II 分担研究報告書

4. マイクロミニピッグ動脈硬化症モデルを用いたスタチンの薬効試験

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【研究要旨】

目的　マイクロミニピッグが、実験動物として有用かどうかを、①アンジオテンシンⅡの２週間静脈内持続投与による高血圧モデルが作成可能かどうか、②アンジオテンシンⅡ静脈内投与終了後に、内因性血管作動物質に対する脳底動脈の血管反応性に変化がみられるかどうか、③変化が見られた際には、その物質の作用機序はどのようになっているか、を検討することにより明らかにする。

方法　アンジオテンシンⅡの静脈内持続(14日間) 投与を行い、前肢より血圧を測定した。投与終了後、脳底動脈を摘出し、内因性血管作動性物質に対する血管反応をマイクロオルガンバスシステムで測定した。さらに、脳底動脈の血管内皮細胞を培養し、変化が見られた反応を引き起こした原因物質の特定およびその変化を生化学的に調べた。

結果　アンジオテンシンⅡの静脈内持続投与は、マイクロミニピッグに持続的高血圧を引き起こした。投与終了後に摘出した脳底動脈では、セロトニンおよびブラジキニンに対する血管反応性に有意な変化が見られた。ブラジキニンにより引き起こされる弛緩に続く収縮の 2 相性反応は、収縮のみの１相性反応となった。ブラジキニンを内皮細胞へ処置すると、一酸化窒素および $PGF_{2\alpha}$が細胞より産生した。アンジオテンシンⅡの内皮細胞への前処置は、ブラジキニンによる一酸化窒素の産生を減少させ、反対に $PGF_{2\alpha}$の産生を増大させた。

考察　アンジオテンシンⅡの静脈内持続投与により、血管平滑筋上の AT_1受容体を介した直接的な作用以外に、血管内皮細胞上の B_2受容体を介した一酸化窒素の産生減少および $PGF_{2\alpha}$の産生増大により、高血圧は引き起こされることが示唆された。

結論　マイクロミニピッグは in vivo, in vitro および培養細胞実験を行うのに適しており、高血圧モデルをはじめ今後、実験動物として期待される。

A. 研究目的

近年、霊長類およびイヌ等を実験動物として使用することは、主要 EU 加盟国やアメリカを中心に、動物福祉やコストの上昇などの点から難しくなりつつあり、研究者からは新たな実験動物の開発が望まれている。マイクロミニピッグはその中でも最も相応しい実験動物の候補の一つに挙げられている。

私達は、これまで日常の食料として供給されている通常のブタの血管を、近くの食肉センター（屠場）から入手後、実験室まで搬入し、結合織や脂肪等を取り除き、張力測定実験やラジオリガンド実験、また内皮細胞を培養して生化学的実験を行ってきた。

今回、マイクロミニピッグを使い①アンジオテンシンⅡの２週間静脈内持続投与が、高血圧症状を引き起こす高血圧モデルの動物として使用することが出来るのかどうか、②脳血管の中でも心臓運動血管中枢等の存在する延髄背面を走行している脳底動脈を摘出し、ノルアドレナリン、ブラジキニン、セロトニンやアンジオテンシンⅡといった内因性血管作動物質に対する血管反応性に、アンジオテンシンⅡ投与終了後、変化がみられるかどうか、③もし変化が見られた際には、その作用機序はどのようになっているか、を in vivo、in vitro および培養細胞実験により明らかにすることを試みた。

B. 研究方法

１）in vivo 実験

メデトミジン水溶液（ドミトール、Orion Corporation, 1 mg/ml, 0.02-0.08 ml/kg）および塩酸ケタミン水溶液（50 mg/ml, 0.08-0.2 ml/kg）の麻酔下でマイクロミニピッグの頸静脈よりカニューレションを行い前大静脈洞に留置する。アンジオテンシンⅡ投与群には、0.2 mg/kg/day で２週間持続的に

投与した。対照群にはアンジオテンシンⅡの代わりに生理食塩液を同様に投与した。
全身性の血圧は、前肢より血圧計を介して測定した。

2）in vitro 実験

アンジオテンシンⅡの静脈内持続投与の最終日に安楽死を行い、脳底動脈を摘出し、直ちに氷冷したKrebs－Ringer 液（119 mM NaCl, 4.7 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4 および10 mM glucose）中に保存して、実験室に搬入した。余分な結合組織や脂肪を取り除いた後、長さ2 mm の血管リング標本を複数個作製した。

作製した血管リング標本に糸のついたステンレス製フックを2本装着し、一方をオルガンバス底部に固定し、他方を等尺性張力トランスデューサ（TB-611T, 日本光電）に接続し、リング標本をオルガンバス内に懸垂した。オルガンバス内は37℃（pH 7.4）に保たれたKrebs－Ringer 液で満たされ、95% O_2 および5% CO_2 の混合ガスを通気した。等尺性張力トランスデューサで感知された血管張力の変化は、増幅アンプ（AP-621G, 日本光電）で増幅後、PowerLab システム（ADInstrument）にてチャートデータとして変換され、パーソナルコンピューターに保存された。

血管リング標本は、オルガンバス内に装着して約30分間静置させた後、静止張力を0.5 g に調節した。この静止張力は、血管リング標本が60 mM KCl により最大収縮反応を起こすことのできる張力である。

全ての実験において、各処理を行う前に安定した再現性のある収縮が得られるまで血管リング標本に対して3回のKCl の適用を行った。これらのKCl 適用のたびにKrebs－Ringer 液にて洗浄し、静止張力を0.5 g に再調節した。内因性血管作動物質の血管反応が収縮する場合、3回目のKCl 反応で得られた最大収縮を100%とした。また、血管反応が弛緩する場合は、実験の最後にニトロプルシド 10^{-4} M を適用し得られた最大弛緩反応を100%とした。

3）培養細胞実験

摘出マイクロミニピッグ脳底動脈にカニューレションを行い、0.05%トリプシン溶液をゆっくりと灌流することにより内皮細胞をチューブに集め、遠心後に上清を除去し、新しい培養液を加える洗浄を行った。0.1%ゼラチンコーティングした90 mm ディッシュに播き、37℃, 5% CO_2-95% air 下で初代培養を行った。培地として、45% DMEM と45% Nutrient mixture 12 HAM それに10% FBS（fetal bovine serum）を添加した混合培地を使用し、コンタミネーションを防ぐために抗菌剤（100 units/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B）を添加した。この細胞を3代目まで継代培養し、4代目以降6代目までの細胞を実験に用いた。なお、培養中は細胞を2あるいは3日毎にHank's Balanced salt solution（Hanks 液）で洗浄し、培地の交換を行った。

4）統計学的処理

得られた実験結果は、平均値±標準誤差で示した。有意差検定には2群間での比較の場合は student paired *t*－test を、多群間での比較の場合は一元配置分散分析を行った後に Bonferroni's multiple *t*-test を用いて検定を行った。危険率5%未満で有意差ありとした。

C. 研究結果

【結果および考察】

1）アンジオテンシンⅡの2週間静脈内投与の全身血圧値へ及ぼす影響

アンジオテンシンⅡを2週間静脈内投与した時のマイクロミニピッグの全身血圧を収縮期血圧（systolic blood pressure）および拡張期血圧（diastolic blood pressure）については、投与1日目からアンジオテンシンⅡ群の収縮期血圧は有意に上昇した。拡張期血圧は対照群と比較すると高い値を示したが一部を除き有意ではなかった。

2）アンジオテンシンⅡを2週間静脈内投与された摘出脳底動脈の内因性血管作動物質に対する血管反応性

内因作動性物質として、①ノルアドレナリン、②アンジオテンシンⅡ、③セロトニン、④ブラジキニン、⑥L-ニトロアルギニン、⑦インドメタシンを用い、得られた反応を下記に示した。

ノルアドレナリン：通常ブタ脳底動脈ではノルアドレナリンに対しては、β受容体（β_1:β_2=7:3）を介して弛緩するが（文献1）、マイクロミニピッグでも通常ブタ同様に弛緩した。アンジオテンシンⅡを持続投与した後の脳底動脈でも、この弛緩反応に有意な影響は認められなかった。

アンジオテンシンⅡ：通常ブタ脳底動脈ではアンジオテンシンⅡに対して、AT_1受容体を介して収縮、AT_2受容体を介して一酸化窒素を遊離するが、AT_1受容体を介した収縮反応が優位である（文献2）。マイクロミニピッグでも通常ブタ同様にアンジオテンシンⅡに対して収縮した。アンジオテンシンⅡを持続投与された後に摘出された脳底動脈でも、この収縮反応に有意な変化は認められなかった。

セロトニン：通常ブタ脳底動脈ではセロトニンに対して、5-HT_1受容体および5-HT_2受容体を介して収縮するが（文献3）、マイクロミニピッグでも通常ブタ同様にセロトニンに対して収縮した。アンジオテンシンⅡを持続投与された後に摘出された脳底動脈では、セロトニンを適用して得られた収縮反応が有意に増強される結果となった。

L-ニトロアルギニン：通常ブタ脳底動脈では一酸化窒素合成酵素阻害剤であるL-ニトロアルギニンに対して、自発的に遊離している一酸化窒素の産生が阻害されるため収縮するが、マイクロミニピッグでも通常ブタ同様に収縮した。アンジオテンシンⅡを持続投与された後のマイクロミニピッグ脳底動脈でも、この収縮反応に有意な影響は認められなかった。

インドメタシン：通常ブタ脳底動脈ではシクロオキシゲナーゼ（COX）阻害剤であるインドメタシンに対して、自発的に遊離しているトロンボキサンA_2の産生が阻害されて弛緩するが、マイクロミニピッグでも通常ブタ同様に弛緩した。アンジオテンシンⅡを持続投与した後の脳底動脈でも、この弛緩反応に有意な影響は認められなかった。

ブラジキニンン：ブタ脳底動脈ではブラジキニンンに対して、内皮細胞上のB_2受容体を介して弛緩および収縮する2双性反応を示すが、マイクロミニピッグでも通常ブタ同様にブラジキニンンに対して2双性の血管反応を示した。アンジオテンシンⅡを持続投与した後に摘出された脳底動脈では、弛緩反応は消失し、収縮反応は逆に大きく増強された。ブラジキニンンに対する血管反応が、アンジオテンシンⅡの持続投与により大きく変化したため、さらにマイクロミニピッグの脳低動脈内皮細胞を培養し、産生される弛緩因子である一酸化窒素および収縮因子のプロスタノイドを測定することとした。既にプロスタノイドはプロスタグランジン(PG)H_2の代謝物であるPGE_2、PGD_2、$PGF_{2\alpha}$でありトロンボキサンA_2ではないことは以前報告している（文献1）。そのため、PGE_2、PGD_2、$PGF_{2\alpha}$のいずれが、ブラジキニンにより、産生が増強されるかを先に検討した。

3）ブラジキニン処置による一酸化窒素産生量への影響

一酸化窒素産生量は用量依存性および時間依存性に増大した。また、増大した一酸化窒素産生量は、B_1拮抗薬であるdes-Arg^9, [Leu^8]-BKでは有意な影響を受けなかったが、B_2拮抗薬であるHOE140および一酸化窒素合成阻害剤であるL-ニトロアルギニンでは完全に消失した。この結果は、ブラジキニン処置により血管内皮細胞上に存在するB_2受容体を介して一酸化窒素が産生され、血管を弛緩していることを示唆している。

4）ブラジキニン処置によるプロスタグランジン産生量への影響

10^{-7} Mブラジキニンの脳底動脈内皮細胞への処置によるPGD_2、PGE_2、$PGF_{2\alpha}$の産生量への影響を示した。その結果、PGD_2およびPGE_2の産生量には有意な変化は認められなかったが、$PGF_{2\alpha}$の産生量は有意に増強された。この結果は、マイクロミニピッグ脳底動脈にブラジキニンン処置をすると、内皮細胞上のB_2受容体を介して$PGF_{2\alpha}$が産生されることにより、収縮反応を引き起こすことを示唆している。

5）アンジオテンシンⅡの48時間脳底動脈内皮細胞への処置が、ブラジキニン処置により産生される一酸化窒素および$PGF_{2\alpha}$産生量へ及ぼす影響

ブラジキニンン処置により一酸化窒素および$PGF_{2\alpha}$が産生されることが内皮細胞を用いて証明されたが、これらの産生量に、アンジオテンシンⅡの48時間前処置がどのような影響を示すかを、次の実験で明らかにしようと試みた。

10^{-7} Mブラジキニンによる一酸化窒素の産生量が、10^{-7} Mアンジオテンシンの前処置（48 hr）によりどのように影響を受けるかを6, 12, 24 hrで検討したところ、アンジオテンシンⅡの前処置は、10^{-7} Mブラジキニンによる一酸化窒素の産生量を時間依存性に増大させた。一方、10^{-7} Mブラジキニンによる$PGF_{2\alpha}$の産生量が、10^{-7} Mアンジオテンシンの前

処置（48 hr）によりどのように影響を受けるかを6, 12, 24 hr で検討すると、アンジオテンシンⅡの前処置は、10^{-7} M ブラジキニンによる $PGF_{2\alpha}$ の産生量を時間依存性に増大させた。この2つの結果は、アンジオテンシンⅡ処置により、ブラジキニンによる一酸化窒素の産生量が減少すると同時に $PGF_{2\alpha}$ の産生量が増大することを示しており、先の in vivo の実験でアンジオテンシンⅡを2週間静脈内投与した後に摘出した脳底動脈で、ブラジキニンによる弛緩の後、収縮反応が起きるという2双性の血管反応の弛緩部分が消失し、収縮部分が増強され1相の収縮反応になったことを細胞レベルで証明したことになる。

D. 考察

マイクロミニピッグにアンギオテンシンⅡを2週間静脈内持続投与すると、高血圧状態を保つことの出来る、いわゆる高血圧モデルを作成することが出来た。このことは、マイクロミニピッグが、高血圧用の実験動物に適していることを示している。

以下、高血圧を引き起こした理由について in vitro および培養細胞実験の結果を基に考察した。

マイクロミニピッグの脳底動脈リング標本の血管反応は、今回内因性血管作動物質とした使用した、ノルアドレナリン、アンジオテンシンⅡ、セロトニン、L-ニトロアルギニンおよびインドメタシンに対し、通常ブタと同様の血管反応を示した。これにより、基本的に通常ブタでこれまでに得られた実験結果を基に、各種実験を行うことが出来ることを示唆している。アンギオテンシンⅡの2週間静脈内持続投与後に摘出された脳底動脈リング標本の血管反応では、セロトニンとブラジキニンで有意な変化が見られた。セロトニンの反応がアンギオテンシンⅡの静脈内持続投与後に増強されることは、既にラットで報告されているが、ブラジキニン反応の変化はこれまでに報告されていないため、ブラジキニンに焦点を当てて、その機序の解明を試みた。

ブラジキニン適用により元々見られた弛緩反応に続く収縮反応は、アンジオテンシンⅡの持続的静脈内投与により、弛緩反応の消失と収縮反応の増強が引き起こされた。培養内皮細胞を用いた実験より、ブラジキニン適用により脳底動脈内皮細胞に存在する B_2 受容体を介して、一酸化窒素と $PGF_{2\alpha}$ が産生されることが示唆された。さらに、この内皮細胞を予めアンジオテンシンⅡで処置しておくと、一酸化窒素の産生量は有意に抑制され、$PGF_{2\alpha}$ の産生量は有意に増大した。この結果は、アンジオテンシンⅡの持続的静脈内投与により、弛緩反応が消失し収縮反応が増強されたかを示したものと思われる。

ブタの内皮細胞の培養は、ウシ内皮細胞と同様、比較的安価な基本の培養液で培養が可能である。このことは実験を行うに当たって大きなアドバンテージと成り得る。In vivo から in vitro, 細胞培養実験、生化学的実験、分子学的実験に至るまで完成できるのがマイクロブタを使った実験の大きな特徴と言える。

E. 結論

今回の実験結果を統括すると、下記のようになる。

①マイクロミニピッグは、動物の大きさ、扱い易さ、コスト、動物倫理および福祉の面は勿論、実際にアンジオテンシンにより高血圧モデルを作成することが出来、また摘出血管実験や内皮細胞培養実験等を行い、実験結果を導くことが可能な動物である。

②摘出した脳底動脈の血管反応は、これまで実験で得られた普通のブタの血管反応とほぼ同一であった。

③アンジオテンシンⅡの2週間持続投与は、摘出した脳底動脈の血管反応の中で、セロトニンとブラジキニンの反応に有意な変化をもたらした。セロトニン反応は有意に増強され、またブラジキニンの2双性反応は、弛緩反応は消失し、収縮反応は有意に増強された。

④上記以外に適用した、ノルアドレナリンおよびアンジオテンシンⅡの反応は有意な影響を受けなかった。

⑤脳底動脈は、トロンボキサン A_2 および一酸化窒素の産生量が他の血管と比べ4倍ほど多いため、シクロオキシゲナーゼ阻害薬であるインドメタシンを適用するとトロンボキサン A_2 の産生が抑制されるため弛緩し、一酸化窒素阻害薬である L-ニトロアルギニンを適用すると収縮するが、この反応にアンジオテンシンⅡの2週間静脈内投与は影響を及ぼさなかった。

⑥マイクロミニピッグ脳底動脈内皮細胞にブラジキニンを適用すると、B_2 受容体を介して一酸化窒素および $PGF_{2\alpha}$ の産生量が有意に増大した。しかし、

PGD_2およびPGE_2の産生量は有意な影響を受けなかった。
⑦アンジオテンシンⅡの前処置は、ブラジキニンによる一酸化窒素の産生量を有意に抑制し、$PGF_{2\alpha}$の産生量を有意に増強した。

以上の結果は、アンジオテンシンⅡの血中濃度が高いと、AT_1受容体を介して血管平滑筋を収縮させ、血圧を上昇させる直接作用の他、ブラジキニンB_2受容体に作用して、一酸化窒素の産生量の減少および$PGF_{2\alpha}$の産生量を増大させる2次的作用により、高血圧を引き起こす可能性を示唆するものである。

高血圧の代表的治療薬にAT_1受容体ブロッカーとアンジオテンシン変換酵素阻害剤があるが、今回の結果は、後者の薬が何故高血圧に有効であるか、理由の一つを示したものと思われる。

今回、マイクロミニピッグが霊長類およびイヌ等の実験動物に代わる候補の実験動物になる可能性を実験結果と共に示すことが出来た。

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F. 健康危険情報

なし

G. 研究発表

1. 論文発表

投稿中

2. 学会発表

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② Md. Zahorul Islam, Miharu Nishimura, Hiroaki Kawaguchi, Naoki Miura, Tomoko Iwanaga, Emi Yamazaki-Himeno, Mitsuya Shiraishi, Noriaki Miyoshi, Akihide Tanimoto, Atsushi Miyamoto: Treatment of angiotensin II decreased NO and increased prostaglandin $PGF_{2\alpha}$ production by bradykinin in cultured porcine basilar arterial endothelial cells. 第156回日本獣医学会学術集会（岐阜大学），2013年9月 (岐阜).

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H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

III 研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kawaguchi H Yamada T Miura N Ayaori M, Uto-Kondo H Ikegawa M, Noguchi M Wang KY Izumi H Tanimoto A	Rapid Development of Atherosclerosis in the World's Smallest Microminipig fed a high-fat/high cholesterol diet: A Useful Animal Model Because of its Size and Similarity to Human Pathophysiology.	J Atheroscler Thromb.	21	186-203	2014
Akioka K Kawaguchi H Kitajima S Miura N Noguchi M Horiuchi M Miyoshi N Tanimoto A	Investigation of Necessity of Sodium Cholate and Minimal Required Amount of Cholesterol for Dietary Induction of Atherosclerosis in Microminipigs.	In Vivo	28	81-90	2014
Kawaguchi H Yamada T Miura N Noguchi M Izumi H Miyoshi N Tanimoto A	Sex Differences in Serum Lipid Profile in Novel Microminipigs.	In Vivo	27	617-621	2013

Ⅳ 研究成果の刊行物・別刷

Original Article

Rapid Development of Atherosclerosis in the World's Smallest Microminipig Fed a High-Fat/High-Cholesterol Diet

A Useful Animal Model Due to its Size and Similarity to Human Pathophysiology

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Aim: **Experimental studies of human atherogenesis require an appropriate animal model that mimics human physiology and pathology. Because swine physiology is similar to human physiology, we developed a hyperlipidemia-induced atherosclerosis model using the recently developed world's smallest Microminipig™.**

Methods: **These animals weigh only 5 kg at 3 months of age, much smaller than any other miniature pig. We found that the administration of a high-fat/high-cholesterol diet containing at least 0.2% cholesterol without cholic acid for as little as eight weeks induces hypercholesterolemia and subsequent atherosclerosis in these animals.**

Results: **The serum levels of low-density lipoprotein cholesterol (LDL-C) and the percent distribution of cholesterol in the LDL fractions were markedly increased. The hepatic expression of LDL receptor and hydroxymethylglutaryl-CoA reductase was coordinately decreased. The cholesteryl ester transfer protein activity, which plays a role in reverse cholesterol transport, was detected in the serum of the Microminipigs. Niemann-Pick C1-like 1 protein was expressed in both the liver and small intestine; however, hepatic apoB mRNA editing enzyme was not expressed. As in humans, and in contrast to that observed in mice, most of the hepatic lipase activity was localized in the liver. These results suggest that the hyperlipidemia-induced gene expression profile linked to cholesterol homeostasis and atherogenesis is similar in Microminipigs and humans.**

Conclusion: **We conclude that the characteristics of the Microminipig, including its easy handling size, make it an appropriate model for studies of atherosclerosis and related conditions.**

J Atheroscler Thromb, 2014; 21:186-203.

Key words: Microminipig, Atherosclerosis, Cholesterol metabolism, CETP

Introduction

Atherosclerosis is a predominant risk factor for cardiovascular and cerebrovascular events and is closely related to serious morbidity and mortality in developed nations. The recent westernization of lifestyle in Japan, especially the increased caloric intake from a fatty diet, may account for the increasing inci-

dence of cerebral and coronary artery disease. An appropriate animal model that reproduces human physiology and pathology would be ideal for investigating atherosclerosis because the pathogenesis of this disease includes both genetic and environmental factors.

Attempts to develop experimental animal models of atherosclerosis have primarily involved mice and rabbits. However, lipid metabolism in mice is quite different from that observed in humans, as mice are originally resistant to high-fat/high-cholesterol diet (HcD)-induced atherosclerosis. As a result, mice that exhibit hyperlipidemia and atherosclerosis due to a lack of apolipoprotein-E (apoE) or low-density lipoprotein cholesterol receptor (LDLr) genes are often used in atherosclerosis studies[1, 2]. The introduction of an additional transgene and/or the use of gene knockout in apoE- or LDLr-deficient mice are good tools for investigating the effects of specific genes in atherosclerosis[3]. Rabbits, which have a similar lipid metabolism to humans and are very sensitive to HcD with respect to the induction of atherosclerosis, are the next most often used animals. LDLr gene-mutated Watanabe heritable hyperlipidemic rabbits are good models of human familial hypercholesterolemia[4]. The recent development of transgenic rabbits has clarified the effects of various specific genes on the development of atherosclerosis[5, 6].

Swine represent another potentially useful animal model because, unlike mice and rabbits, their anatomy, physiology and habits of feeding and sleep are very similar to those of humans[7]. In general, domestic pigs are often used in medical training and education regarding vascular surgery techniques, arterial intervention, etc. However, their large size hampers handling and maintenance; therefore, they are unsuitable for experimental use in ordinary laboratories. Commercially available experimental miniature pigs (minipigs), such as Clawn, Göttingen, Chinese Bama and Yucatan minipigs are smaller than domestic pigs. Many studies have reported that HcD can induce atherosclerosis in Göttingen, Chinese Bama and Yucatan minipigs as well as domestic pigs[8-12]. These animals, which weigh less than 100 kg by definition, are still too large to be widely used in life science research. The Microminipig™ (MMPig, Fuji Micra Inc., Shizuoka, Japan) has recently been established as an experimental animal[13]. The MMPig is the world's smallest pig, with a body weight of only 5 kg at 3 months of age that remains at less than 10 kg at 7 months of age. Our preliminary study demonstrated that hyperlipidemia-induced atherosclerosis develops in MMPigs fed HcD containing sodium cholate (SC)[14]. Dietary SC is required to accelerate the progression of hyperlipidemia and atherosclerosis, presumably by inhibiting cholesterol excretion into bile; however, it is also known to cause hepatotoxicity[15].

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Aim

The specific aims of the present study were to further expand upon our previous research and detect evidence of close similarities between MMPigs and humans with respect to lipid metabolism and atherogenesis. We specifically investigated whether HcD alone (with no SC) induces atherosclerosis in the MMPigs and determined the minimum cholesterol content required to cause disease. We performed a histological evaluation of hyperlipidemia-induced atherosclerosis and assessed the expression of genes regulating cholesterol metabolism, including LDLr, class B scavenger receptor type I (SR-BI), hydroxymethylglutaryl-CoA reductase (HMGCR), apoB mRNA editing enzyme catalytic polypeptide 1 (APOBEC-1) and Niemann-Pick C1-like 1 protein (NPC1L1), which is expressed in the mammalian small intestine and liver and is critical for intestinal cholesterol absorption[16, 17]. The activity and localization of cholesteryl ester transfer protein (CETP) and hepatic lipase (HL) in the MMPigs were also evaluated and compared with those observed in humans.

Methods

Animals and Diet

Male MMPigs 3 months of age were maintained in a special room under environmental conditions with a room temperature of 24±3℃, a relative humidity of 50%±20% and a 12-hour light/dark cycle. Tap water was available *ad libitum*, and the animals were provided a special diet on a daily basis. The body weight was measured once a week. All protocols were approved by the Ethics Committee of Animal Care and Experimentation at Kagoshima University and performed according to the laws (no. 105) and notifications (no. 6) of the Japanese Government. This study was also performed in accordance with the animal welfare bylaws of Shin Nippon Biomedical Laboratories Ltd., a facility fully accredited by the Associa-

tion for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and approved by the International Animal Care and Use Committee.

Twenty-two MMPigs were divided into four groups: seven control animals fed a normal chow diet (NcD) and three groups (five animals in each group) fed HcD for eight weeks. The HcD was composed of 12% lard (Miyoshi Oil & Fat, Tokyo, Japan) and 0.2%, 0.5% or 1.5% cholesterol (Wako Pure Chemical Industries, Osaka, Japan) mixed with NcD (Kodakara 73; Marubeni Nisshin Feed, Tokyo, Japan). After eight weeks, all MMPigs were anesthetized and sacrificed via bilateral axillary artery exsanguination.

Blood Pressure

The arterial systolic and diastolic blood pressures were measured at the foreleg with an apparatus meant for human pediatric use according to the Manchette method.

Hematology and Biochemical Analysis

Blood samples were collected once every two weeks for general hematology, biochemistry and lipoprotein profiling. The biochemical parameters measured in the blood samples included the levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, γ-glutamyl transpeptidase, glucose and total bilirubin. The levels of total cholesterol (TC), chylomicrons (CM), very low-density lipoprotein cholesterol (VLDL-C), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were analyzed using an automated agarose gel electrophoresis apparatus (Epalyzer 2, Helena Laboratories, Saitama, Japan).

For measurement of the HL activity, blood was collected from the NcD-fed MMPigs before and 10 minutes after the intravenous injection of sodium heparin (50 unit/kg BW) at 5 and 25 months of age. Serum samples of the pre- and post-heparin fractions were then analyzed using an HL activity assay kit (Progen Biotechnik GmBH, Heidelberg, Germany). The serum CETP expression and activity in the HDL fractions were assayed in the NcD-fed control MMPigs (See **Supplemental text**).

Serum ApoB Profiles in the CM and VLDL Fractions

The similarity between the human and MMPig plasma proteomes was investigated using a method for cross-species detection of lipoproteins (**Supplemental text**). In brief, the CM and VLDL fractions were separated via ultracentrifugation. The samples were then alkylated, digested with trypsin and analyzed on a nano HPLC system coupled with a triple quadrupole mass spectrometer.

Evaluation of Visceral and Subcutaneous Fat

The weight of visceral tissue fat was measured at necropsy. The accumulation of subcutaneous fatty tissue (back fat thickness) was evaluated on computed tomography (CT) performed at the beginning and end of the study. The back fat thickness was measured at the midportion of the level between the lower angles of both scapulae, and the percentage increase in thickness after the eight-week dietary treatment was calculated.

Pathological Examination

At necropsy, the aorta, arteries, heart, liver, kidneys, spleen and small intestine were removed from each animal. The heart, liver, kidneys, spleen and visceral (omental and mesenteric) adipose tissue were weighed. All organs were fixed in 10% phosphate-buffered formalin and routinely processed as paraffin-embedded, 5-μm-thick tissue sections stained with hematoxylin and eosin (H&E) and Elastica-Masson stains. The aortas were longitudinally incised and fixed with 10% buffered formalin for 24 hours, followed by staining with Oil-red O stain for an *en face* analysis. The Oil-red O-stained area relative to the entire surface was calculated using the Image J software program. Immunostaining for atherosclerotic lesions was performed (Envision kit, Dako Cytomation, Kyoto, Japan) on the paraffin-embedded sections using antibodies against smooth muscle actin (anti-α-SMA clone 1A4, ×100; Dako Cytomation) and macrophages (anti-lysozyme rabbit polyclonal antibody, × 2,000; Dako Cytomation).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The liver and small intestine were stored in RNAlater immediately following sample collection, and total RNA was extracted using the mirVana miRNA isolation kit (Invitrogen, Carlsbad, CA). The mRNA expression was quantified using qRT-PCR with a TaqMan quantitative PCR analysis (Applied Biosystems, Oyster Bay, NY). **Supplemental Table 1** lists the genes investigated and the primers/probes used for PCR. The primers and probes were either obtained from predesigned gene expression assays or designed based on the sequence information of domestic swine (Applied Biosystems). The expression level of GAPDH mRNA was used as an internal control.

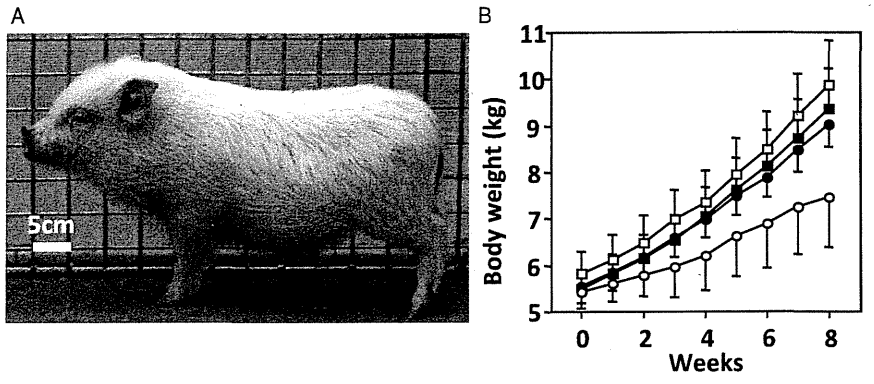

Fig. 1. Growth curves of the MMPigs

(A) Profile of a 5-month-old NcD-fed MMPig (body weight, 7 kg; body length, 60 cm). (B) Growth curves of the NcD-fed control and HcD-fed animals over eight weeks. All animals experienced an increase in body weight; however, no significant differences were observed among the experimental groups. Note that the body weights of HcD-fed animals remained < 10 kg following the administration of the HcD for eight weeks (7 months of age). Open circle, NcD control; closed circle, HcD with 0.2% cholesterol; open rectangle, HcD with 0.5% cholesterol; closed rectangle, HcD with 1.5% cholesterol.

Table 1. Visceral and subcutaneous adiposity in the MMPigs

% cho in diet	0	0.2	0.5	1.5
Relative weight (g/kg)				
Omentum	0.80±0.25	1.78±0.30*	1.96±0.19**	1.78±0.36*
Mesenterium	3.05±0.47	4.30±0.64	4.40±0.35	4.22±0.88
% increase of BFT	147.7±38.8	214.0±18.8	211.2±26.4	234.8±21.8*

The data are presented as the mean±SE. cho, cholesterol; BFT, back fat thickness *$p<0.05$ and **$p<0.01$ vs. the control (0 % cho diet)

Statistical Analysis

All results are expressed as the mean±SE. The statistical analysis of the differences between groups was performed using Student's *t*-test, and the results were considered to be significant at $p<0.05$.

Results

Body Weight, Adiposity and Blood Pressure

A 5-month-old male NcD-fed MMPig, 7 kg in body weight, is shown in **Fig. 1A**. The body weight values increased in all groups during the eight-week experimental period. Compared with the NcD-fed MMPigs, the HcD-fed MMPigs showed a more rapid increase in body weight. In contrast, no significant differences in body weight were observed between the NcD- and HcD-fed groups (**Fig. 1B**). The omental fat weight was higher in the HcD-fed animals than in the NcD-fed animals; however, no significant differences in mesenteric adipose tissues were observed. The back fat thickness was significantly increased after eight weeks in the 1.5% cholesterol-fed MMPigs, as compared with that observed in the control MMPigs (**Table 1**). The blood pressure levels, both systolic and diastolic, were similar in the HcD-fed and NcD-fed control MMPigs.

Hematology and Blood Biochemistry

Considering the normal reference data for MMPigs[18, 19], no animals exhibited leukopenia, leukocytosis or anemia after being fed NcD or HcD for eight weeks. No increases in the levels of AST, ALT or LDH occurred in either the NcD- or HcD-fed animals. The levels of ALP, γ-GTP and total bilirubin were moderately increased at scattered time points in the animals fed 0.5% or 1.5% cholesterol. No differences in blood glucose were noted between the HcD-fed and NcD-fed MMPigs.

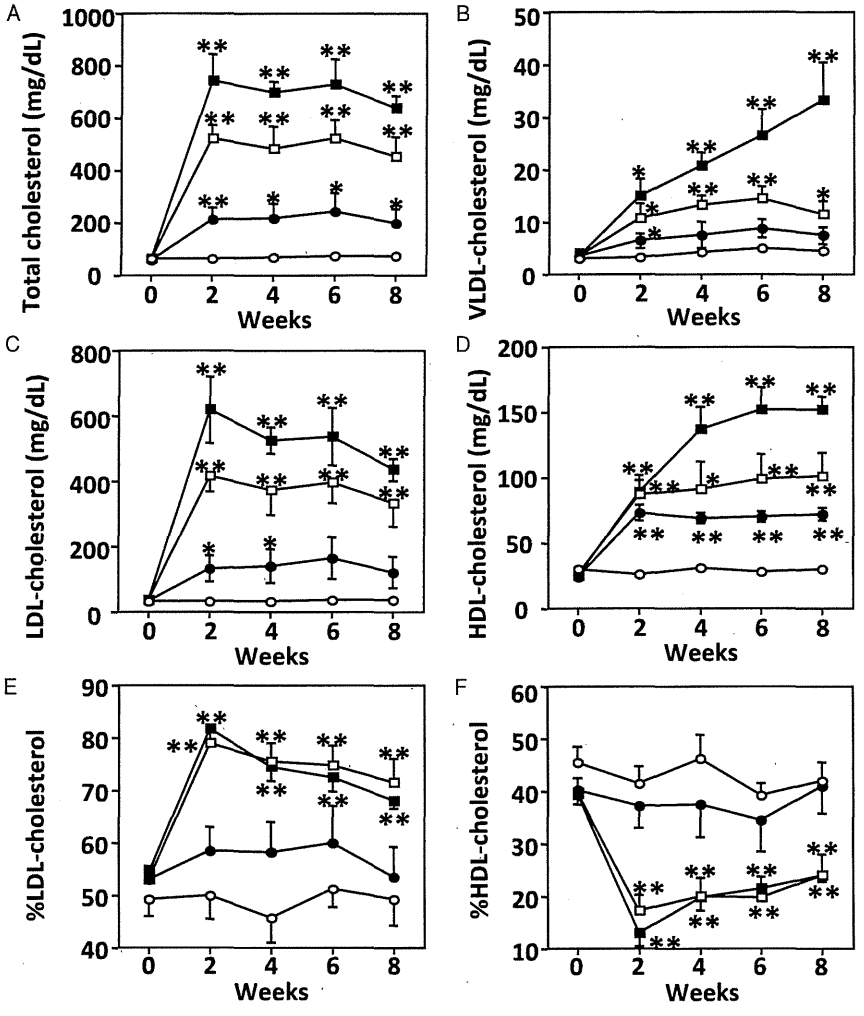

Fig. 2. Serum lipoprotein profiles

(A) Total cholesterol (TC). (B) VLDL-C. (C) LDL-C. (D) HDL-C. TC and each cholesterol fraction in the HcD-fed MMPigs exhibited dose-dependent increases during the eight-week experimental period. The percentages of the cholesterol fractions increased in LDL-C (E) and decreased in HDL-C (F) in the MMPigs fed HcD with 0.5% and 1.5% cholesterol during the eight-week experimental period. Open circle, NcD control; closed circle, HcD with 0.2% cholesterol; open rectangle, HcD with 0.5% cholesterol; closed rectangle, HcD with 1.5% cholesterol. The data are presented as the mean ± SE. $^{*}p<0.05$, $^{**}p<0.01$ vs. the control

Serum Lipoprotein Profile

The MMPigs became hypercholesterolemic after two weeks of the HcD at all cholesterol concentrations (0.2%, 0.5% and 1.5% cholesterol) compared with that observed in the NcD-fed controls (**Fig. 2A**). The TC levels plateaued after two weeks. The VLDL-C and LDL-C levels were increased in the groups fed higher concentrations of cholesterol (0.5% and 1.5% cholesterol;**Fig. 2B** and **2C**), whereas the HDL-C levels increased in all HcD-fed groups during the eight-week experimental period (**Fig. 2D**). The percent distribution of cholesterol with 0.5% and 1.5% cholesterol loading was increased in the LDL fractions and decreased in the HDL fractions at every time point during the experiment (**Fig. 2E** and **2F**). The serum TG levels were similar in all groups.

Expression of LDLr, SR-BI, HMGCR, SREBP-2 and NPC1L1

The hepatic expression of LDLr and HMGCR

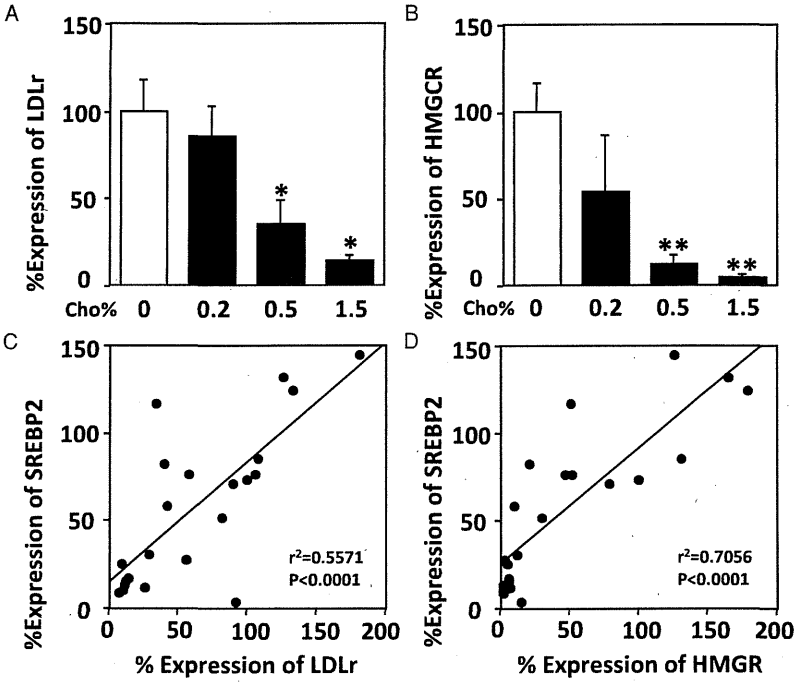

Fig. 3. Hepatic expression of LDLr, HMGCR and SREBP-2

The hepatic expression of LDLr, HMG-CoA reductase and SREBP-2 was analyzed using real-time RT-PCR at the end of the eight-week experiment. The expression of (B) was significantly decreased by HcD containing 0.5% and 1.5% cholesterol. (C) (D) The expression level of the SREBP-2 gene was highly correlated with the LDLr and HMGCR expression. The gene expression levels were calculated as the percentage expression over the level observed in the control NcD-fed group. The data are presented as the mean ± SE. $*p < 0.05$, $**p < 0.01$ vs. the control

was downregulated in the MMPigs fed HcD for eight weeks at higher dietary cholesterol concentrations (0.5% and 1.5% cholesterol; **Fig. 3A** and **3B**). The expression levels of these two genes were highly correlated with that of SREBP-2, irrespective of diet (**Fig. 3C** and **3D**). The expression levels of SR-BI in the liver were unchanged during HcD consumption (**Fig. 4A**). The expression of NPC1L1 in the small intestine was not decreased in the jejunum or ileum in the animals fed the HcD compared with that observed in the controls (data not shown); however, the hepatic expression was markedly reduced in the HcD-fed MMPigs (**Fig. 4B**).

HL and CETP Activity

The HL activity was much higher in the post-heparin fractions vs. the pre-heparin fractions in the NcD-fed MMPigs, thus demonstrating an activity of more than 90% in the circulation following the administration of sodium heparin (**Table 2**). For CETP, the protein expression, detected using Western blotting with antibodies against human CETP, and activity were detected in the HDL fraction in the serum of the NcD-fed MMPigs (**Supplemental Fig. 1**).

Expression of apoB mRNA Editing Enzyme and Serum apoB Profile

Hepatic APOBEC-1 is expressed in mice but not in humans or rabbits, and the editing enzymatic action generates apoB48 to form VLDL, including both apoB48 and apoB100[21]. Under our experimental conditions, the APOBEC-1 expression was detected using qRT-PCR in the small intestine alone and not in the liver in the MMPigs (**Fig. 4C**). We detected apoA1 and apoB48/100 peptides in both the human and swine VLDL and CM fractions (**Supplemental Fig. 2**).

Hyperlipidemia-Induced Atherosclerosis

The *en face* analysis demonstrated that aortic atherosclerotic lesions were significantly increased in the HcD-fed MMPigs at all dietary cholesterol concentra-

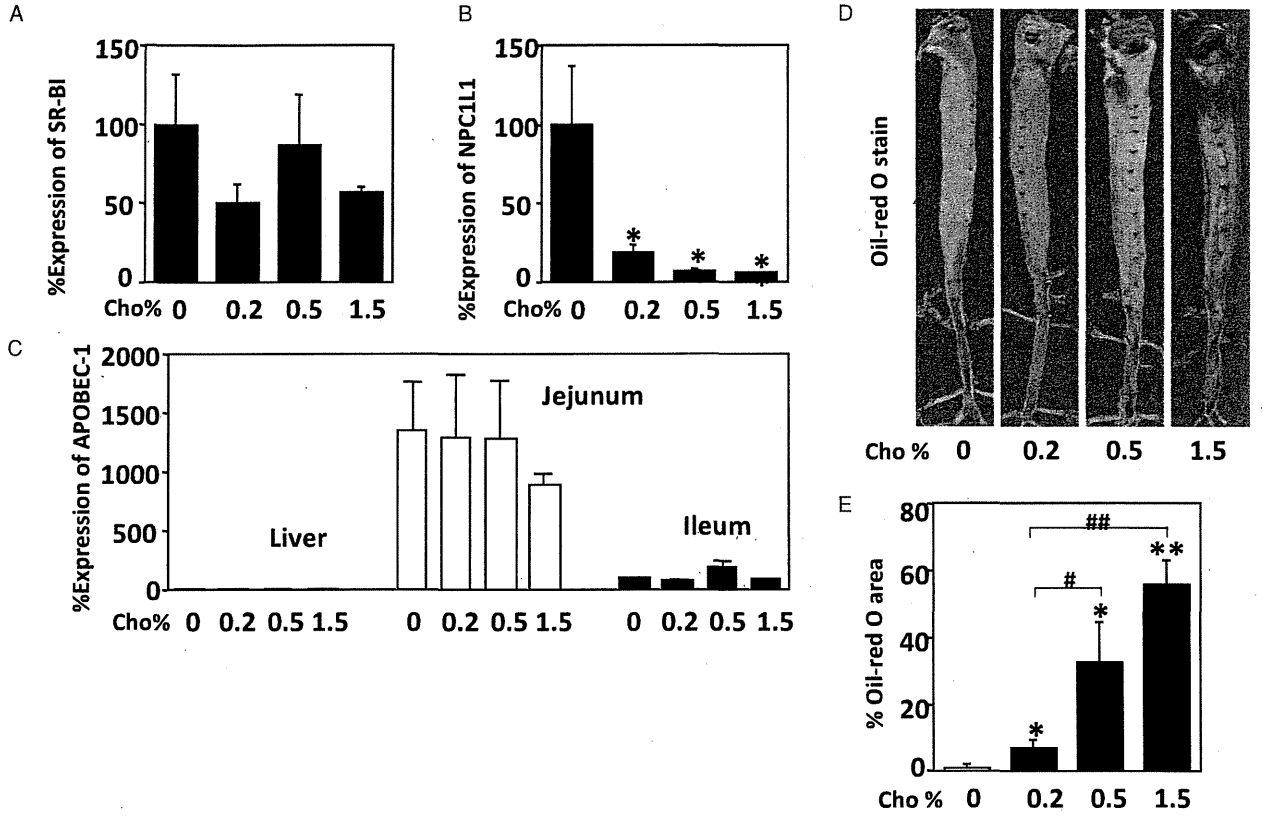

Fig. 4. Hepatic expression of SR-BI, NPC1L1 and APOBEC-1 and quantitative analysis of hyperlipidemia-induced atherosclerosis

(A) The hepatic expression of SR-BI in the HcD-fed groups was similar to that observed in the NcD-fed controls. (B) The hepatic NPC1L1 expression was markedly reduced by HcD feeding. (C) The APOBEC-1 expression was detected in the jejunum and ileum but not in the liver. The gene expression levels were calculated as the percentage expression over the level observed in the control NcD-fed group. (D) Oil-red O-stained atherosclerotic lesions in the NcD-fed and HcD-fed MMPigs after eight weeks. (E) The number of atherosclerotic areas stained with Oil-red-O stain was increased in the HcD-fed MMPigs. The values are presented as the mean ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ between each group.

Table 2. Plasma hepatic lipase activity in the NcD-fed MMPigs

age (month)	25					5			
animal No.	1	2	3	4	mean ± SE	5	6	7	mean ± SE
B.W. (kg)	24.0	24.0	28.0	25.0	25.3 ± 1.6	5.7	5.8	5.7	5.73 ± 0.05
HL activity (pmol/ml/min)									
pre-heparin	0.07	0.24	0.08	0.09	0.12 ± 0.07	0.11	0.15	0.14	0.13 ± 0.02
post-heparin	2.47	2.37	2.66	3.42	2.73 ± 0.41	2.89	1.98	2.93	2.60 ± 0.44
liver-bound	2.40	2.13	2.58	3.33	2.61 ± 0.46	2.78	1.83	2.79	2.47 ± 0.45
% in liver	97.2	89.9	97.0	97.4	95.4 ± 2.8	96.2	92.4	95.2	94.6 ± 1.6

NcD, normal chow diet; B.W., body weight; HL, hepatic lipase

tions but unchanged in the NcD-fed controls (**Fig. 4D** and **4E**). The atherosclerotic lesions developed first at the aortic arch and the entry of the spinal arteries and abdominal aorta and progressed to involve the entire aorta as the cholesterol content in the HcD increased. The aortic atherosclerotic lesions were located in the intima (**Fig. 5A**) and primarily composed of infiltration of foam cells (**Fig. 5B**). Elastica-Masson Tri-

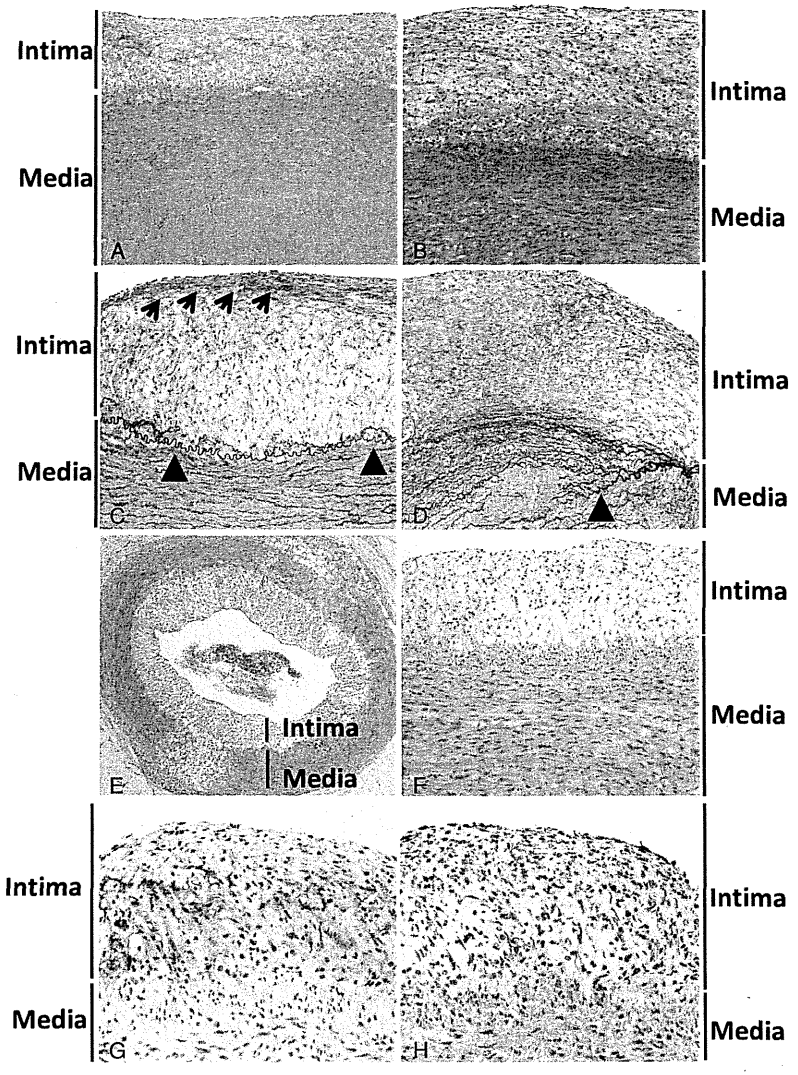

Fig. 5. Atherosclerotic lesions in the aorta and coronary arteries

Aortic atherosclerotic lesions of an MMPig fed HcD for eight weeks. (A) (B) Atheromatous plaque in the abdominal aorta with intimal infiltration of foam cells (H&E stain). (C) The internal elastic laminae are duplicated (arrowheads) and the plaque is covered with a fibrous cap (arrows; Elastica-Masson Trichrome stain). (D) An advanced lesion exhibits disruption of the internal elastic lamina and stratification of newly formed laminae (arrowhead; Elastica-Masson Trichrome stain). (E) Atherosclerotic lesions in the coronary artery of an MMPig fed HcD for eight weeks. (F) Light microscopy of an atherosclerotic lesion in the left main trunk at low and high power (H&E stain). (G) The plaque lesion consists of the accumulation of foam cells positive for the macrophage marker lysozyme. (H) A small number of intimal cells are positive for α -SMA. Medial smooth muscle cells positive for α-SMA were used as an internal control.

chrome staining showed duplication and disruption of the internal elastic lamina (**Fig. 5C** and **5D**). The advanced lesions exhibited fibrous cap formation covering the atheromatous plaques (**Fig. 5C**). The stages of atherosclerosis were varied in the sections examined; however, in general, the abdominal aortas showed a large amount of advanced lesions. The atherosclerotic lesions in the carotid, coronary and femoral arteries were identical to those observed in the aorta (**Fig. 5E** and **5F**). In the coronary arteries, the

proximal portions, located immediately after branching from the aorta, were severely involved in the atherosclerosis; however, a few lesions in the distal and intramuscular arteries were also observed, especially in the higher cholesterol diet-fed groups. The infiltrating foam cells were positive for the macrophage marker (lysozyme) (**Fig. 5G**). The atheromatous lesions also demonstrated small numbers of α-SMA-positive smooth muscle cells (**Fig. 5H**).

Histopathology of other Organs

No significant histological changes were observed in the heart, lungs, spleen or kidneys. Mild fatty degeneration was detected in the liver in the HcD-fed MMPigs at 0.5% and 1.5% cholesterol concentrations (data not shown). Despite the presence of atherosclerotic lesions, no HcD-fed MMPigs experienced thrombosis or spontaneous myocardial or cerebral infarction during the eight-week experiment.

Discussion

Diet-Induced Hypercholesterolemia and Atherosclerosis

This study demonstrated the presence of eight-week HcD-induced hypercholesterolemia and atherosclerosis accompanied by moderate visceral and subcutaneous adiposity in MMPigs. After eight weeks of the administration of HcD containing 1.5% cholesterol, the serum levels of TC reached approximately 600 mg/dL and atherosclerotic areas were seen in approximately 50% of the aortas. The relatively low 0.2% dietary cholesterol concentration was sufficient to induce the formation of aortic atherosclerotic areas in 7.5% of the animals, with TC levels of approximately 200 mg/dL. The consumption of dietary SC, which is often administered to mice in order to induce atherosclerosis[20], was not necessary to enhance the development of hyperlipidemia-induced atherosclerosis in the MMPigs. It is noteworthy that a period of only eight weeks was sufficient to induce atherosclerosis in MMPigs, whereas other miniature pigs require more than three months to develop this condition[8-12].

Serum Lipid Profiles

Both the serum LDL-C and HDL-C levels markedly increased after the administration of the HcD, with a greater increase observed in the LDL-C levels. The percentage distribution of cholesterol into HDL-C was therefore significantly reduced, indicating a proatherogenic lipid profile. This paradoxical increase in HDL-C induced by dietary fat has been reported in both humans and rabbits[21, 22]. Human metabolic studies have shown that the administration of HcD decreases the catabolic rate while increasing the transport rate of apoA-I, resulting in increased HDL-C levels[23]. Similar observations have been made in human apoA-I transgenic mice, in which the administration of HcD increases the HDL-C levels, whereas metabolic turnover studies indicate a decreased catabolic rate and increased transport rate of HDL-cholesteryl ester (CE) and apoA-I[24]. The diet-induced increase in the HDL-C levels may represent an adaptation for enhancing reverse cholesterol transport mediated via the HDL pathway, although the exact mechanism is unclear. The detection of the enzymatic activity of CETP, which regulates a portion of RCT, in the HDL fraction in the MMPigs supports the presence of this possible adaptive mechanism(s).

Serum ApoB Profiles

In this study, the MMPigs, similar to humans and rabbits, did not express hepatic APOBEC-1. The VLDL-C fraction therefore included only apoB100 and not apoB48. In contrast, mice express hepatic APOBEC-1 to generate apoB48-containing VLDL-C and subsequently lower the LDL-C levels[25]. The transfer of the APOBEC-1 gene in the liver in New Zealand White rabbits and Watanabe heritable hyperlipidemic rabbits results in the production of apoB48-containing VLDL-C and a reduction in LDL-C formation[26]. Serum VLDL-C cannot be converted to LDL-C in humans with hypobetalipoproteinemia because it contains truncated apoB50 due to a premature stop codon in the apoB gene[27]. Therefore, the regulation of the size of apoB by hepatic apoB mRNA editing represents a fundamental mechanism for limiting the generation of atherogenic apoB100-containing lipoproteins. The hepatic expression of APOBEC-1 corresponds closely with a low ratio of [VLDL-C+LDL-C] to HDL-C (<0.5) in dogs (0.26), rats (0.41), mice (0.25) and horses (0.44). Mammals that do not express hepatic APOBEC-1 exhibit higher ratios, including humans (1.92), monkeys (0.91) and pigs (1.4). Rabbits (0.32) are an exception to this rule[25]. In this study, the ratio in the NcD-fed MMPigs was 1.31, indicating a proatherogenic lipid profile in this group.

Expression of LDLr, HMGCR and NPC1L1

The blood cholesterol levels are largely determined by LDL-C removal mediated by LDLr and cholesterol synthesis via the HMGCR activity in the liver[28, 29]. The hepatic expression of LDLr and HMGCR is coordinately regulated by the sterol regulatory element-binding protein (SREBP)-2 signaling

pathway; the transcriptional activity of SREBP-2 is usually inhibited when cellular cholesterol is abundant[30-32]. In the present study, the hepatic expression of LDLr, HMGCR and SREBP-2 was correlated and markedly downregulated following the consumption of HcD. The regulation of these genes and the LDL-C-rich lipid profile in MMPigs is therefore very similar to that observed in humans and is in contrast with that observed in mice, in which downregulation gene responses are minimal[30].

Intestinal absorption and biliary excretion are additional closely regulated mechanisms of cholesterol homeostasis[33, 34]. A recently identified NPC1L1 protein that regulates intestinal cholesterol absorption is highly expressed in the small intestine in various species, including humans, rabbits and mice[16, 35]. The NPC1L1 protein is also expressed in the human liver, where it partially regulates biliary cholesterol excretion; however, it is not expressed in the murine liver[33]. In the present study, the NPC1L1 gene was expressed in both the small intestine and liver in the MMPigs. The regulatory gene mechanisms of the NPC1L1 expression in both organs include the SREBP-2 pathway, suggesting that a high level of cholesterol suppresses SREBP-2-regulatory genes, such as LDLr, HMGCR and NPC1L1[36, 37]. Although the NPC1L1 expression was not clearly reduced following the administration of the HcD, the detection of the expression of NPC1L1 in both the liver and intestine supports the conclusion that the gene expression profile linked to cholesterol homeostasis is very similar between MMPigs and humans.

Expression of the CETP and HL Activity

CETP catalyzes the transfer of CE from HDL to apoB-containing lipoproteins and is considered to be a key protein for reverse cholesterol transport[38, 39]. Humans and animals, including rabbits and chickens, with documented atherosclerosis susceptibility have a higher level of CETP activity than atherosclerosis-resistant animals, such as cats, dogs, rats and mice[40]. The development of atherosclerosis is accelerated by an atherogenic diet when CETP is genetically introduced in mice, which are naturally deficient in CETP[41, 42]. CETP and apoB100 double transgenic mice also show increased levels of atherosclerosis[43]. However, the overexpression of CETP in apoC-III or lecithin cholesterol acyltransferase transgenic mice inhibits atherosclerosis[44, 45]. Assessments of the effects of CETP on atherogenesis in mice are limited because the lipoprotein metabolism in mice differs markedly from that observed in humans. The relationship between the CETP activity and the development of human atherosclerosis is also controversial at present. Patients with coronary heart disease have lower levels of large HDL particles and higher levels of small, dense (sd) LDL particles[46-48]. CETP inhibitors, which significantly increase the level of large HDL particles and decrease the level of sdLDL particles, are thought to be effective for reducing the development of atherosclerotic cardiovascular disease[49]. In humans, a deficiency of the CETP activity results in increased plasma HDL-C levels with the generation of large CE-rich HDL particles, supporting this hypothesis[50]. Cases of CETP polymorphism or genetic variation, characterized by a diminished CETP activity, are associated with increased levels of HDL-C, a reduced incidence of coronary heart disease and greater longevity[51, 52]. However, other studies have reported an increased incidence of atherosclerotic cardiovascular and cerebrovascular diseases in CETP-deficient human subjects[53-55]. Furthermore, a recent clinical study showed that the administration of a CETP inhibitor (dalcetrapib) did not reduce the risk of recurrent cardiovascular events even though it increased the HDL cholesterol levels in patients with a recent history of acute coronary syndrome[56]. Whether CETP inhibitors are effective in preventing cardiovascular disease remains a matter of controversy.

Swine express no or very low levels of CETP activity, although they are susceptible to developing atherosclerosis[40]. In the present study, the CETP gene expression was not detected in the MMPigs using RT-PCR with primers designed from human and rabbit CETP cDNA sequences (data not shown). However, the MMPigs expressed CETP-like proteins and the CE transfer activity. Although the presence of a similar CETP gene remains a matter of debate, MMPigs appear to be a useful model for evaluating the relationship between the CETP activity and atherogenesis.

HL hydrolyzes TG in HDL to convert HDL_2 to HDL_3 and is involved in the conversion of IDL to LDL. In this study, the HL activity in the MMPigs was mostly detected in the post-heparin plasma, similar to findings observed in humans and rabbits. This suggests that most HL activity is localized on the surface of hepatic sinusoidal endothelial cells, in contrast to that observed in mice, in which most of the activity is detected in the circulation[57]. HL- and apoE-deficient mice exhibit a lesser degree of atherosclerosis than apoE-deficient mice, indicating the proatherogenic role of murine HL[58]. In LDLr and murine endogenous HL-deficient mice with the transgenic overexpression of human HL, which can bind to the surface of hepatic sinusoidal endothelial cells, the levels of VLDL, IDL and LDL are decreased, subse-

Table 3. Comparison of lipoprotein metabolism in mice, humans, rabbits and MMPigs

	mice	rabbits	humans	MMPigs
Lipoprotein	HDL-rich	LDL-rich	LDL-rich	LDL-rich
CETP	no	yes	yes	yes
HL	70% in circulation	liver-bound	liver-bound	liver-bound
Hepatic apoB editing	yes	no	no	no
Hepatic LDLr	high	down-regulated	down-regulated	down-regulated
Hepatic NPC1L1	no	yes	yes	yes
Diet-induced atherosclerosis	resistant	sensitive	sensitive	sensitive

CETP, cholesteryl ester transfer protein; HL, hepatic lipase; LDLr, LDL receptor; NPC1L1, Niemann-Pick C1-like 1 protein
This table was modified from ref. no. 5 by Fan J. and Watanabe T.

quently reducing atherosclerosis[59]. The overexpression of human HL results in the reduction of HDL and IDL in rabbits[60]. The function of HL appears to be affected by the location in which it is primarily localized (the liver or the circulation).

Conclusion

We herein established a novel swine model of hyperlipidemia-induced atherosclerosis in the world's smallest MMPig, an animal with a cholesterol metabolism very similar to that observed in humans (**Table 3**). We believe that MMPigs represent a potential alternative animal model suitable for studies of metabolic syndrome because the alteration of lipid metabolism is one of the key events in this condition.

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Conflicts of Interest

None.

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