5B).

We have previously reported that $p21^{Cip1}$ could regulate mediators of inflammation in a CDK- independent manner (8). In the present study, we show that both CDK and its substrate RB can regulate them independently. Thus, cell cycle proteins are closely associated with the expression of inflammatory molecules through multiple pathways. It might be interesting to speculate

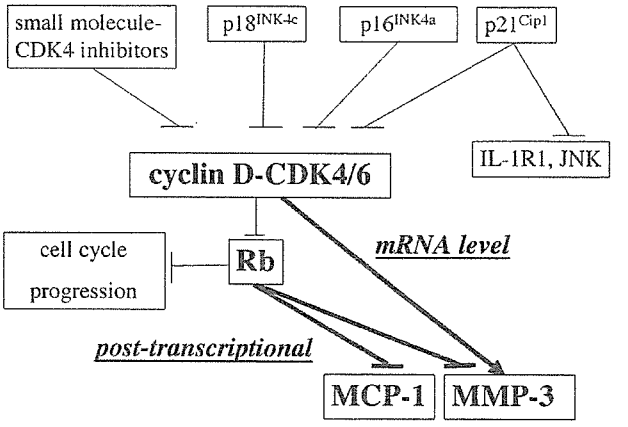

Figure 6. Multiple pathways of regulation of rheumatoid arthritis synovial fibroblast (RASF) production of mediators of inflammation by proteins of the cyclin-dependent kinase (CDK)–retinoblastoma (RB) axis. Inhibition of cyclin D–CDK-4/6 activity by p16^{INK4a}, p18^{INK4c}, or small-molecule CDK-4 inhibitors suppresses the production of matrix metalloproteinase 3 (MMP-3) and monocyte chemoattractant protein 1 (MCP-1) by RASFs. Inhibition of CDK-4/6 suppresses MMP-3 mRNA, but not MCP-1 mRNA. Active RB reduces the expression of MMP-3 and MCP-1 by posttranscriptional regulation. We have also previously found that $p21^{Cip1}$ can exert antiinflammatory effects outside the CDK–RB axis (8). IL-1RI = type I interleukin-1 receptor.

that unknown evolutional selections have imposed secure control of inflammation by cell cycle regulators.

MMP-3 degrades proteoglycans, gelatins, fibronectins, and collagens. Since it also activates other MMPs, it is the master proteinase in the cascade of tissue-degrading enzymes in the rheumatoid joint (28–30). Moreover, MMP-3 was found to be essential for joint destruction in an animal model of RA (31). In other models of RA, administration of MMP inhibitors that suppress the proteinases that are activated by MMP-3 prevented joint destruction (32–34). MCP-1 evokes both the migration and activation of lymphocytes and macrophages in RA synovial tissues (35). Administration of an MCP-1 antagonist was shown to be an effective treatment in an animal model of RA (36). Thus, mediators of inflammation that are down-regulated by the inhibition of CDK-4/6 play important roles in the inflammation that occurs in RA. Nevertheless, $p16^{INK4a}$ did not completely abrogate the production of MMP-3 and MCP-1. IL-1β– and TNFα-stimulated RASFs expressing $p16^{INK4a}$ produced more mediators of inflammation than did unstimulated RASFs. We assume that the antiinflammatory effects of CDK-4/6 inhibition might assist the antiproliferative, therapeutic effects of CDKI in CDKI gene therapy.

In HeLa cells, $p16^{INK4a}$ interacts with NF-κB to inhibit its transcriptional activity (37). Our preliminary studies suggest that $p16^{INK4a}$ in RASFs can regulate the expression of other cytokines in a CDK-4/6–independent manner. However, we found that overexpression of $p16^{INK4a}$ in RASFs did not suppress AP-1 and NF-κB DNA binding activities. Since the Ets family transcription factors Ets-1 and Ets-2 up-regulate the expression of MMP-3, their suppression might account for the down-regulation of MMP-3 expression (38,39).

We showed that $p16^{INK4a}$, $p18^{INK4c}$, and $p21^{Cip1}$ gene transfer into RASFs can suppress their production of mediators of inflammation and proteinases via inhibition of CDK-4/6 activity. A small-molecule CDKI compound also down-regulated the expression of these molecules. For clinical application, modulation of cyclin–CDK activity by small-molecule CDK inhibitors is more feasible than CDKI gene transfer. Many small-molecule CDK inhibitors have already been developed and tested as oncostatics in clinical trials (40), and they might prove useful in the treatment of RA. However, since inhibition of the inflammatory molecules could also be independent of RB, each inhibitor may have a unique balance of the RB-dependent antiproliferative and RB-independent antiinflammatory effects. Thus, in the treatment of RA patients with CDK inhibitors, the two effects need to be balanced.

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