2. 再生医療材料の安全性と臨床研究への応用

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再生医療を臨床応用，普及するためには，使用する細胞製剤の品質および安全性の確保が重要である．欧米諸国とくらべた場合，日本では，実用化に至った再生医療製品の数は圧倒的に数が少ない．本稿では，再生医療の応用化に際して，その製品化に向けた規制の国際状況，ガイドラインや安全性評価の現状比較を行い，また，国内における最近の動向に関して概説する．

はじめに

わが国における世界をリードする基礎研究の成果を十分に活用することにより，再生医療の早期実用化・産業化を達成することが大いに望まれている．本分野における日本の独自技術は水準が高いため，医療製造分野としては，従来の輸入過多から輸出型産業へ転換する初めての領域となることが期待されている．ゆえに，グローバルな産業化を目指した研究戦略も視野に入れることで，日本の経済再生の新たな原動力となることが予測される．しかしながら，再生医療の臨床応用に関しては，海外と比較したとき，現状では決して進んでいるとは言えない．再生医療材料の医薬品，生物製剤としての規制や安全性の評価という観点からは，特に，細胞や組織を利用するため，感染症伝播の可能性を完全に排除しきれないこと，また移植した細胞自体が腫瘍を形成するリスクがあることなどが懸念事項となっている．

1 再生医療分野における規制，レギュラトリーサイエンスの理解

レギュラトリーサイエンスとは，医薬品，食品，環

[キーワード&略語]
FDA，IND，CMC，EMEA

CAT：committee for advanced therapies（先端治療品委員会）
CBER：center for biologics evaluation and Research（生物製剤評価研究センター）
cGTP：current good tissue practice
CMC：chemistry, manufacturing, and control
EMEA：european medicines agency（欧州医薬品審査庁）
FDA：food and drug administration（米国食品医薬品庁）
ICH：international conference for harmonization（日米EU医薬品規制調和国際会議）
IND：investigational new drug
MCB：master cell bank
NDA：new drug application（新薬承認申請）
WCB：working cell bank

Safety issues of cellular-based regenerative medicine products and clinical development

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境物質など，人体などに影響がある物質の適正かつ安全な使用のために，その基準値，安全性・有効性の評価，対応，上位では行政施策やシステムのあり方について，実験室での研究（ウェット研究）や社会学的研究・疫学研究（ドライ研究），臨床研究を通じて検討していく分野である．ゆえに，行政施策や社会に対してきちんと正確な知見を情報発信していくことも重要である．

さて，レギュラトリーサイエンスというと，和訳直訳すると「規制科学」と訳されることから，規制をしてイノベーションの確度を落としてしまうような印象を与えることもあるが，これはまったくの誤りである．再生医療などに用いられる新規性の高い細胞を医療応用化する場合，その細胞が本当に目的臓器を形成するのか，癌化しないのか，感染症のリスクはどうなっていくのかといった懸念事項をクリアしない限り，規制当局からの承認を受けることはできない．そのため，研究開発の各段階において，同じ時間軸でその評価系も構築し，動物実験や臨床試験データから安全性の情報を取得していく，またその科学的結果を行政・規制のガイドラインへと反映し，承認を迅速化していくという考え方は，国際的にも推進されているところである．わが国においても，レギュラトリーサイエンスの真の重要性を理解し，この領域を産官学ともに推進していかない限りは，せっかく日本発の優れた研究があっても，応用化の出口部分で時間をとられてしまって国際競争に敗北してしまうことになる[1]．

2 日米欧における再生医療の応用化のための規制環境

1）日本

現在，わが国では，再生医療などに使用する細胞製剤，組織製品は医療機器の範疇で規制をうけている．薬事法上の治験として臨床試験を企画する場合，1999年の医薬発第906号「細胞・組織を利用した医療用具又は医薬品の品質及び安全性の確保について」，2000年の医薬発第1314号「ヒト又は動物由来成分を原料として製造される医薬品等の品質及び安全性確保について」といったガイドラインを遵守して，治験計画の届出と審査に先立って厚生労働大臣に確認申請を行い，医薬品医療機器総合機構（PMDA）による審査を受ける必要がある（図1）．一方で，医師法上での臨床研究においても，遺伝子治療や体性幹細胞を用いる臨床研究に際しては，厚生労働省に申請，審査を受け，その実施に当たって大臣からの許可が必要となった．

薬事法上での承認を受けて日本国内で使用する場合には，いうまでもなく，治験として臨床試験を行い，PMDAにて承認申請の審査を受けて，その後厚生労働大臣名で承認を受ける必要がある[1]．

2）米国

米国では，大学など研究機関や製薬企業といった区別なく，ヒトを対象とした臨床試験を計画，実施する場合には，必ず米国食品医薬品庁（FDA）に対してIND申請を実施し，臨床試験実施の認可を受ける必要がある．再生医療製品に関しては，21世紀に入ってからは，FDAのうち生物製剤評価研究センター（CBER）が所管となり，安全性や有効性の評価や行政指導を行うようになっている．臨床試験における細胞製剤の安全性にかかるCMCの水準は，連邦政府の規制集に従う必要がある．

しかしながら，特に再生医療用途や癌ワクチンなどに用いられる細胞製剤に関しては，"Guidance for reviewers : Instructions and template for chemistry, manufacturing, and control (CMC) reviewers of human somatic cell therapy INDs[2]" が発表され，規制側（FDA審査官）および開発者（大学や製薬企業）双方に対しての基本的な安全性評価の考え方について記されている[3]．また，医療や移植に用いられる組織（臍帯血も含む）の感染症対策や追跡対応については，'04年11月にcGTPが公表され，現在は本基準に基づいた規制と行政対応が行われている．

3）欧州

欧州では，'04年5月以降は臨床試験指令が施行されており，再生医療製品を含む医薬品や生物製剤を用いた臨床試験を実施する場合には，EU（欧州連合）に加盟する各国の規制当局に実施計画を届け出し，審査，認可を受ける必要がある．臨床試験の終了後は，欧州の医薬品行政機関である欧州医薬品審査庁（EMEA）において承認申請審査を実施する．'07年11月には先進治療製剤に関する規制（Regulation on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation 726/2001）が新た

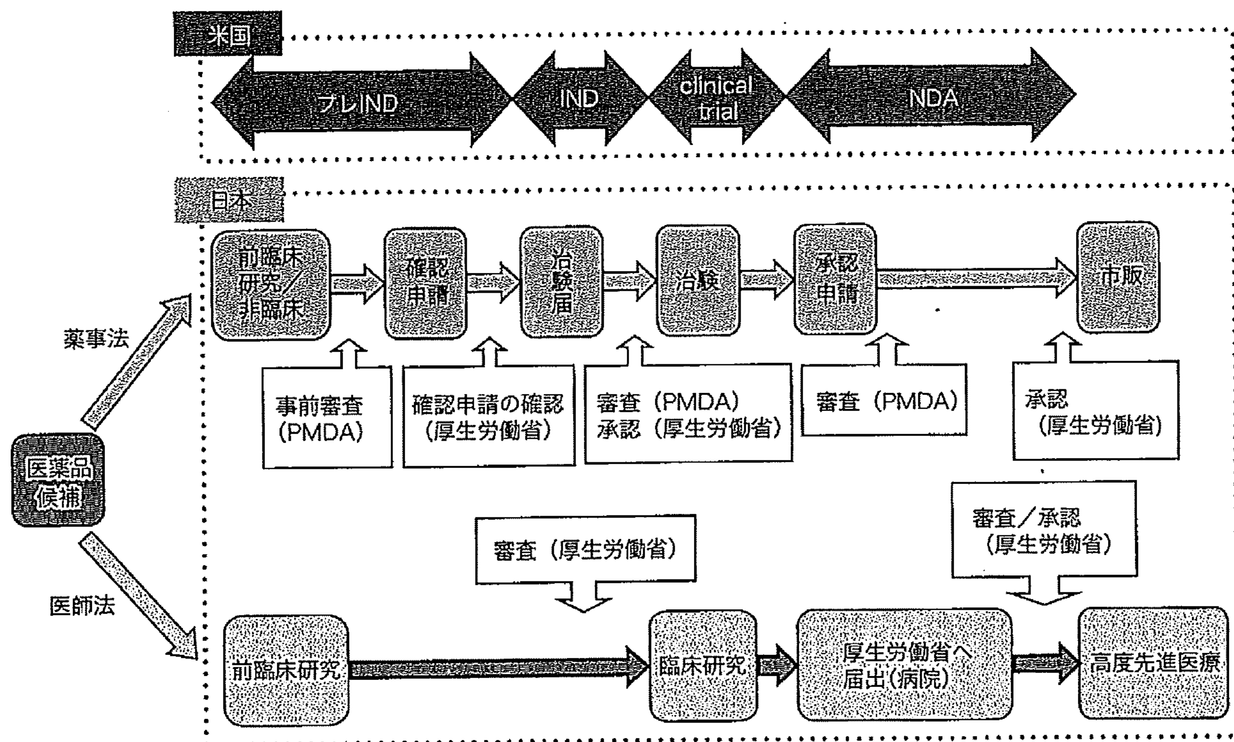

図1　日米における細胞・組織製品の開発・承認の流れ
米国においてはIND制度のなかとFDAにて一元的に管理，審査，承認を受ける．日本においては，治験と臨床研究の2つのトラックがあり，複雑なしくみとなっている〔IND：investigational new drug（治験薬），NDA：new drug application（新薬承認申請），PMDA：pharmaceuticals and medical devices agency（医薬品医療機器総合機構）〕

に発出され，再生医療，細胞治療に関連する各種ガイドラインが整備されている状況である．

先進治療製剤は，遺伝子医薬，細胞製剤，組織工学製品の3つと定義されているが，遺伝子治療薬と細胞治療薬については，従来から通常の医薬品規制の範疇内に分類されてきたが，組織工学製品に関しては，最近明確化された．その他の関連する規制としては，細胞・組織製品の品質及び安全性基準を定めたDirective 2004/23/EC[4]がある．これらのガイドラインをもとに，EMEAに先端治療品委員会（CAT）が新設され，審査にあたっている．

4）日米欧の規制の比較

表1に示すように，日米欧の再生医療の安全性評価や製造にかかるCMC関連ガイドラインに含まれる項目を比較すると，種類や様式は異なっているもののカバーするフレームワークはほぼ同様であることがわかる[5]．

また，実際に実用化の阻害となっている要因を調査するために，国内の再生医療関連企業を対象とした質問票調査を行ったところ，最も多くの回答者が安全性の指針，審査・承認体制の整備を再生医療の早期実用化に対する最も重要な課題点とあげた[6]．

さらに日本における法規制に関する問題点として，治験と臨床研究の並存する制度が指摘され，審査に関しては，審査時間の長さや確認申請のハードルの高さを問題とする企業が多いことが判明した．

3 細胞製剤の癌化可能性についての評価

再生医療に用いられる細胞製剤の癌化は，臨床応用にかかる安全性の最も重要な関心事である．細胞株の培養工程における癌化の危険性の評価，特に免疫不全マウスなどの試験系を用いて細胞が移植された際の腫瘍形成の有無や程度などを検討する，いわゆる造腫瘍性（tumorigenicity）試験については，細胞製品の品質管理上の特性解析の1つの指標として，その実施が求められている．日米欧により構成される日米EU医

表1　日本，米国，欧州における再生医療に関連するガイドラインの記載項目

項目	日本			米国	欧州
	医薬発第1314号	医薬発第906号	ヒト幹細胞臨床研究の指針*	Guidance for CMC reviewers+	Regulation on advanced therapy§
基本原則（適用範囲など）	○	○	○	○	○
原材料に関する項目					
細胞・組織の起源・特性・適格性など	○	○	○	○	
細胞・組織の採取機関・方法・安全対策など	○	○	○※	○	○‡
細胞・組織の保存・出荷・運搬など	○	○	○※		
ドナースクリーニング	○		○	○	○‡
ドナーへのインフォームドコンセント	○		○		
対価について	○		○		○
ドナー・原材料などの記録	○		○		
製造・調整に関する項目					○†
製造・調整方法/工程（ロット構成の有無・妥当性・記録など）	○	○	○※		
培養条件・安定性・血清成分など	○		○※	○	
セルバンク	○	○		○	
分離・加工方法など	○			○	
細胞・組織などの同一性・均一性の評価	○				
遺伝子工学的改変	○	○			
細胞・組織以外の原材料の特性・試験項目など	○	○	○	○	
SOP	○				
品質管理・安全対策に関する項目			○※		○†
システム構築・試験方法など	○	○		○	
試験項目（微生物学的検査・同一性・純度・活性・ウイルス検査・力価など）	○			○	
安定性試験の実施・限界・運搬方法など	○	○		○	
最終製品の出荷規格試験	○			○	
原材料・試薬の受け入れ試験	○			○	
検疫・出荷・配送などの要件	○			○	
製品・調整物および使用段階の安全対策に関する項目					
効果効能の裏付け試験	○	○	○		
体内動態	○	○			
複合製品				○	○
最終製品の回収方法・組成・剤型など				○	○†
患者へのインフォームドコンセント	○		○		
製品の追跡				○	○
容器・表示	○			○	○
前臨床・非臨床について					○†
試験項目（安全性試験など）	○	○			
前臨床or非臨床の総括	○	○	○	○	
その他					
組織・管理体制（職員の教育など）	○			○	
製造施設・設備		○		○	

＊：ヒト幹細胞を用いる臨床研究に関する指針に準ずる

＋：Guidance for reviewers: instructions and template for chemistry, manufacturing, and control（CMC）reviewers of human somatic cell therapy estigational new drug applications（INDs）に準ずる

§：Regulation of the European parliament and of the council: on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation（EC）No 726/2004 に準ずる

※：医薬発第 1314 号に準ずる

‡：ヒト組織・細胞の寄付・採取・試験に関しては "Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004: on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissue." に準ずる

†："Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001: on the Community code relating to medicinal products for human use" に準ずる

薬品規制調和国際会議（ICH）による文書の関連項目を以下に述べる．

1）米国FDAにおける考え方

米国FDA-CBERの考え方を示すものとして，'93年に生物製剤全般を対象としたPTC "Points to consider in the characterization of cell lines used to produce biologicals[7)]"（以下1993 draft PTC）がドラフト版として発行されている．その後，'06年には感染症の予防と治療に用いられるウイルスワクチンを対象としたドラフトガイダンスとして，"Guidance for industry : characterization and qualification of cell substrates and other biological starting materials used in the production of viral vaccines for the prevention and treatment of infectious diseases"が公布されている．1993 draft PTCによれば，米国FDAでは，適当な動物試験系として，ヌードマウスを含む3種の試験系を，また，*in vitro*試験としてアガロースにおけるコロニー形成と培養増殖をあげている．特に，体細胞治療については，「特別な場合において造腫瘍性試験が必要であるかもしれない」との記述があり，すべての細胞製剤について造腫瘍性試験の実施を求めているわけではない．

2）欧州EMEAにおける考え方

欧州EMEAにおいては，'07年1月に新たに発行された細胞医薬品に関するドラフトガイドライン "draft guideline on human cell-based medicinal products[8)]"（EMEA/CHMP/410869/2006）（以下，2007 CBMP draft guideline）が存在する．本ガイドラインにおいて，細胞培養やセルバンクシステムからの細胞医薬については，造腫瘍性試験が必要であるかもしれないと述べられている．本文書中では具体的な方法については述べられていないが，ICH Q5Dガイドラインが参照されている．

3）ICHにおける考え方

ICHにおける関連ガイドラインとして，生物製剤の製造に用いられる細胞基材の調製，ならびにセルバンクの調整，特性解析に関する勧告を示した，Q5Dガイドライン "Derivation and characterisation of cell substrates used for production of biotechnological/biological products"（'97年）がある．ここでは，精製操作をほとんど行わない医薬品の製造に用いられる新規細胞基材について，造腫瘍性試験の有用性はケースバイケースで評価すべきであると述べられている．

造腫瘍性試験の方法については，生物製剤の製造用として動物細胞の利用に関して述べたWHO文書 "WHO requirements for the use of animal cells as *in vitro* substrates for the production of biologicals"（in WHO Expert Committee on Biological standardization 47th Report, WHO technical Report Series No. 878, 1998）が参考とされている．

4 ウイルス否定試験について

ヒト個体にはさまざまな感染因子が潜伏または持続感染しているため，生体から採取した再生医療材料にもともと病原体が存在する可能性がある．さらに，再生医療において，細胞の調製には*in vitro*での培養工程を伴うことから，培養工程中に外来ウイルスが混入する危険性も十分に考慮しておく必要がある．したがって，製造に用いる細胞剤材料については，原材料のスクリーニングとともに，培養工程中の品質検査において病原性ウイルス試験の陽性結果が出た場合は，それが原材料に起因するものなのか，あるいは培養工程での外部からのウイルス混入によるものなのか，培養工程での感染因子の動態，原因を十分に把握し，それに応じた対策を立てることが必要となる．

海外において細胞医薬の培養工程で求められているウイルス否定試験の要求種および方法を以下に示す．

1）米国FDAにおける考え方

米国FDAからは数種の関連ガイドラインが発出されているが，なかでも'03年8月，FDA担当官が体細胞医薬品のIND申請を審査する際の指針として作成された "draft guidance for Reviewer : Instructions and template for chemistry, manufacturing and control (CMC) reviewers of human somatic cell therapy investigational new drug application (IND)" が存在する．本文書は，生物製剤全般を対象とする1993 draft PTCを基盤として作成されている．ここでは，MCBの段階で外来性ウイルスの否定試験を行うこと，さらに他家の細胞を用いる場合には，ヒト由来ウイルス（CMV, HIV-1, HIV-2, HTLV-1, HTLV-2, EBV, HBV, HCVなど）の試験を行うことが求めら

れている．外来性ウイルス試験の詳細については，ICH-Q5Aと1993 draft PTCを参照することとされている．

2）欧州EMEAにおける考え方

欧州EMEAにおいては，関連ガイドラインとして，生物製剤の臨床開発前あるいは臨床開発中に必要とされるウイルス安全性試験に関するドラフトガイドライン"Guideline on virus safety evaluation of biotechnological investigational medicinal products"（EMEA/CHMP/BWP/398498/2005-corr：'06年6月）と，'07年1月に発行された2007 CBMP draft guidelineがある．前者では，MCB，WCB，さらに製造条件で規定されている細胞齢の上限まで培養した細胞の各段階でのウイルス試験については，ICH-Q5Aガイドラインに従って実施するとのみ記載されている．一方，後者では，細胞の受入れ検査として，ヒト由来感染性ウイルスに適切な感度をもつ検出系を用いて，細胞種ごとに特有のウイルススクリーニング検査を行うこと，さらにその後の細胞培養工程の適切な段階で外来性ウイルスの否定試験を行うことが記載されている．試験すべき具体的ウイルス種についての記述はない．

3）日米欧ICHにおける考え方

ICHにおける関連ガイドラインとして，生物製剤の承認申請時に必要なウイルス安全性データに関して述べたQ5A（R1）ガイドライン"Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin"（'99年）がある．ここでは，MCBでは内在性および非内在性ウイルスについて，WCBでは外来性ウイルスについて，さらに*in vitro*細胞齢の上限の細胞では内在性ウイルスと外来性ウイルスに関する試験を行うことが推奨されている．試験すべき具体的ウイルス種はあげられていないが，ヒト由来の細胞を用いる場合には，免疫不全や肝炎などの疾患を引き起こす可能性のあるウイルスに関する試験を行うべきとの記述がある．

誌面の都合から記載は割愛するが，細胞医薬品におけるマイコプラズマ否定試験についても国際的に各種ガイドラインなどが発出されている．

5 日本における当該分野の最近の動向

'07年から実施されている新エネルギー・産業技術総合開発機構（NEDO）による基礎研究から臨床研究への橋渡し促進技術開発・レギュラトリーサイエンス支援のための実証研究「再生医療材料の安全性の確立と規格化及び臨床研究への応用」では，京都大学，アルブラスト，三菱化学メディエンス，先端医療振興財団が中心となり，すでに臨床研究に使われている骨髄由来の間葉系細胞，臍帯由来の間葉系細胞を対象とした安全性に関する実証研究を行っている．

間葉系細胞を利用した再生医療は，神経，免疫，血管，筋肉，肝臓，膵臓，軟骨，骨などの広範囲の疾患領域での臨床応用が期待されている．この新規医療に用いられる細胞医薬の安全性の確認のために，ウイルス，マイコプラズマ試験の標準的な試験系を確立し，培養工程中の動態を解析すること，また，染色体及び遺伝子変異と細胞増殖の関係を明らかにすることで，より安全性の高い培養方法の確立を目指している．現在のところ，このような試験を多検体で大規模に実施し，統計学的に意味のある実データを蓄積している．近い将来には，これらのデータから安全性ガイドラインに使用できるような科学的エビデンスを発表することを目標としている．

文献

1）川上浩司：ファルマシア，43：1195-1200, 2007
2）Guidance for FDA Reviewers and Sponsors：Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs) - 4/9/2008：http://www.fda.gov/cber/gdlns/cmcsomcell.htm
3）Kawakami, K. & Puri, R.K.：Dev. Biol., 116：53-59, 2004
4）The tissues and cells directive (2004/23/EC)：http://www.who.int/ethics/en/ETH_EU_Directive_2004_23_EC.pdf
5）Tsubouchi, M. et al.：Health Policy, 88：62-72, 2008
6）Tsubouchi, M. et al.：Regen. Med., 3：497-504, 2008
7）Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals- 7/12/1993：http://www.fda.gov/cber/gdlns/ptccell.pdf
8）Guideline on human cell-based medicinal products (EMEA/CHMP)：http://www.emea.europa.eu/pdfs/human/cpwp/41086906en.pdf

<著者プロフィール>
川上浩司：筑波大学医学専門学群卒，横浜市立大学大学院医学研究科卒（医学博士，医師）．米国連邦政府食品医薬品庁（FDA）生物製剤評価研究センター（CBER）にて細胞遺伝子治療部臨床試験（IND）審査官，研究官を歴任し，臨床試験の審査業務および行政指導に従事．東京大学客員助教授を経て，2006年より現職（京都大学教授）．ほかに慶應義塾大学医学部客員教授などを兼務．
堀部智久：立命館大学理工学部卒，同大学院卒（理学博士）．同大学 総合理工学研究機構 博士研究員後，La Trobe大学生化学部門・Australian Research Council博士研究員を経て現在に至る（京都大学医学研究科・特定助教）．

A Handbook of Statistical Analyses Using R

Second Edition

Brian S. Everitt
and
Torsten Hothorn

Rによる統計解析ハンドブック

第2版

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ナースのための臨床試験入門

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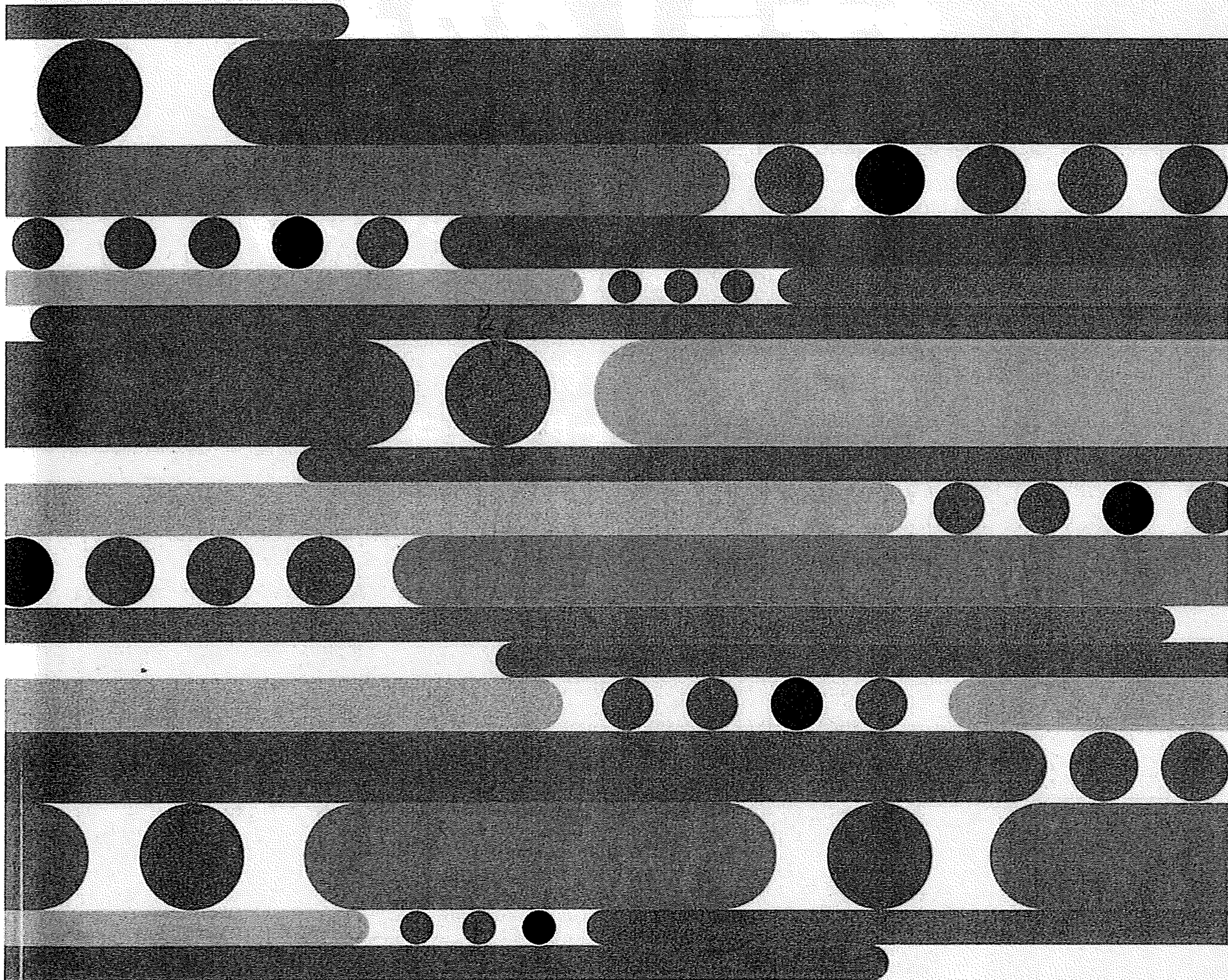

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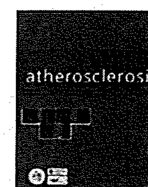

Mulberry leaf ameliorates the expression profile of adipocytokines by inhibiting oxidative stress in white adipose tissue in db/db mice

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ABSTRACT

Previous study showed that mulberry (*Morus Alba* L.) leaf (ML) ameliorates atherosclerosis in apo$E^{-/-}$ mice. Although the adipocytokine dysregulation is an important risk factor for atherosclerotic cardiovascular disease, the effect of ML on metabolic disorders related to adipocytokine dysregulation and inflammation has not been studied. Therefore, we studied the effects of ML in metabolic disorders and examined the mechanisms by which ML ameliorates metabolic disorders in db/db mice. We treated db/db mice with ML, pioglitazone, or both for 12 weeks and found that ML decreased blood glucose and plasma triglyceride. Co-treatment with ML and pioglitazone showed additive effects compared with pioglitazone. Moreover, their co-treatment attenuated the body weight increase observed under the pioglitazone treatment. ML treatment also increased the expression of adiponectin, and decreased the expression of TNF-α, MCP-1, and macrophage markers in white adipose tissue (WAT). Furthermore, ML decreased lipid peroxides and the expression of NADPH oxidase subunits in WAT and liver. Their co-treatment enhanced these effects. Thus, ML ameliorates adipocytokine dysregulation at least in part through inhibiting oxidative stress in WAT of db/db mice, and that ML may be a basis for a pharmaceutical for the treatment of the metabolic syndrome as well as reducing adverse effects of pioglitazone.

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1. Introduction

Recent study showed that white adipose tissue (WAT) produces and secretes a variety of adipocytokines involved in metabolic syndrome [1,2]. Increased production of monocyte chemoattractant protein-1 (MCP-1) from WAT contributes to macrophage infiltration into WAT and causes inflammation [3], while tumor necrosis factor-α (TNF-α) causes insulin resistance [4]. In contrast, adiponectin, which is an adipocyte-specific endocrine protein, exhibits anti-atherogenic and anti-diabetic properties, and its plasma level is decreased in visceral obesity [5,6].

In addition to inflammation, oxidative stress also plays critical roles in the metabolic syndrome [7]. Oxidative stress is shown to be increased in obesity via NADPH oxidase activation [8]. NADPH oxidase is a major source of reactive oxygen species (ROS) in various organs, especially in WAT [8]. NADPH oxidase consists of membrane-associated flavocytochrome b558 family of proteins, which include $gp91^{phox}$ and $p22^{phox}$ as well as cytosolic components $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ [9]. Because macrophages are also known to produce ROS in addition to inflammatory adipocytokines, such as TNF-α [10], infiltrated macrophages might be involved in augmented NADPH oxidase and elevate ROS production in WAT. Furthermore, in adipocytes ROS themselves have been shown to augment expression of NADPH oxidase subunits as well as PU.1, a member of the ETS family of transcription factors required for the development of multiple hematopoietic lineages [8]. Thus, increased oxidative stress in WAT might cause dysregulated production of adipocytokines, which induces macrophage infiltration into WAT, causing more inflammation, and induction of oxidative stress. Furthermore, previous study showed that anti-oxidants such

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as vitamin C, E, and α-lipoic acid ameliorate insulin resistance [11]. Thus anti-oxidant may be a potential agent for the metabolic syndrome.

We have studied the role of mulberry leaf (ML), because it contains various nutritional components, such as flavonoids. Dietary ML also shows hypoglycemic [12] and anti-atherogenic effects [13,14] in certain animal models. Recently, we demonstrated that ML treatment reduced atherosclerotic lesions in apo$E^{-/-}$ mice by inhibiting lipoprotein oxidation [13]. We also showed that ML-derived aqueous fractions (MLAF) inhibit TNF-α-induced nuclear factor κB activation and lectin-like oxidized low-density lipoprotein receptor-1 expression in vascular endothelial cells [15]. However, roles and mechanisms of ML in metabolic disorders and inflammation in WAT have not been investigated.

Therefore, in this study, we examined the effects of ML on the expression profile of adipocytokines and related metabolic disorders in obese diabetic db/db mice, and compared its effect with that of a PPAR-γ agonist, pioglitazone. We also investigated the mechanisms by which ML improves development of metabolic disorders.

2. Materials and methods

2.1. Mulberry leaves

Mulberry trees were cultured in mulberry plantation of Center for Bioresource Field Science, Kyoto Institute of Technology by a standard method in Japan. Mulberry (*Morus Alba* L.) race used was "Shin-Ichinose". Mulberry leaves were harvested in July 2006 and immediately dried by air flush at 180 °C for 7 s. The average diameter of the dried powder used in this experiment was 20 μm.

2.2. Animals and experimental protocol

All animals were obtained from Oriental Bio-Service (Kyoto, Japan) and housed in a temperature-, humidity-, and light-controlled room (14-h light and 10-h dark cycle) and had free access to water and chow. In db/db mice studies, male mice at 9 weeks of age were treated with each diet for 12 weeks (n = 5–6 in each group). Briefly, mice in the ML group were fed with regular chow containing 3% (w/w) ML powder, mice in the Pio group were fed with regular chow containing 0.01% (w/w) pioglitazone (Takeda Pharmaceutical, Osaka, Japan) and mice in the ML+Pio group were fed with regular chow containing both 3% ML powder and 0.01% pioglitazone. Mice at 21 weeks of age were euthanized, blood was collected, and epididymal WAT and liver tissue were dissected out and frozen in liquid nitrogen. Samples were stored at −80 °C until use. All animal experiments were performed according to the guidelines of Kyoto University Animal Research Committee.

2.3. Body fat composition analysis

For computed tomography (CT) analysis of body fat composition, mice were anesthetized and then scanned using a LaTheta (LCT-100 M) experimental animal CT system (Aloka, Tokyo, Japan). Body fat mass was analyzed quantitatively using LaTheta software (version 1.00), as previously described [16].

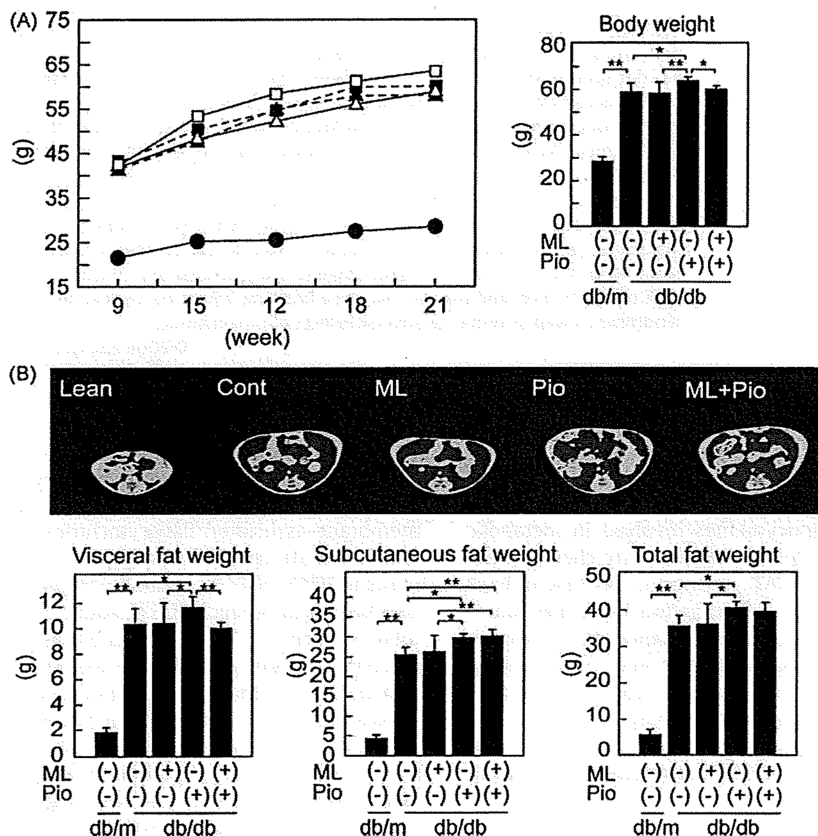

Fig. 1. Effect of mulberry leaf, pioglitazone, or both treatment for 12 weeks on body and fat weight. (A) Growth curve during experiment and body weight at the end of experiment of db/m (Lean) and db/db mice on control (Cont), 3% ML-supplemented (ML), 0.01% pioglitazone-supplemented (Pio), or co-supplemented (ML+Pio) diet for 12 weeks, respectively. Closed circle, Lean (n = 6); open triangle, Cont (n = 5); closed triangle, ML (n = 5); open square, Pio (n = 6); closed square, ML+Pio (n = 6). (B) Representative CT sections of abdominal regions and weight of visceral, subcutaneous, and total fat in db/m and db/db mice on each treatment calculated from CT scan data. Pink areas show visceral fat, while yellow areas show subcutaneous fat. Data are expressed as means ± SD. n = 5 or 6. $^{*}P$ < 0.05; $^{**}P$ < 0.01.

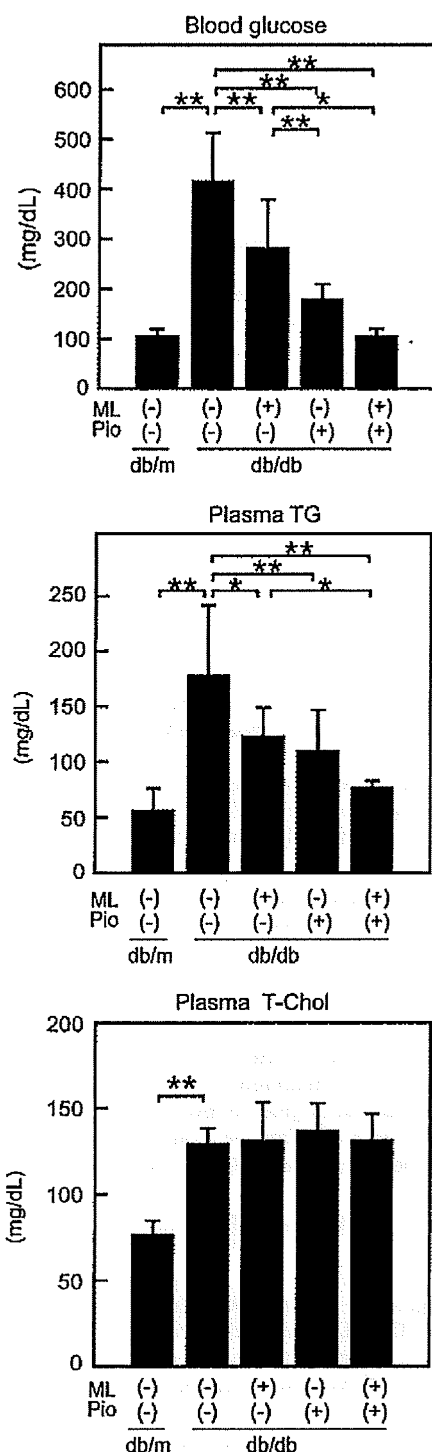

Fig. 2. Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on glucose and lipid metabolism. Blood glucose levels and plasma concentrations of triglyceride and total cholesterol in each group of mice are shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{*}P < 0.05$; $^{**}P < 0.01$.

2.4. Analysis of metabolic parameters

All blood samples were collected after overnight fasting. Blood glucose level and plasma concentrations of triglyceride (TG), total cholesterol (T-Chol) and adiponectin were measured by automatic glucometer (Glutest Ace, Sanwa Chemical, Hiratsuka, Japan), triglyceride E-test Wako, cholesterol E-test Wako (Wako Pure Chemical), and adiponectin ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan), respectively. For intraperitoneal glucose tolerance tests (IPGTT), mice were starved for 16 h and then injected with 1.5 mg/kg body weight of glucose. Blood samples were collected before and after injection, and plasma glucose concentration was measured with an automatic glucometer.

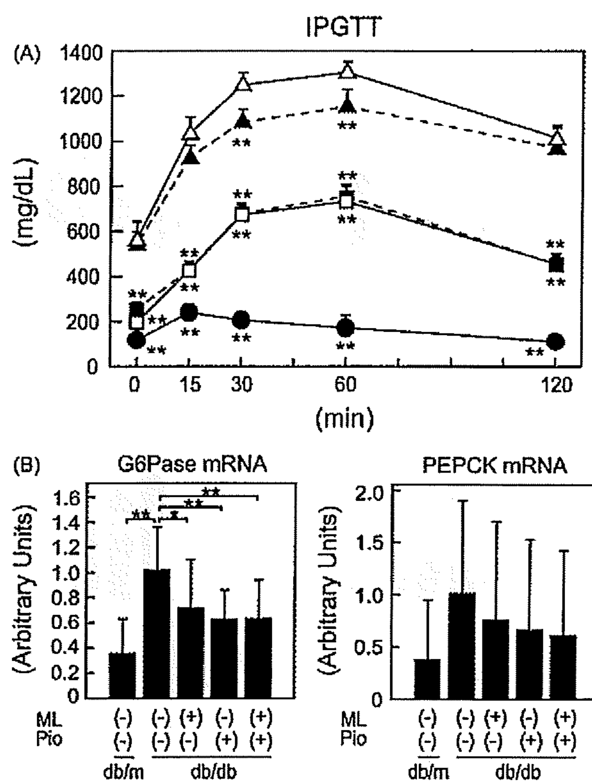

Fig. 3. Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on abnormal glucose tolerance and the expression of genes related to gluconeogenesis. (A) Plasma glucose levels were determined in each group of mice are shown. $n = 5$ or 6. $^{**}P < 0.01$ versus db/db mice on control diet. (B) Expression of G6Pase and PEPCK in the liver of db/m and db/db mice on each diet after 12 weeks is shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{**}P < 0.01$.

2.5. Quantitative real time polymerase chain reaction (PCR)

Total RNA was extracted from WAT and liver tissue using RNeasy lipid tissue kit (Qiagen, Valencia, CA). Real time PCR was performed on an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) using the SYBR GREEN PCR Master Mix (Applied Biosystems). Primer sets used for quantitative real time PCR are shown in Supplementary Table. mRNA levels were normalized relative to the amount of 18S rRNA and expressed in arbitrary units.

2.6. Lipid peroxide concentration

WAT and liver tissue were homogenized in lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Nondient-P40, 0.25% SDS). Tissue suspension was centrifuged at 1600 × *g* for 10 min at 4 °C, and the supernatants were collected and used for assay. The levels of lipid peroxide in tissue homogenate were measured as thiobarbituric acid reactive substance (TBARS) using the TBARS Assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacture's recommendation.

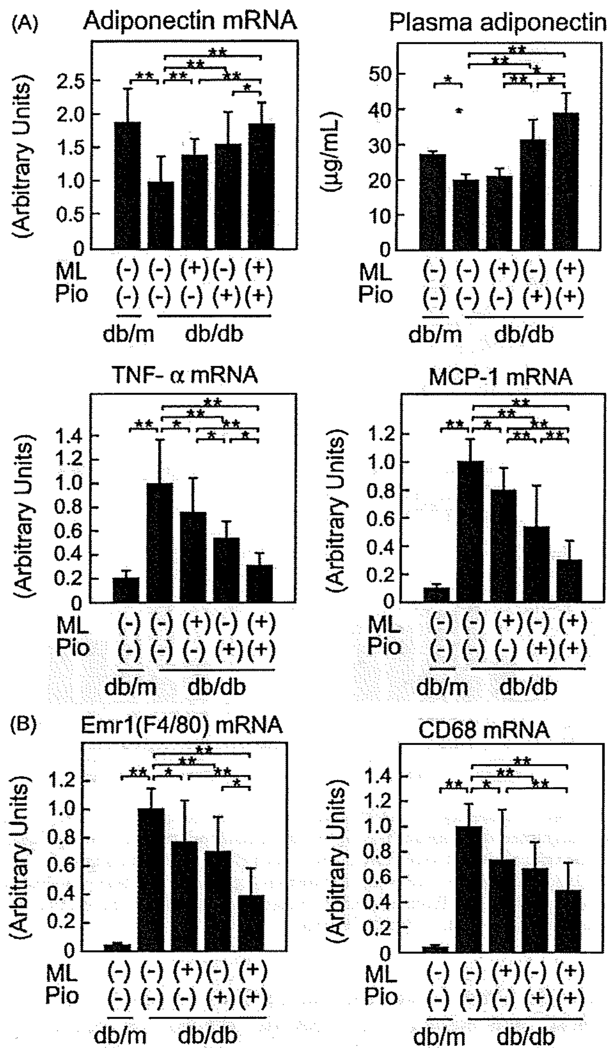

Fig. 4. Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on the expression of adipocytokines and macrophage infiltration in white adipose tissue. (A) Levels of adiponectin in WAT and in plasma in each group of mice are shown. (B) Expression of TNF-α and MCP-1 mRNAs and Emr1 (F4/80) and CD68 mRNAs in WAT in each group of mice are shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{*}P < 0.05$; $^{**}P < 0.01$.

2.7. Hepatic TG content

Hepatic TG content was measured as previously described [17].

2.8. NADPH oxidase activity

Liver tissue was homogenized on ice in ice-cold Tris–sucrose buffer (10 mM Tris (pH 7.6), 340 mM sucrose, 1 mM EDTA, 1 mM PMSF, 0.5% Protease inhibitor cocktail (Sigma–Aldrich)). The tissue suspension was centrifuged at 15,000 × g for 20 min at 4 °C, and the supernatant was collected and used for assay. NADPH oxidase activity was measured as previously described [18]. NADPH oxidase activity was expressed as relative NADPH oxidase activity versus the rate of NADPH consumption of non-treated db/db mice.

2.9. Statistical analysis

The results are expressed as means ± SD. The statistical significances of differences among multiple groups were evaluated using ANOVA and post hoc Fischer's PLSD tests. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Effect of ML, pioglitazone, and their co-treatment on body weight and body fat mass

Db/db mice (9 weeks of age) were treated with ML, pioglitazone, or both for 12 weeks and the changes of body weight were examined. ML did not affect the body weight gain of db/db mice, whereas pioglitazone slightly but significantly increased it by 7% compared with non-treated db/db mice. Their co-treatment significantly attenuated the body weight gain induced by pioglitazone (Fig. 1A). Next, we analyzed the body fat composition by CT scan. As previously shown, pioglitazone significantly increases visceral, subcutaneous, and total fat mass. Interestingly, the addition of ML to pioglitazone inhibited the increase of visceral fat mass induced by pioglitazone, while ML did not affect the visceral, subcutaneous, or total fat mass (Fig. 1B).

3.2. Effect of ML, pioglitazone, and their co-treatment on energy homeostasis and lipolysis

To investigate the effect of ML on energy homeostasis and lipolysis, we next measured the expression of uncoupling protein (UCP)-1, 2, and β3-adorenoceptor (β3AR), which regulate energy expenditure and lipolysis [19] in WAT and liver. However, ML had no effect on the expression of UCP-1, 2 or β3AR in WAT, or UCP-2 in the liver (Supplementary Fig. 1). In addition, co-treatment of ML and pioglitazone did not affect total fat mass.

3.3. Effect of ML, pioglitazone, and their co-treatment on blood glucose, plasma TG and T-Chol

Next, we measured the changes in blood glucose, plasma TG and T-Chol levels. Although all these blood parameters were higher in db/db mice than in db/m mice, ML decreased blood glucose level by 32% and plasma TG level by 30% compared with non-treated db/db mice. Furthermore, co-treatment of ML and pioglitazone showed further 40% reduction in glucose level, and 30% reduction in TG level compared with pioglitazone alone. On the other hand, any treatment did not affect plasma T-Chol levels (Fig. 2).

3.4. Effect of ML, pioglitazone, and their co-treatment on glucose homeostasis

To investigate the effect of ML on glucose homeostasis, we performed IPGTT. ML significantly improved abnormal glucose tolerance, while pioglitazone, or their co-treatment markedly improved it (Fig. 3A). We also measured the expression of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), both of which regulate gluconeogenesis in the liver. Although the expression of G6Pase was significantly higher in db/db mice than in db/m mice, ML, pioglitazone, or their co-treatment significantly decreased the expression of G6Pase by 24, 37, and 31%, respectively. However, any treatment did not affect the expression of PEPCK (Fig. 3B).

3.5. Effect of ML, pioglitazone, and their co-treatment on adipocytokine expression

We next measured the levels of adipocytokines in epididymal WAT and plasma. Adiponectin levels in WAT and in plasma were

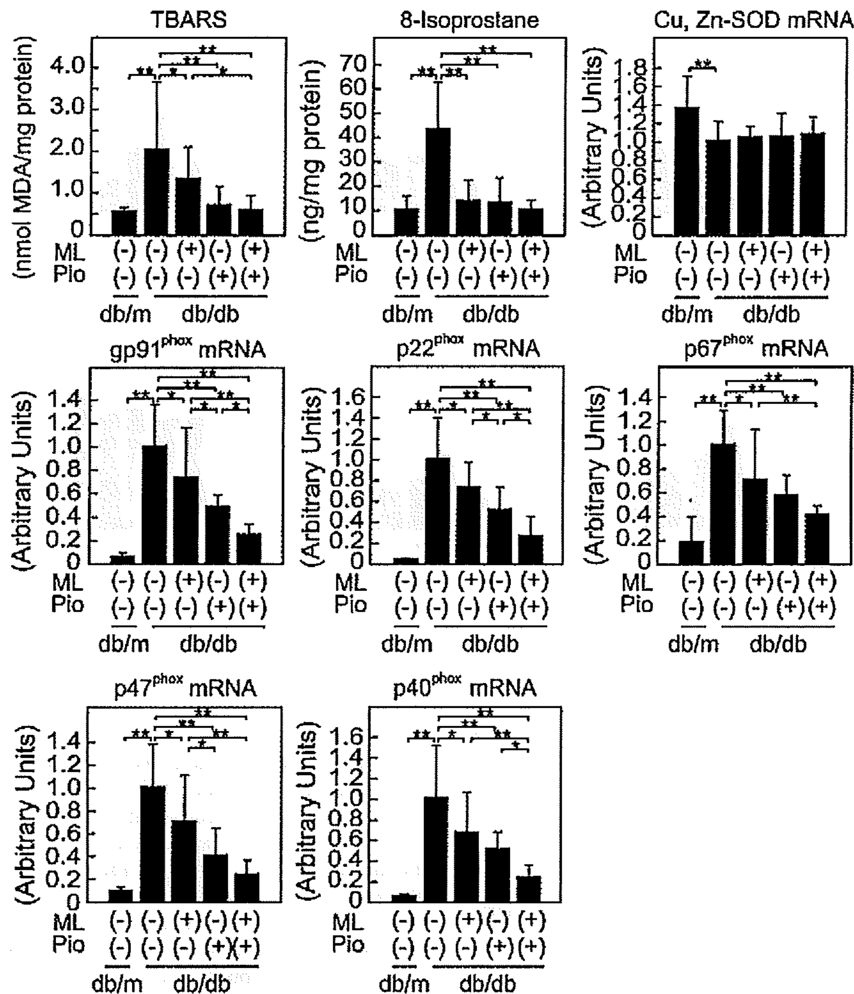

Fig. 5. Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on oxidative stress in white adipose tissue. Levels of TBARS, Cu, Zn-SOD mRNA, and NADPH oxidase subunits, gp91^{phox}, p22^{phox}, p67^{phox}, p47^{phox} and p40^{phox} and PU.1 mRNAs in WAT from each group of mice are shown. Data are expressed as means ± SD. n = 5 or 6. $^{*}P<0.05$; $^{**}P<0.01$.

significantly lower in db/db mice than in db/m mice. ML significantly increased adiponectin levels in WAT by 40% compared with non-treated db/db mice, but not in plasma. Co-treatment further increased adiponectin levels by 17% in WAT and by 25% in plasma compared with pioglitazone alone. In contrast, the expression of inflammatory adipocytokines, such as TNF-α and MCP-1 in WAT was markedly increased in db/db mice than in db/m mice. However, ML decreased the expression of TNF-α and MCP-1 by 25 and 20% in db/db mice, respectively. In addition, co-treatment resulted in a further decrease by approximately 45% compared with pioglitazone alone (Fig. 4A).

We also measured the expression of two macrophage markers, F4/80 antigen, Emr1, and CD68 in WAT. Expression of Emr1 and CD68 was markedly increased in db/db mice than in db/m mice. However, ML significantly decreased the expression of Emr1 and CD68 by 13 and 16% in WAT, respectively. Co-treatment further decreased the expression of Emr1 and CD68 by 46 and 26%, respectively, compared with pioglitazone alone (Fig. 4B).

3.6. *Effect of ML, pioglitazone, and their co-treatment on oxidative stress in WAT and liver*

We next measured adipose TBARS concentrations to investigate the effect of ML on oxidative stress. Although adipose TBARS concentrations were markedly higher in db/db mice than in db/m mice, treatment with ML, pioglitazone, or both significantly decreased them in db/db mice by 43, 62, and 72%, respectively.

We also investigated the effects of ML, pioglitazone, or their co-treatment on gene expression related to the production and removal of ROS in WAT. Expression of genes related to the production of ROS, including all NADPH oxidase subunits and PU.1 was markedly increased in epididymal WAT of db/db mice, but ML significantly decreased them. Further, co-treatment consistently decreased the expression of these genes compared with pioglitazone alone. On the other hand, expression of Cu, Zn-SOD, the ROS-elimination system, was decreased in db/db mice compared with that in db/m mice. However, any treatment did not affect the expression of Cu, Zn-SOD (Fig. 5).

In the liver TG accumulation was higher in db/db mice than in db/m mice. ML significantly decreased hepatic TG content by 44% in db/db mice. TBARS concentrations and NADPH oxidase activity were also higher in db/db mice than in db/m mice. Treatment with ML, pioglitazone, or both markedly decreased hepatic TBARS concentrations by 35, 33, and 59%, respectively, and NADPH oxidase activity by 37, 65, and 74%, respectively in db/db mice. Furthermore, although we could not show a significant effect of each treatment on the expression of NADPH oxidase subunits, gp91^{phox} and p47^{phox}, and Cu, Zn-SOD, tendencies were

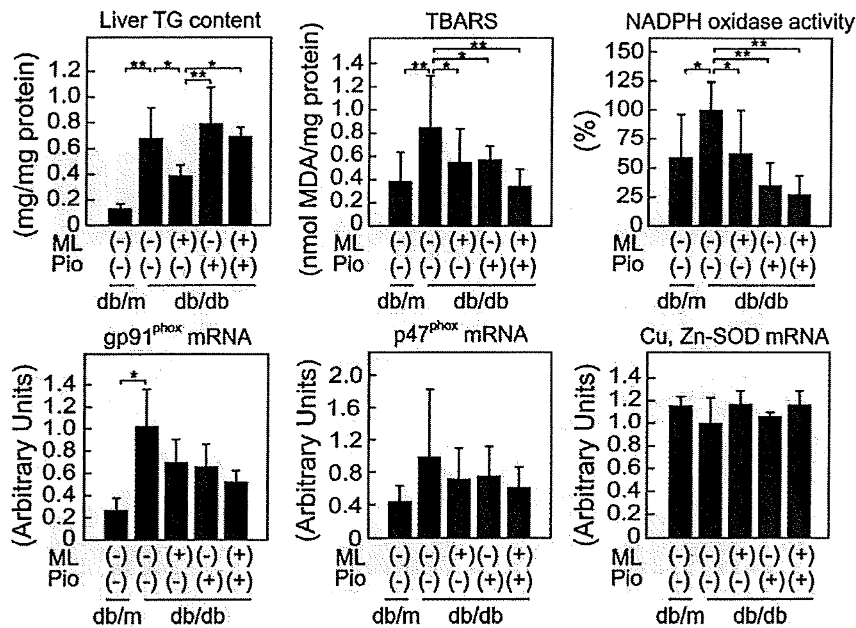

Fig. 6. Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on oxidative stress in the liver. Levels of TBARS, Cu, Zn-SOD mRNA, and NADPH oxidase from each group of mice are shown. Data are expressed as means ± SD. n = 5 or 6. $^{*}P$ < 0.05; $^{**}P$ < 0.01.

observed to show the effect of ML, Pio, and their co-treatment (Fig. 6).

3.7. *Effect of MLAF on glucose metabolism, oxidative stress and macrophage infiltration*

We previously showed that MLAF shows the strongest anti-oxidant effect [13,15]. Therefore, we treated db/db mice with MLAF for 5 weeks. We found that MLAF ameliorated result of IPGTT (Supplementary Fig. 2A) and IP insulin tolerance test (ITT) (Supplementary Fig. 2B). Furthermore, MLAF decreased plasma and urine 8-isoprostane levels (Supplementary Fig. 3A), and TBARS levels in skeletal muscle (Supplementary Fig. 3B), which are markers of lipid peroxides. In addition, MLAF also significantly decreased the ration of F4/80 positive cells in WAT (Supplementary Fig. 4).

4. Discussion

In the present study, we have shown that ML ameliorates metabolic disorders and adipocytokine dysregulation in db/db mice. Intriguingly, these effects of ML are additive to the effects of pioglitazone. We also proposed that these effects are mediated, at least in part through inhibiting oxidative stress in WAT and liver of obese mice.

Our results indicate that ML ameliorates adipocytokine dysregulation and suppresses macrophage infiltration, which are involved in the development of obesity [8]. We also demonstrated that ML, pioglitazone, and their co-treatment attenuated TBARS concentrations and the expression of NADPH oxidase subunits in WAT. The oxidation of fatty acids is an important source of ROS in fatty liver [20]. ML also decreased hepatic TG and TBARS concentrations by inhibiting NADPH oxidase activity through decreased expression of NADPH oxidase subunits and induction of the expression of Cu, Zn-SOD in the liver. These results could indicate that the inhibition of ROS generation via NADPH oxidase in WAT and liver may be one of the mechanisms by which ML can ameliorate metabolic disorders.

In accordance with the effects of ML on adipocytokine dysregulation, ML decreased blood glucose and plasma TG levels as previously described [12]. Previous study demonstrated that ML contains α-glucosidase inhibitor, 1-deoxynojirimycin (1-DNJ) [21]. Therefore, we expected that the effect of ML on glucose metabolism can be partly attributed to the inhibition of glucose absorption from intestine by 1-DNJ. However, as a novel mechanism, we propose that ML ameliorates adipocytokine dysregulation and ROS production through inhibiting oxidative stress in WAT, because we previously showed that MLAF shows the strongest anti-oxidant effect. Furthermore, we also found that MLAF ameliorated result of IPGTT and IPITT. MLAF also decreased plasma and urine 8-isoprostane levels, and TBARS levels in skeletal muscle and macrophage infiltration into WAT. These data may strengthen that ML ameliorates metabolic disorders and inflammation through its anti-oxidative effect in addition to the inhibition of glucose absorption from the gut by 1-DNJ.

We showed that administration of ML in addition to pioglitazone attenuated the body weight gain observed under pioglitazone treatment. Clinical study shows that treatment with thiazolidinediones such as pioglitazone is associated with edema and weight gain [22]. Previous study showed that pioglitazone induces fat mass by increasing the number of small adipocytes in Zucker (*fa*/*fa*) rats by an activation of PPAR-γ [23]. Another study showed that mice treated with pioglitazone experience weight gain from epithelial Na^+ channel (ENaC)-mediated renal salt absorption [24]. In this study, ML attenuated pioglitazone-induced visceral fat mass gain. Therefore, we speculated that ML might attenuate visceral fat gain through promotion of energy consumption by increasing adiponectin. However, ML had no effect on the expression of UCP-1, 2 or β3AR in WAT, or UCP-2 in the liver. In addition, co-treatment of ML and pioglitazone did not affect total fat mass. Thus, the ameliorative effect of ML on body weight gain might depend on another mechanism. Although we did not study the effect of ML on ENaC or urine volume, previous study shows diuretic effects of γ-aminobutyric acid [25], which is abundantly contained in ML [26]. Therefore, although we did not study the brown adipose tissue, which mainly regulates thermogenesis, this effect of ML might be

caused by amelioration of edema through its diuretic action more than promotion of energy expenditure.

The present study clearly demonstrated that ML ameliorates metabolic disorders including diabetes and dyslipidemia, and shows additive effects with pioglitazone. As an expected mechanism, we propose that ML could ameliorate adipocytokine dysregulation at least in part through inhibiting oxidative stress in WAT. In addition, we showed that ML attenuated the body weight gain caused by pioglitazone treatment. Thus, our study implicates that ML may be a basis for a pharmaceutical for the treatment of the metabolic syndrome as well as inducing effects of pioglitazone while reducing its adverse effects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2008.10.021.

References

[1] Spiegelman BM, Flier JS. Obesity and the regulation of energy balance. Cell 2001;104:531–43.
[2] Matsuzawa Y, Funahashi T, Kihara S, et al. Adiponectin and metabolic syndrome. Arterioscler Thromb Vasc Biol 2004;24:29–33.
[3] Kanda H, Tateya S, Tamori Y, et al. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J Clin Invest 2006;116:1494–505.
[4] Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 1993;259:87–91.
[5] Okamoto Y, Kihara S, Ouchi N, et al. Adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice. Circulation 2002;106:2767–70.
[6] Yamauchi T, Kamon J, Minokoshi Y, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 2002;8:1288–95.
[7] Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 2006;440:944–8.
[8] Furukawa S, Fujita T, Shimabukuro M, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 2004;114:1752–61.
[9] Babior BM. NADPH oxidase. Curr Opin Immunol 2004;16:42–7.
[10] Weisberg SP, McCann D, Desai M, et al. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003;112:1796–808.
[11] Evans JL, Goldfine ID. Alpha-lipoic acid: a multifunctional antioxidant that improves insulin sensitivity in patients with type 2 diabetes. Diabetes Technol Ther 2000;2:401–13.
[12] Park MY, Lee KS, Sung MK. Effects of dietary mulberry, Korean red ginseng, and banaba on glucose homeostasis in relation to PPAR-alpha, PPAR-gamma, and LPL mRNA expressions. Life Sci 2005;77:3344–54.
[13] Harauma A, Murayama T, Ikeyama K, et al. Mulberry leaf powder prevents atherosclerosis in apolipoprotein E-deficient mice. Biochem Biophys Res Commun 2007;358:751–6.
[14] Enkhmaa B, Shiwaku K, Katsube T, et al. Mulberry (*Morus alba* L.) leaves and their major flavonol quercetin 3-(6-malonylglucoside) attenuate atherosclerotic lesion development in LDL receptor-deficient mice. J Nutr 2005;135:729–34.
[15] Shibata Y, Kume N, Arai H, et al. Mulberry leaf aqueous fractions inhibit TNF-alpha-induced nuclear factor kappaB (NF-kappaB) activation and lectin-like oxidized LDL receptor-1 (LOX-1) expression in vascular endothelial cells. Atherosclerosis 2007;193:20–7.
[16] Oike Y, Akao M, Yasunaga K, et al. Angiopoietin-related growth factor antagonizes obesity and insulin resistance. Nat Med 2005;11:400–8.
[17] Tomimoto S, Tsujita M, Okazaki M, et al. Effect of probucol in lecithin-cholesterol acyltransferase-deficient mice: inhibition of 2 independent cellular cholesterol-releasing pathways in vivo. Arterioscler Thromb Vasc Biol 2001;21:394–400.
[18] Tsuda M, Iwai M, Li JM, et al. Inhibitory effects of AT1 receptor blocker, olmesartan, and estrogen on atherosclerosis via anti-oxidative stress. Hypertension 2005;45:545–51.
[19] Enerback S, Jacobsson A, Simpson EM, et al. Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. Nature 1997;387:90–4.
[20] Browning JD, Horton JD. Molecular mediators of hepatic steatosis and liver injury. J Clin Invest 2004;114:147–52.
[21] Kong WH, Oh SH, Ahn YR, et al. Antiobesity effects and improvement of insulin sensitivity by 1-deoxynojirimycin in animal models. J Agric Food Chem 2008;56:2613–9.
[22] Fonseca V. Effect of thiazolidinediones on body weight in patients with diabetes mellitus. Am J Med 2003;115(Suppl. 8A):42S–8S.
[23] de Souza CJ, Eckhardt M, Gagen K, et al. Effects of pioglitazone on adipose tissue remodeling within the setting of obesity and insulin resistance. Diabetes 2001;50:1863–71.
[24] Guan Y, Hao C, Cha DR, et al. Thiazolidinediones expand body fluid volume through PPARgamma stimulation of ENaC-mediated renal salt absorption. Nat Med 2005;11:861–6.
[25] Yamakoshi J, Fukuda S, Satoh T, et al. Antihypertensive and natriuretic effects of less-sodium soy sauce containing gamma-aminobutyric acid in spontaneously hypertensive rats. Biosci Biotechnol Biochem 2007;71:165–73.
[26] Kang TH, Oh HR, Jung SM, et al. Enhancement of neuroprotection of mulberry leaves (*Morus alba* L.) prepared by the anaerobic treatment against ischemic damage. Biol Pharm Bull 2006;29:270–4.

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N-Acetylcysteine Reduces the Severity of Atherosclerosis in Apolipoprotein E-Deficient Mice by Reducing Superoxide Production

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Background: Oxidative stress may play an important role in the development of atherosclerosis. Because N-acetylcysteine (NAC) is able to reduce oxidative stress, the present study assessed the hypothesis that NAC may reduce the severity of atherosclerosis in apolipoprotein (apo) E-deficient mice.
Methods and Results: Atherosclerosis was induced in apoE-deficient mice fed a high-fat diet containing 0.3% cholesterol. Mice were injected intraperitoneally with NAC (20 mg·kg^{-1}·day^{-1}) 3 times per week over 8 weeks. Fatty streak plaque developed in the apoE-deficient mice, but not in mice treated with NAC. In addition, NAC reduced superoxide production in the aortic walls, as detected by ethidium staining. NAC treatment did not significantly modify the serum lipid profiles.
Conclusions: In this animal model NAC may suppress atherosclerosis via reducing superoxide production. (*Circ J* 2009; **73:** 1337–1341)

Key Words: Atherosclerosis; Free radicals; N-acetylcysteine; Oxidative stress

Many kind of stresses, especially oxidative stress and free radicals, may be key factors in the development of atherosclerosis.[1,2] In addition, the significance of systemic inflammation in the development of atherosclerosis is now well known.[1,2] For example, angiotensin II is a major mediator of oxidative stress by activating NADH/NAD(P)H oxidase via the type 1 receptor, which results in the production of the superoxide anion.[3,4] Thus, angiotensin II has deleterious effects on vessel walls. Recent reports indicate that N-acetylcysteine (NAC), a potent antioxidant, inhibits inflammatory cardiovascular diseases[5–7] and it has also been reported that NAC inhibits not only inflammatory cytokines, but also free radical production. These results imply that NAC may be an effective agent against inflammatory reactions and oxidative stress in the vessel walls. However, the effects of NAC on atherosclerosis are still unknown, irrespective of the previous reports.[8,9]

Methods

Experimental Atherosclerosis

ApoE-deficient 129ola×C57BL/6 hybrid mice were the generous gift of Dr Edward M. Rubin (University of California, Berkeley, CA, USA). They were mated with C57BL/6 mice to produce F_1 hybrids. The F_1 apo $E^{+/-}$ mice were then backcrossed to C57BL/6 mice for 10 generations. Mice homogeneous for the apoE-null allele on a C57BL/6 background were subsequently generated. Male mice were used in the subsequent experiments. They were kept in a temperature-controlled facility on a 14.10-h light–dark cycle with free access to food and water.

After being weaned at 4 weeks of age, mice were fed a normal chow diet (Oriental Yeast) until 6 weeks of age, when they were switched to a high-fat diet containing 20% fat and 0.3% cholesterol as previously described.[10,11]

The experimental protocols were approved by the Ethics Committee for Animal Experiments of Kyoto University.

Treatment Protocol

At 6 weeks of age, mice given a daily intraperitoneal injection of either saline (control group, n=9) or 20 mg·kg^{-1}·day^{-1} of NAC (NAC group, n=9) 3 times per week on alternate days for 8 weeks. The dosage of the drug was determined from previous reports.[5–7] At 14 weeks the mice were killed by puncture of the ventricle under ether anesthesia. The organs were weighed, and the ratio of heart weight (HW) to body weight (BW) was calculated.

Tissue Processing

Mice were killed by exsanguination after puncturing the ventricle. The aortas were removed 36 h after the last injection of NAC. The vasculature was perfused with sterile phosphate-buffered saline and 6.8% sucrose. The root of the aorta was dissected under a microscope and frozen in OCT embedding medium for serial cryosectioning covering 1.0 mm of the root. The first section was harvested when the first cusp became visible in the lumen of the aorta. Four sections of 6-μm thickness were harvested per slide, and thus 8 slides per mouse were prepared. All sections

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were immersed for 15 s in 60% isopropanol, stained for 30 min in a saturated oil-red-O solution at room temperature, counterstained with hematoxylin, and then mounted under coverslips with glycerol gelatin.[11,12]

In Situ Detection of Superoxide Production

To evaluate in situ superoxide production by the aorta, unfixed frozen cross-sections of the specimens were stained with dihydroethidium (DHE; Molecular Probe, OR, USA) according to a previously validated method.[13–15] In the presence of superoxide, DHE is converted to the fluorescent molecule, ethidium, which can then label nuclei by intercalating with DNA. Briefly, the unfixed frozen tissues were cut into 10-μm sections, and incubated with 10 μmol/L DHE at 37°C for 30 min in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope. Superoxide production was demonstrated by red fluorescence labeling.

For quantification of ethidium fluorescence from the aortas, fluorescence (intensity×area) was measured using a high-power image.

Immunohistochemistry

Anti-macrophage (anti-Mϕ, M 3184, 1:400, PharMigen) and anti intercellular adhesion molecule-1 (ICAM-1) (M-19, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies were applied to acetone-fixed cryosections of the aortic roots. After being washed, the sections were then exposed to the second antibody (horseradish peroxidase-conjugated antibody), and antibody binding was visualized with diaminobenzidine. Sections were counterstained with methyl green or Mayer's hematoxylin.

The positive-staining macrophages were counted in several fields at ×400 magnification (within a 1-mm² grid), and the percentage of positive-staining cells/total infiltrating cells was calculated.

Western Blotting

Protein expression of ICAM-1 was examined as described previously.[8] Protein samples were probed with the anti-ICAM-1 antibody. The β-actin samples were probed in the blots as internal controls for loading. Resulting bands were quantified as optical density×band area by the image analysis system.

Lipid Measurement

Serum was separated by centrifugation and stored at –80°C. Serum total cholesterol and triglyceride levels were measured.

Statistical Analysis

Values are expressed as means±SD. Student's t-test was performed, and $P<0.05$ was considered statistically significant.

Table 1. Organ Weights

	n	BW (g)	HW (g)	HW/BW (mg/g)
Control	9	25.3±4.1	0.15±0.03	6.09±1.02
NAC	9	24.0±2.4	0.15±0.02	6.06±0.46

Mean±SD.
BW, body weight; HW, heart weight; NAC, N-acetylcysteine.

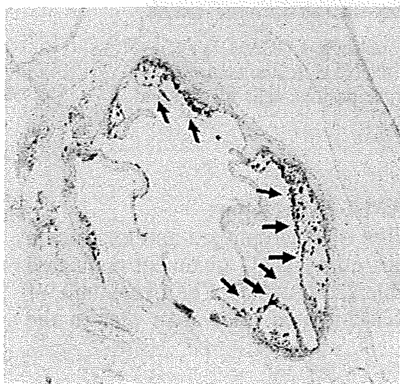
Results

Effects of NAC on Organ Weights

HW, BW and the HW/BW ratio were not significantly different between the NAC group and the control group (**Table 1**).

Effects of NAC on Atherosclerotic Lesions

ApoE-deficient mice were kept on a cholesterol-rich diet for 8 weeks to induce fatty streak formation. The surface



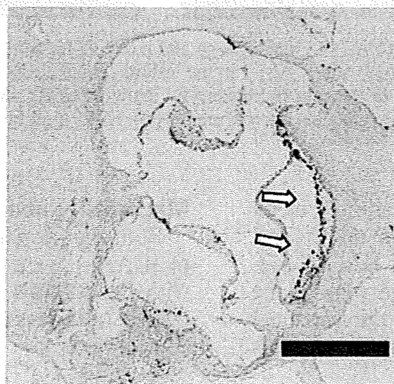


Figure 1. Effects of N-acetylcysteine (NAC) treatment on atherosclerotic lesions in the NAC-treated mouse (**B**, white arrows), which were smaller and covered less of the inner circumference of the aortic root than those (black arrows) in the control mouse (A). Bar=500 μm. Oil-red-O stain.

Table 2. Lesion Area

	n	Lesion area, μm² (%)	Mϕ-positive cells (%)	Ethidium fluorescence (units)
Control	9	65.91±20.60×10³ (8.45±2.64)	14.3±4.2 (n=5)	1.00±0.12 (n=5)
NAC	9	35.49±19.97×10³ (4.55±2.56)*	4.5±4.0* (n=5)	0.84±0.04* (n=5)

Mean±SD.
*P<0.05 vs Control group.
Mϕ, macrophage. Other abbreviation see in Table 1.

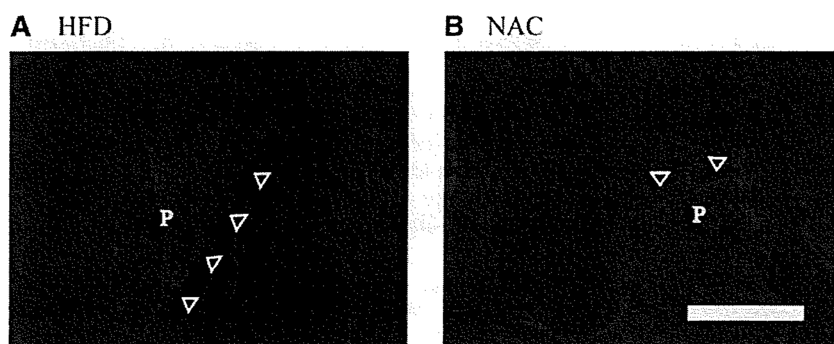


Figure 2. Effects of N-acetylcysteine (NAC) on superoxide production. The degree of ethidium fluorescence (arrowheads) in the NAC group is less than that in the control group, suggesting less superoxide production after NAC treatment. HFD, high-fat diet; P, plaque. Bar=50 μm (dihydroethidium stain; ×100).

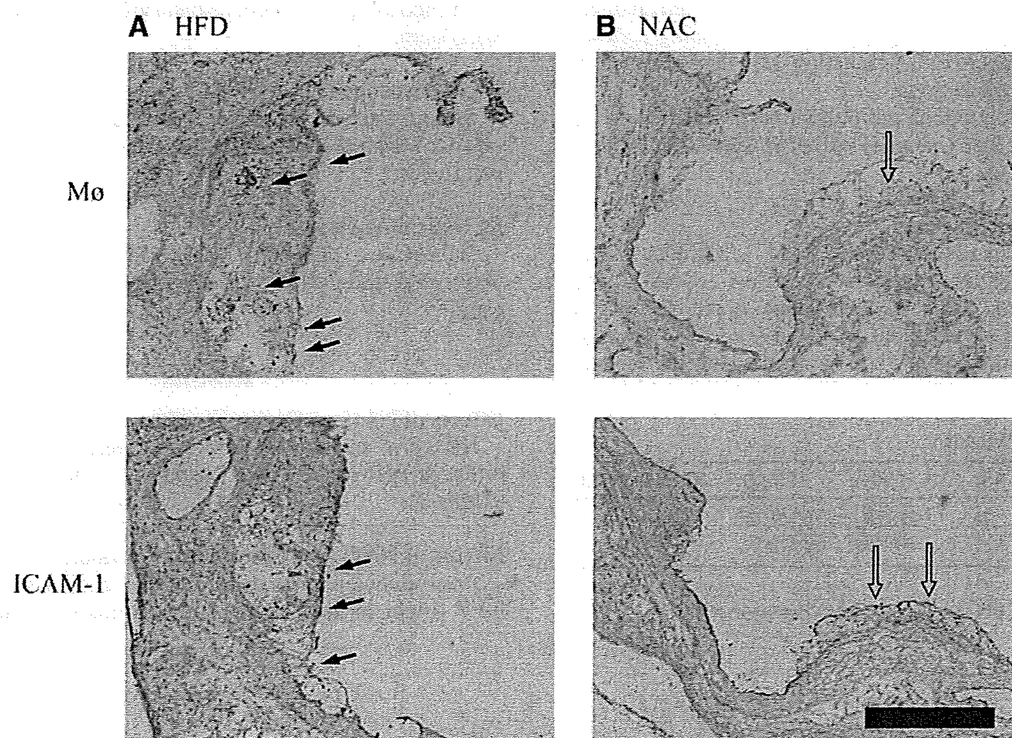


Figure 3. Effects of N-acetylcysteine (NAC) on macrophage and intercellular adhesion molecule-1 (ICAM-1) expressions. The expression of macrophages (Mϕ) and ICAM-1 in the lesions (white arrows) of the NAC-treated mouse (B) is less compared with that (black arrows) of the control mouse (A). Brown staining shows the positive cells for Mϕ and ICAM-1 expressions in the atherosclerotic plaques. Bar=50 μm (×100). HFD, high-fat diet.

area covered by fatty streak lesions was quantified in oil red-O-stained samples, and specimens from the control group were compared with those from the NAC group. Controls developed extensive lesions in the root of the aorta (**Figure 1**). In mice treated with NAC, the fractional area of the lesions was reduced compared with the controls (**Table 2, Figure 1**).

Superoxide Production

Ethidium fluorescence in the NAC group was significantly weaker than that in the control group (**Table 2, Figure 2**). Namely, the brightness of the DHE-stained lesions from NAC treated mice was less than that from control mice. The origin of the superoxide might have been mainly from macrophages, but also partly from endothelial cells, considering the results of both a previous report[14] and the current study.

Macrophage and ICAM-1 Expressions

Both the accumulation of macrophages and expression of ICAM-1 were decreased by NAC treatment compared with the controls (**Table 2, Figure 3**). The percentage of lesions was less in NAC-treated mice than that in the control mice.

Western Blotting

Decreased ICAM-1 expression in NAC-treated mice was confirmed by Western blot analysis (**Figure 4**).

Lipid Profiles

NAC treatment did not significantly modify the serum lipid profiles (**Table 3**).

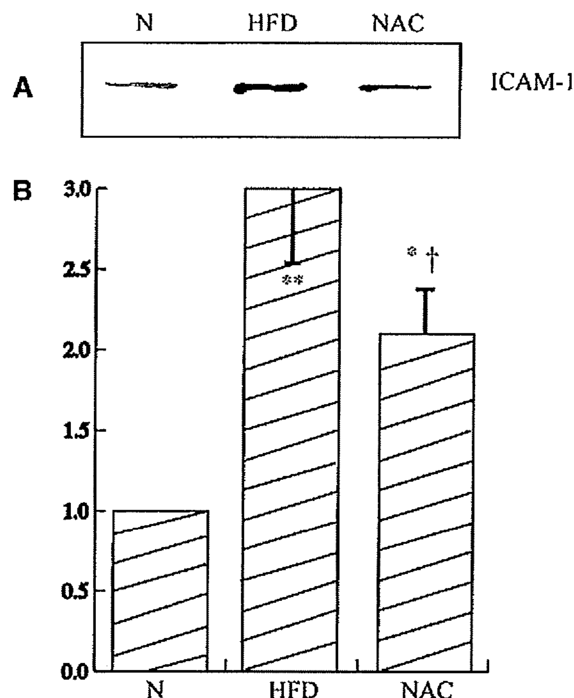


Figure 4. (A) Western analysis of intercellular adhesion molecule-1 (ICAM-1) expression. Representative Western blot analysis showed decreased expression of ICAM-1 in N-acetylcysteine (NAC)-treated mice compared with control mice. (B) Densitometric analysis of relative protein levels. In mice with the control (high-fat diet (HFD)), ICAM-1 expression was increased, but was decreased by NAC treatment. Values are derived from 5 animals and represent a percentage of controls. *P<0.05, **P<0.01 vs Normal (N); †P<0.05 vs HFD.

Table 3. Lipid Profiles

	n	TC (mg/dl)	TG (mg/dl)
Control	9	648.8±97.5	93.2±92.6
NAC	9	644.0±174.1	29.0±20.0

Mean±SD.
TC, total cholesterol; TG, triglycerides. Other abbreviation see in Table 1.

Discussion

The results of the current study show that NAC treatment suppressed the development of experimental atherosclerosis in apoE-deficient mice by reducing both superoxide production and macrophage accumulation in the aortic walls.

Chronic inflammation is thought to be of central importance in atherosclerosis.[1,16] It has been shown that regular and chronic exercise can suppress overt and subclinical inflammation,[17,18] based on the fact that atherosclerosis can be considered as a generalized manifestation of an inflammatory disease.[1] We and other investigators had already reported that experimental atherosclerosis in apoE-deficient mice was markedly suppressed by the Fcγ portion of immunoglobulin administration, possibly by an antiinflammatory action via the inhibitory Fcγ receptor IIB.[19–21]

There is also increasing evidence to support the critical role of both free radicals and oxidative stress in the development of atherosclerosis[22–25] and in heart failure.[26,27] We had already demonstrated that MCI-186, a free radical scavenger, and olmesartan, an angiotensin type 1 receptor antagonist, suppress the severity of experimental atherosclerosis.[11] Indeed, angiotensin stimulation has been reported to produce free radicals from various cells.[28] Free radicals from vessel walls are thought to play a critical role in atherogenesis. It is considered that free radicals induce the expression of adhesion molecules and chemokines, accelerate atherosclerotic plaque formation, increase matrix metalloprotease production, and cause vulnerable plaques.[29] The superoxide anion is a free radical.

Psychological and behavioral stress are now recognized as important contributors to inflammatory and free radical mediated cardiovascular diseases. Both clinical and experimental evidence support the hypothesis that oxidative stress is linked to hypertension, heart failure, and atherosclerosis. Accordingly, the present study determined the protective effects of NAC, a potent, antioxidative agent,[5–7] on experimental atherosclerosis, irrespective of the results of previous reports,[8,9] and we found that NAC treatment clearly suppressed the severity of atherosclerosis in apoE-deficient mice.

As an antioxidant, NAC, has a chelating capacity, free radical scavenging activity, and peroxidation inhibiting activity, and together these result in a potent reducing power. The antiatherosclerotic effects of NAC in animal models have been reported in part, which were related to the nitric oxide system[8] as well as its anti-oxidative effects.[9] In the present study, we clearly demonstrated that NAC not only suppressed superoxide production but also macrophage accumulation, assessed by ICAM-1 expression, in the aortic walls. Our immunohistochemical study showed that macrophage accumulation and the intensity of ICAM-1 staining in the aortic wall were very similar. It has already been established that the degree of instability of plaque correlates with the amount of macrophages, and thus ICAM-1 expression.[29] Therefore, a decrease in the intensity of macrophage and ICAM-1 expressions in the aortic walls may reflect a decrease in oxidative stress by NAC treatment.

NAC is a water-soluble material and may be smoothly metabolized from the plasma. However, the pharmacokinetics of NAC may differ between mice and humans, as previously reported.[5–9,30–32] Further studies are needed to determine the optimal dose–effect relationship in humans. In the current study, however, we chose an intraperitoneal injection of 20 $mg \cdot kg^{-1} \cdot day^{-1}$ of NAC, guided by previous reports.[5–7]

Conclusion

NAC treatment protected against experimental atherosclerosis in apoE-deficient mice by suppressing superoxide production in the atherosclerotic lesions. Our results suggest that anti-oxidative agents, food and antioxidant behavior may be beneficial for protecting against atherosclerosis in the clinical setting.

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Disclosure

There is no conflict of interest.