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心筋梗塞、脳梗塞の予知因子の同定と予知法の開発に関する研究

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主任研究者　北　徹

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Ⅰ. 総合研究報告

厚生労働科学研究費補助金（長寿科学総合研究事業）
（総合）研究報告書

心筋梗塞、脳硬塞の予知因子の同定と予知法の開発に関する研究

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研究要旨　心筋梗塞や脳梗塞は高齢者の生命予後・QOLを規定する疾患である。本研究はそれらの疾患の発症予知因子を開発し、臨床検査として確立することを目的として研究を進めた。そのような予知因子があり、発症を予測できると、強力な危険因子の管理などにより発症が回避できるであろう。近年血中CRP値等の上昇と急性冠症候群発症との間に相関が示されたが、どれとして臨床上広く用いられている指標はない。

我々の見いだした酸化LDL受容体LOX-1は動脈硬化巣に発現誘導され、可溶型が存在する。我々は血清LOX-1が予知因子になる可能性を考え研究を進めてきた。平成14年度、ELISA法による測定系を確立し、血清LOX-1値を測定した結果、血清LOX-1値は安定型狭心症での上昇はわずかであるが、急性冠症候群（急性心筋梗塞症・不安定狭心症）では著明に上昇していることを見いだした（Circulation in press, 2005）。血清LOX-1値は、これまでにいわれているどの指標よりも感度、特異度が高く、急性冠症候群の予知因子となる可能性がある。閉塞性動脈硬化症（ASO）患者において、血清LOX-1値および共同研究者、近藤らの開発した酸化ストレスマーカーであるthiol化蛋白質を測定したが、両者とも、ASO患者で上昇しており、また、血清LOX-1値はASOの重症度との相関が認められた。ASOにおいても、血清LOX-1値は有用な検査法と成る可能性がある。今後、多くの症例で血清LOX-1値を測定し、動脈硬化性疾患の臨床検査として確立していく計画である。

心筋梗塞や脳梗塞発症時に血小板は活性化するとされているが、血小板活性化指標の測定は採血後に影響を受けやすく、煩雑かつ熟練を要するので、臨床的に広く用いられていない。我々は全血および濃厚血小板血漿を用いた凝集計により、安定して血小板被活性化を解析できる条件を整えた。本指標を用いて、心血管疾患のリスクが高いことが示されている睡眠時無呼吸症候群（SAS）患者の血小板被凝集性が亢進していることを見いだした。さらに、SASではCPAP（持続性陽圧気道内圧法）が有効な治療法であるが、CPAP療法直後は、さらに凝集性が亢進し、3ヶ月後に前値より改善することを見いだした（投稿準備中）。今後、このシステムを用いて血小板活性化指標が心筋梗塞・脳梗塞の予知因子になるか、さらに、抗血小板療法は広く施行されているにもかかわらずその効果判定のためのシステムはないので用量調節性の抗血小板療法が可能かどうか検討するため、前向き研究を開始した。

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A．研究目的

本研究の目的は心筋梗塞と脳梗塞の臨床応用可能な予知法の開発である。日本人の死因は、心疾患15%、脳血管障害15%であり、高齢になるに従い増加する。これらの疾患は高齢者の生命予後を規定するとともに、回復後も後遺症を残すため高齢者のQOLを損なう。もし、その発症が予知できれば、バイパス手術やカテーテル治療等の侵襲的治療法や、強力な抗血小板療法、脂質低下療法等の内科的治療によりその発症は回避可能であろう。そのため、国民医療・高齢者医療の向上のためには、心筋梗塞・脳梗塞発症予知法の確立は急務である。心筋梗塞発症は粥腫の破裂に引き続く血栓形成が原因とされる。心筋梗塞は必ずしも冠動脈の高度狭窄部位で発症するものではなく、狭窄度の低い部位からの発症が多いとされている。動脈硬化を基盤に発症するのではあるが、一般に、冠動脈造影では予知は困難であると考えられている。特に危険因子が重複する高齢者などでは、心筋梗塞や脳梗塞を予知できる指標があれば定期的に測定することで、治療のタイミングや治療強度を決定することが可能となる。脳梗塞は、その発症メカニズムについていまだ不明な点が多いが、脳梗塞予知法が開発されれば、より質の高い医療を実施することが可能となるであろう。

なお、抗血小板療法の心筋梗塞・脳梗塞予防に対する有効性が確立され、多くの患者が治療されているが、同時に副作用として脳出血などの出血性合併症の頻度を増加させる。現在、広く臨床の場で用いられている効果モニター法がないが、もし、そのような方法が確立できれば、効果をモニターしながらより有効でかつ安全な抗血小板療法を施行できるであろう。本研究で確立しようとしている血小板活性化指標は予知因子としてばかりでなく、抗血小板療法の効果モニターに広く応用される可能性がある。

B．研究方法

北は分担研究者、久米等とともに見いだした酸化LDL受容体LOX-1、SR-PSOXに、両者とも動脈硬化巣に発現し、可溶型があることを見出した。ELISA法を用いて、LOX-1の血中濃度測定系を確立できたので、急性冠症候群を中心に多くの症例において測定した。また、LOX-1、SR-PSOXの細胞生物学的な機能解析を行った。さらに、分担研究者、近藤らは、酸化ストレスのマーカーとして、thiol化蛋白質の定量測定系を確立し、多くの血管疾患をもつ症例で測定した。血小板活性化指標がない理由のひとつは、血小板活性化機構に不明な点が多いためである。分担研究者、堀内等とともに独自の、形質膜を透過型にした血小板を用いた血小板顆粒放出および凝集解析系を確立し、血小板活性化の分子機構の解明に取り組んだ。また、予知因子としての可能性および、抗血小板療法の効果モニターのため、血小板凝集能測定システムを確立した。本年度は健常者のコントロールを解析し、また、睡眠時無呼吸症候群患者で測定し解析している。さらに、抗血小板療法の効果と心血管イベント発症との関係を明らかにすべく、大規模前向き研究を計画した。

C．研究結果

動脈硬化発症には酸化低比重リポ蛋白質（LDL）が重要な働きをしているが、我々は２つの酸化LDL受容体、LOX-1（Sawamura et al., Nature, 1997）およびSR-PSOX（Shimaoka et al., JBC, 2000）を見出している。LOX-1は可溶型が存在し、測定法を確立し得た。ヒト血清において測定可能なことを確認し、急性心筋梗塞等を含む急性冠症候群の患者では高値となることを見出した（Hayashida et al., Circulation in press, 2005）。CRP等の炎症マーカーや心筋障害マーカーとも異なる動きをいした。すなわち、トロポニンTなどの心筋障害マーカーは発症後２−3時間で上昇してくるが、LOX-1は発症時にはすでに最高値に達しているという結果であった。かLOX-1は現在のところ、最良の急性冠症候群の予知因子である可能性が高い（Circulation in press, 2005）。さらに多くの症例を重ね、時間経過の詳細、さらに、安定型および不安定狭心症での位置づけ等まで踏み込み、心筋梗塞、脳梗塞予知因子として確立していく計画である。種々の疾患において、多くの症例で可溶型LOX-1値を測定し、今後、血清診断法としての位置づけを確立していく計画である。また、LOX-1に関しては、PPARγ（Hayashida et al, BBRC, 2004）および、HB-EGF（Mukai et al., Atherosclerosis, 2004）がLOX-1を発現誘導することを見出し、さらに、単球の細胞接着に重要な役割を演ずることを明らかにしている（Hayashida et al., FEBS Letter, 2002）。

近藤らは、酸化ストレスのマーカーとして、thiol化蛋白質の定量法を確立した。その方法を用い、多くの症例で、可溶型LOX-1値とともにその指標を測定した。多くの閉塞性動脈硬化症でも測定したが、thol化タンパク量および可溶型LOX-1値ともに、コントロールに比べ明らかに閉塞性動脈硬化症症例で上昇しており。可溶型LOX-1は重症度に従って上昇していることを見いだした（投稿準備中）。興味深いことに、両者の間には相関は認められなかったが、それは、両者の上昇もメカニズムの違いによるものであろうと考えられる。

我々が同定した別の酸化LDL受容体SR-PSOX（CXCL16と同一）が感染性心内膜炎等で心臓の弁内皮細胞に強く発現してCD８T細胞のVCAM-1への接着を促進し、IFNγの産生を増加させることを発見した（Yamauchi et al., ATVB, 2003）。可溶型SR-PSOXは感染性心内膜炎等の診断マーカーになる可能性がある。早急な測定法の確立が望まれる。また、細菌が抗原提示細胞に貪食される際に重要な役割を果たすことを明らかに

している (Shimaoka et al., J. Immunol., 2003)。動脈硬化の最終段階は血栓形成による動脈の閉塞である。血小板活性化の分子機構は多くのことが不明であるが、核のない血小板で分子生物学的手法を用いることが困難であることが大きな原因である。我々は透過型血小板を用いた凝集 (Nishioka et al., BBRC, 2001)・顆粒放出 (Shirakawa et al., JBC, 2000) 解析系を確立することで、その障壁を克服し、血小板活性化の分子機構を解析している。その系を用い、細胞質分画より、顆粒放出必須因子を生化学的に生成し、PKCαと同定した (Yoshioka et al., JBC, 2001)。最近では、small GTPase Rab27 が濃染顆粒放出を制御していること、血小板細胞質中の Rab27 の標的タンパク質を Munc13-4 と同定し、両者が血小板濃染顆粒放出を制御していることを見いだした (Shirakawa et al., JBC, 2004)。フランスのグループより、Munc13-4 が家族性血球貪食症候群 (FHL3) の原因であることが報告されたが、NK 細胞や細胞障害性T細胞でのperforin等を含む顆粒放出が障害されているためである。我々は、我が国における遺伝性血球貪食症候群を解析し、Munc13-4 の異常によるものは、perforin の non-sense mutation よりは症状が軽いことを明らかにした (Ishii et al., Blood, 2005)。Perforin の場合、'殺し屋'そのものであるが、Munc13-4 は顆粒放出の調節因子という違いが臨床症状に強弱をつけた可能性が考えられる。血小板凝集では、PKCαが凝集を制御していることを直接的に証明した (Tabuchi et al., JBC, 2003)。さらに、アダプター蛋白質 ShcA が重要な働きをしていることも明らかにした (Higashi et al., BBRC, 2004)。我々の確立した系を用いて研究をすすめ、血小板活性化の分子メカニズムを明らかにし、血栓性疾患の予防、治療に貢献していきたいと考えている。平成１５年度には、安定かつ信頼性の高い血小板凝集指標の測定法を確立した。京都大学医の倫理委員会の承認を得て平成 16 年度には、約 50 名の健常人での基礎データを採取した。さらに、呼吸器内科との共同研究で 60 名の睡眠時無呼吸症候群患者およびコントロール 60 名の血小板機能を測定し、睡眠時無呼吸症候群患者で血小板凝集性が亢進していることを明らかにした (Sumi et al., 投稿準備中)。現在、抗血小板療法を受けているハイリスク患者 1,000 例規模の血小板機能や LOX-1 値を測定し、前向きに３年間追跡するという APTEST Trial をまさに始めようとしている。

D．考察

動脈硬化巣には、コレステロールを蓄えたマクロファージ由来の泡沫細胞の集簇が認められる。コレステロールは血清中を LDL の形で運ばれるが、マクロファージは、その酸化産物である酸化 LDL の形で、コレステロールを貪食する。酸化 LDL 受容体は既にいくつか同定されたが、我々も LOX-1 および SR-PSOX の２分子を同定した。LOX-1 は、血管内皮細胞や、平滑筋細胞に発現しており、種々の炎症刺激で発現誘導される。さらに、動脈硬化巣に強く発現していることも確認している。SR-PSOX は、ケモカイン CXCL16 と同一分子であり、リンパ球に発現していることから免疫学的側面から興味が持たれている。

LOX-1 は動脈硬化巣で強く発現しており、予備実験では、血栓形成とともに可溶型が増加する。その両者の効果が相乗的に作用し、急性冠症候群で増加しているのであろう。LOX-1 は、hsCRP より感度、特異度とも大きく優れていた。さらに、急性冠症候群時、トロポニン T は発症２−３時間で上昇するが、血清可溶型 LOX-1 値は、急性冠症候群発症時、すでにピークと成っていた。すなわち、血清可溶型 LOX-1 値は、現在の、最も優れた急性冠症候群予知因子の可能性がある。さらに、急性冠症候群時、臨床の現場ではトロポニン T 値がしばしば測定されるが、血清可溶型 LOX-1 値を組み合わせることで、さらに診断能力が上昇する可能性がある。

SR-PSOX は、感染性心内膜炎の弁で発現が著明に増加していることを見いだした。また、SR-PSOX にも可溶型があることを見いだしている。これまでは感染精心内膜炎の特異的マーカーは皆無であったが、血清可溶型 SR-PSOX が感染性心内膜炎の特異的マーカーと成る可能性がある。更なる症例の積み重ねを計画している。

血小板は、動脈血栓のトリッガーである。抗血小板療法の心血管イベントに対する予防効果が証明されており、その重要性が広く認識されている。血小板の指標としては、血小板特異的因子であるβTG や PF4 などの血清値の測定による血小板の活性化の指標があり、また、血小板等を体外に取り出し、刺激に応じた血小板凝集の解析から得られるような血小板被活性化の指標がある。両者はある程度の相関が予想されるが、同一のものではない。また、糖尿病などで、血清βTG 値の上昇などが明らかにされているが、予後との関連を明らかにしたような前向き研究はほとんどない。

我々は、血小板被活性化を精密に測定する方法を確立し、多くの症例で測定した。そして、本研究中に睡眠時無呼吸症候群患者で血小板被活性可能が亢進していることを見いだした。また、CPAP 治療により３ヶ月まではさらに活性化していることを見いだしている、今後、睡眠時無呼吸症候群患者および、CPAP 導入より３ヶ月以内の睡眠時無呼吸症候群患者には、抗血小板療法を施すべきかもしれない。

最近、アスピリンの抗血小板作用には個人差があり、アスピリンに不応性の血小板を持つ症例が含まれていることが明らかになった。さらに、アスピリン不応性の患者に心血管イベント発症の頻度が高いことも示されている。我々の構築した血小板被活性化能を解析するシステムは、アスピリン不応性の解析に有用である。我々は、本システムを利用して、大規模前向き研究を計

画したが、その研究により、日本人におけるアスピリン不応性の頻度等を明らかにし、さらに前向きに追跡することにより、その予後との相関も明らかにできよう。

E．結論

本研究を通じ、血清可溶型 LOX-1 値が、現在では、最も信頼のおける心筋梗塞予知因子に成る可能性を示せた。さらに、多くの疾患、症例で検討を進め、可溶型 LOX-1 値の診断学的意義を確立する。また、血小板活性化指標を確立できた、本指標が予知因子と成るか、さらに、抗血小板療法効果判定指標と成るか、今後、検証していく。

F．研究業績

[北　徹、横出正之、木村剛、久米典昭、堀内久徳、荒井秀典、田中誠]

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II. 研究成果の刊行に関する一覧表

雑誌

研究成果の刊行に関する一覧表

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inevitable, and the future remains bleak for heart transplantation and probably for transplantation in general in this country.

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Coxsackie B virus and myocardial infarction

Sir—Nicholls and Thomas[1] claimed that ten of 38 patients with acute myocardial infarction, admitted to King Edward VII Hospital in Midhurst, UK, during a 2-month period in 1977, showed serological evidence of very recent Coxsackie B virus infection. Many workers have confirmed this apparent link.

Patients with myocardial infarction also frequently show evidence of previous depleted selenium status[2] and raised plasma glutathione peroxidase activity, which suggests a role for this trace element. Indeed, mortality from myocardial infarction may be notably reduced by selenium supplementation during the recovery period.[3]

The Coxsackie B virus causes Keshan disease,[4] an endemic cardiomyopathy that occurs in China but only in the selenium-deficiency belt that crosses that country from northeast to southwest. Taylor and co-workers[5] have established that this virus possesses a gene that is a homologue of the selenoenzyme glutathione peroxidase. The Coxsackie B virus, therefore, cannot replicate without obtaining selenium from its host. In a subgroup of patients, acute myocardial infarction seems to be triggered by a selenium deficiency, exacerbated by replication of Coxsackie B virus.

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Hypertension in Japanese old-old

Sir—We have studied community-dwelling elderly people aged 65 years and older in two towns in Japan, Kahoku and Urausu, and in a suburb town of Seoul, Korea, for many years, and have included elderly people in Singapore since 2001.

The study was based on the principles of comprehensive geriatric assessment[1] and used a self-reported questionnaire in addition to various tests, including neuropsychiatric performance-based function and blood tests. Some issues have arisen from our preliminary Singapore data that may be of wider interests.

Our data show that, with the exception of diastolic pressure in the Korean population, the systolic and diastolic blood pressure of community-dwelling elderly people in Singapore were significantly higher than those in the other populations. Most participants, other than in Korea, were older than 75 years (old-old).

In Singapore, members of senior citizens clubs within the area of a certain polyclinic were invited to participate, and a third responded. In the two Japanese towns, in which 34% and 26%, respectively, of the population is elderly, 40% of all old-old people responded. In Korea, elderly people in the participating town volunteered for the study.

Blood pressure measurements were the average of two readings taken while the patients were sitting. We chose to define normotensive instead of hypertensive patients to avoid the controversy of defining hypertension in the old-old, and possible ambiguity of self-reported information about diagnosis.

Lifestyle differences, especially dietary factors and exercise, would certainly account for part of the difference. The diet of Japanese elderly people living away from major cities is still traditional. Dietary habits of Singaporean elderly people are varied and still very much race-dependent. Going to the farm, walking or cycling long distances to the nearest shop is a part of daily lives for elderly people in Japan or Korea, but not in the city lifestyle of those in Singapore.

In the Singapore National Health Survey,[2] hypertension is prevalent (64·3% in the 60–69-year age-group) but frequently not previously diagnosed (53·0%). 49% of our sample who were older than 75 years had hypertension for the first time. This finding emphasises the importance of screening the old-old from a younger age.[3]

An ambitious national screening programme has already been started in Singapore and the next issue seems to be treatment. The current WHO guidelines for management of hypertension[4] include clear statements for elderly people up to age 80 years, and cite promising trials for older people. In practice, successful treatment hypertension in elderly people is commonly made more difficult by side-effects to drugs, coexisting illnesses, and adherence to treatment. However, as the elderly population in Singapore and many other parts of the world increases towards the level of Japan, we will need to refine the method of screening as well as the level of control of hypertension in community-dwelling elderly people to avoid the morbidity and mortality.

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detected. Theoretically, these parasitaemic patients should show symptomatic visceral leishmaniasis, but the number with this disorder is currently low in Spain. However, in one study,[4] leishmania was detected in peripheral blood samples from a striking proportion of blood donors living in the Mediterranean area. According to these results, frequent occurrence of leishmania parasitaemia might be identified among HIV-1-seronegative asymptomatic injecting drug users living in Spain if a sensitive PCR is used, but this hypothesis has not yet been investigated.

In an epidemiological survey, we collected serum samples from 93 asymptomatic HIV-1 seronegative injecting drug users who shared equipment. All participants lived in Seville. We tested for leishmania antibodies with an immunofluorescent antibody test. 23 (25%) of these individuals had antibody titres of 1/40 or more. Whole blood samples, cryopreserved at −70°C, from these leishmania seropositive individuals were tested for *Leishmania infantum* kDNA by PCR,[5] and seven were positive. Thus, 7·5% of the total injecting drug users group harboured *L infantum* in blood without symptoms of overt visceral leishmaniasis. Since all of them shared injection equipment routinely, they could be spreading the infection.

These results are in line with those of Cruz and colleagues and explain why a high proportion of shared syringes contain leishmania, whereas the frequency of overt visceral leishmaniasis declines. Likewise, the previous finding of a high prevalence of indirect markers of leishmania infection[2] would also be explained. Since asymptomatic parasitemic injecting drug users who share injecting devices seem to be a suitable reservoir for *L infantum*, an artificial anthroponotic cycle would be completed. Needles and syringes would be the vectors and uninfected injecting drug users the receptors.

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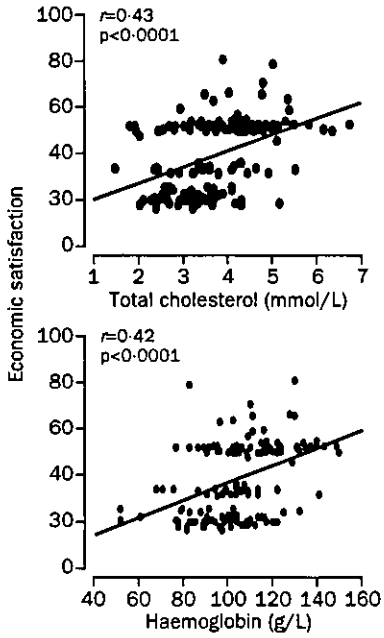
Health status and subjective economic satisfaction in West Papua

Sir—Richard Horton's March 23 Commentary[1] leads us to ponder the concept of Americanised globalisation.

We have done a medical assessment, in cooperation with a local Christian Hospital, in indigenous people living in Senggo, a rural village in West Papua/Irian Jaya, Indonesia, where the population is about 2000. Senggo is a tidal swampland on the south coast of West Papua, with abundant sago palms, birds, and fish. Economic conditions are generally poor. Birth rates seem to be high but average life expectancy is low.

We used questionnaires to assess subjective quality of life, did physical examinations, and measured total cholesterol and haemoglobin concentrations. Subjective quality of life was rated on a 100 mm visual analogue scale (worst on the left, best on the right). We asked participants to mark on the scale the level of their economic condition. We defined the distance from the left (mm) to the marked position as the quality of life score.[2,3]



Association of total cholesterol and haemoglobin concentrations with subjective economic status

227 indigenous people (115 men, 112 women, mean age 36·4 years [SD 13·5]) participated. 153 agreed to undergo blood tests. The mean concentrations of total cholesterol (3·7 mmol/L [1·0]) and haemoglobin (104 g/L [19]) were lower than those of healthy Japanese men aged 30–39 years (5·2 mmol/L [0·9], and 151 g/L [9·7]).

One of the explanations of lower concentrations of total cholesterol and haemoglobin may be widespread and chronic infections of malaria, tuberculosis, and parasites on top of general poor nutrition. There was significant linear association between total cholesterol and haemoglobin concentrations, and, more surprisingly, they both correlated significantly with subjective economical satisfaction (figure). These correlations were stronger among men than women. Total cholesterol and haemoglobin, therefore, seem to be indicators of objective nutritional and chronic infectious state in such regions.

Although economical conditions are recognised as being associated with health and nutritional state, asymptomatic medical disturbances such as cholesterol or haemoglobin concentrations were closely associated with subjective economical satisfaction in West Papua. Although our findings relate to a specific area, they may lead to a more global way of thinking when designing strategy for public health in developing countries.

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LOX-1 pathway affects the extent of myocardial ischemia-reperfusion injury

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Abstract

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) was originally identified as a receptor for oxidized low-density lipoprotein predominantly expressed in endothelial cells. LOX-1 expression can be induced in cardiomyocytes and that activation of LOX-1 is involved in apoptosis. To investigate possible roles of LOX-1 in myocardial ischemia-reperfusion injury, rats were subjected to coronary artery ligation for 1 h followed by reperfusion for 2 h. Immunohistochemistry revealed that expression of LOX-1 in cardiac myocytes was induced following ischemia-reperfusion but not ischemia alone. Administration of anti-LOX-1 monoclonal antibody resulted in a nearly 50% reduction in myocardial infarction size compared with that of normal IgG or saline ($P < 0.05$). These findings suggest that activation of the LOX-1 pathway is involved in determining the extent of myocardial ischemia-reperfusion injury and that inhibition of the LOX-1 pathway may provide a novel strategy for treatment of acute myocardial infarction in humans.

Keywords: Myocardial infarction; Ischemia-reperfusion; Oxidized low-density lipoprotein LOX-1

Myocardial reperfusion by thrombolysis or primary angioplasty has been widely performed for the management of acute myocardial infarction. Although early reperfusion can salvage jeopardized myocardium, reperfusion itself causes myocardial cell injury [1]. Possible mechanisms of this injury include increase of inflammatory responses such as neutrophil accumulation and production of reactive oxygen species [2,3]. However, the strategies that inhibit ischemia-reperfusion injury have yet to be established.

Lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) is a type II membrane protein that belongs to the C-type lectin family and was originally identified as a receptor for oxidatively modified low density lipoprotein (LDL) [4]. LOX-1 is expressed in highly vascularized organs such as the placenta and lung, and is predominantly expressed in vascular endothelial cells. LOX-1 expression can be induced in macrophages and smooth muscle cells as well as endothelial cells by various kinds of stimuli including oxidized LDL, angiotensin II, shear stress, and tumor necrosis factor-α. Previous studies have demonstrated that LOX-1 is involved in oxidized LDL-induced apoptosis through intracellular production of reactive oxygen species in vascular endothelial and smooth muscle cells [5,6]. We have recently demonstrated that expression of LOX-1 can be induced in cardiac myocytes and that the activation of this pathway induces apoptosis via p38 mitogen-activated protein kinase pathway [7]. Since myocardial cell apoptosis has been implicated as possible mechanisms of myocardial ischemia-reperfusion injury [3,8], LOX-1 might be involved in determining the extent of this injury. Therefore, in the present study we investigated whether administration of anti-LOX-1 antibody reduces the myocardial infarction size following ischemia-reperfusion.

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Materials and methods

Generation of anti-rat LOX-1 monoclonal antibodies. Anti-rat LOX-1 monoclonal antibodies were generated by immunizing Balb/c mice with Chinese hamster ovary-K1 cells stably expressing rat LOX-1 (rLOX-1-CHO) [9]. Hybridomas were generated by standard procedure and screened by the activity to block the uptake of oxidized LDL in the stable cell line.

Immunohistochemistry. Primary ventricular cardiac myocytes were prepared as previously described [7,10]. The cardiac myocytes were then grown on flask-style glass slides (Nalgen Nunc, Naperville, IL) and stimulated with hydrogen peroxide (H_2O_2, 10^{-5} M) or saline as a control for 24 h. Then the cells were fixed with Bouin's solution for 10 min at room temperature and subjected to immunocytochemistry for LOX-1 using the indirect immunoperoxidase method, as previously described [7]. Briefly, the sections were incubated with 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity, followed by further incubation with normal goat serum for 30 min to block nonspecific bindings. They were then incubated with anti-rat LOX-1 antibody at a final dilution of 1:50 for 16 h at 4 °C. In the second step, they were treated with a 1:200 dilution of peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 45 min. Peroxidase activity was visualized by use of diaminobenzidine and hydrogen peroxide. The sections were counterstained with hematoxylin and evaluated microscopically.

The dissected myocardium was kept submerged in a bath of ice-cold PBS and cleaned thoroughly. The tissues were then embedded in OCT compound (Miles Laboratories) and snap-frozen in acetone cooled with dry ice. The samples were sectioned serially at 6-μm thickness. The sections were subjected to immunohistochemical staining as described previously [7].

Animal preparation and experimental protocols. All experiments in this study were performed in accordance with the Guidelines for Animal Experiments of Kyoto University established in 1988.

Male Wistar rats, weighing 250–300 g, were anesthetized by intraperitoneal injection of sodium pentobarbital (20 mg/kg). Rats were tracheotomized, intubated, and mechanically ventilated on a rodent respirator (Harvard Apparatus, Southnatik, MA) at 60 breaths per minute and a tidal volume of 4.0 mL per breath. Polyethylene catheters were placed in the right internal carotid artery and the right femoral vein for measurement of arterial blood pressure and infusion of the drugs. The arterial blood pressure and the electrocardiogram were monitored continuously.

Coronary arteries were ligated by a previously described technique [11]. Briefly, a left thoracotomy was performed through the 4th or 5th intercostal space and the heart was exposed. The left coronary artery (LCA) was ligated near its origin with 6-0 silk. Ischemia was produced by tightening the previously placed ligature around the LCA to completely occlude the vessel. Ischemia was confirmed in all rats by discoloration of the ischemic area under direct vision and the ST segment elevation on the monitor electrocardiogram. The anti-LOX-1 monoclonal antibody (Group A; $n = 7$) or normal immunoglobulin G (IgG) (Group B; $n = 7$) was administered 15 min before and 30 min after coronary ligation in a total dose of 5 mg/kg. The ligature was untied after 1 h and the ischemic myocardium was reperfused for 2 h. Reperfusion was confirmed by the return of color to the ischemic area. Control rats has received normal saline (Group C; $n = 7$).

Detection of DNA fragmentation. The cells were subjected to the terminal deoxynucleotidyl transfer-mediated end labeling of fragmented nuclei (TUNE assay) and TUNEL-positive cells were quantified as previously described [7,10].

Measurement of myocardial infarct size. On completion of the 2-h reperfusion period, the ligature around the LCA was reoccluded and 1 mL of 2.5% patent blue violet (Wako Pure Chemical Industries, Osaka, Japan) was injected via the femoral catheter to differentiate the area at risk. The heart was rapidly removed and placed in ice-cold 0.9% saline, and atria, right ventricle, and great vessels were removed. The left ventricle was sliced transversely into sections 2 mm thick. The unstained portion of the myocardium, which represents the risk area, was separated from the stained portion of the myocardium. The heart was sectioned further into 1 × 1-mm pieces, followed by incubation with 1% solution of triphenyltetrazolium chloride (Wako Pure Chemical Industries, Osaka, Japan) in phosphate buffer for 20 min at 37 °C, pH 7.4. The irreversibly injured or necrotic portion (i.e., unstained) of the myocardium in the risk area was separated from the stained portion of the myocardium (i.e., ischemic but non-necrotic area). All three portions of the myocardium (i.e., non-ischemic, ischemic non-necrotic, and ischemic necrotic) were weighed individually. The results are expressed as the percentage of risk area to the total left ventricle and infarct area to the risk area.

Statistical analysis. All values are expressed as means ± SD. Comparisons were assessed by Student's t test, the paired t test, or the multiple comparison test with post hoc analysis. Differences were considered significant at $P < 0.05$.

Results and discussion

Expression of LOX-1 in rat cardiomyocytes following ischemia-reperfusion

Since oxidant stress is a major contributor in ischemia-reperfusion injury, we examined whether oxidant stress by hydrogen peroxide can express LOX-1 in cardiac myocytes. Neonatal rat cardiac myocytes were incubated in the presence of hydrogen peroxide (10^{-5} M) or saline for 24 h and subjected to immunocytochemical staining using anti-LOX-1 monoclonal antibody. As shown in Figs. 1A–C, positive brown signals indicating LOX-1 were observed in cardiac myocytes stimulated with hydrogen peroxide (B) but not in cells treated with saline (A). The immunoreactive signals were mainly localized in the cytoplasm of cardiac myocytes. Brown signals were not observed in cells stimulated with hydrogen peroxide when we substituted normal mouse IgG for the primary antibody (C). In our preparation of primary cardiac myocytes from neonatal rats, nearly 5% of cells are non-myocytes, mainly fibroblasts. However, all reactive cells possessed myofibrils, indicating that the LOX-1-expressing cells were cardiac myocytes. Non-myocytes appeared to be negative for LOX-1. We observed heterogeneous staining with anti-LOX-1 antibody at 24 h after the stimulation with hydrogen peroxide. At this stage, few cells exhibited morphological characteristics of apoptosis or positivity for TUNEL assay. However, significant percentages of these cells underwent apoptosis at 48 h after the stimulation. It should be clarified whether heterogeneous immunostaining for LOX-1 observed at 24 h after the

Fig. 1. Immunohistochemical staining of LOX-1 in rat cardiac myocytes. (A–C) Neonatal rat cardiac myocytes were incubated in the presence of hydrogen peroxide (10^{-5} M; B) or saline (A) for 24 h and subjected to immunocytochemical staining using anti-LOX-1 monoclonal antibody. In C, we substituted normal mouse IgG for the primary antibody. Rats were subjected to ischemia alone (E), ischemia-reperfusion (F) or sham operation (D). LV myocardium from these rats were subjected to immunohistochemistry for LOX-1. Arrow heads indicate an intramyocardial vessel wall.

stimulation represents cell fate (apoptosis or survival) at later stages.

Next, we examined whether LOX-1 expression in cardiac myocytes is actually induced in ischemia-reperfused rat myocardium in vivo. As shown in Figs. 1D–G, immunohistochemistry demonstrated that positive brown signals indicating LOX-1 immunoreactivity were observed in the myocardium subjected to ischemia-reperfusion (F and G) but not in the myocardium subjected to ischemia alone (E) or sham operation (D). In the ischemia-reperfused heart, almost all cardiac myocytes possess positive signals (F). However, cells constituting vessel walls and cells in the perivascular fibrosis area were barely positive for LOX-1 (G).

The question of the identity of the stimulus that induces LOX-1 expression after reperfusion then arises. Several studies have shown that a large quantity of reactive oxygen species is produced following reperfusion [2,3]. We demonstrate here that stimulation with hydrogen peroxide markedly induces LOX-1 expression in cultured neonatal rat cardiac myocytes. These findings suggest that oxidant stress is one of the stimuli that induce LOX-1 expression after reperfusion.

Effect of anti-LOX-1 monoclonal antibody on myocardial ischemia-reperfusion injury

We previously reported that stimulation of cardiac myocytes with hydrogen peroxide induces apoptosis at 48 h after the stimulation [10]. To investigate the role of LOX-1 in the development of hydrogen peroxide-induced apoptosis, cardiac myocytes were treated with saline or hydrogen peroxide (10^{-5} M) in the presence of 3.9×10^{-2} mg/mL of normal mouse IgG or anti-LOX-1 antibody for 48 h. Then these cells were subjected to TUNEL assay for the detection of DNA fragmentation, a hallmark of apoptosis. As shown in Fig. 2, stimulation with hydrogen peroxide increased the percentage of TUNEL-positive cells, compatible with our previous report [10]. Administration of anti-LOX-1 antibody largely inhibited this increase, suggesting a requirement of LOX-1-dependent pathway for hydrogen peroxide-induced apoptosis in cardiac myocytes.

In order to clarify the role of LOX-1 in the extent of myocardial infarction size following ischemia-reperfusion, we examined whether administration of anti-LOX-1 antibody reduces infarction size. Rats were randomly divided into the following three groups; administration of anti-LOX-1 antibody (group A), normal mouse IgG (group B) or saline (group C). We have previously shown that the administration of this anti-LOX-1 antibody blocks apoptosis induced by LOX-1 expression in cultured neonatal rat cardiac myocytes [7]. Hemodynamic data for the three groups are summarized in Table 1. Although premature ventricular beats are common during the occlusion and reperfusion periods, only 6 rats died before completion of the full experimental protocol. Mortality rates were equal among groups A, B, and C (22%). The presented data come from 21 rats that completed the full experimental protocol. There were no significant differences in heart rate, mean arterial blood pressure, or rate-pressure product among the three groups.

As shown in Fig. 3, the average risk area was $37.6 \pm 8.7\%$ of the LV mass for Group A, $34.0 \pm 8.9\%$ for Group B, and $39.2 \pm 12.2\%$ for Group C, with no significant difference among the three groups. Rats treated with normal IgG (Group B) exhibited an infarct size ($40.6 \pm 10.2\%$ of the risk area) similar to that of saline-treated rats (Group C, $38.3 \pm 11.5\%$). However, treatment with anti-LOX-1 monoclonal antibody (Group A) resulted in a significant decrease ($P < 0.05$) in infarct size to $21.2 \pm 17.2\%$, representing a nearly 50% reduction in the extent of infarction compared with treatment with normal IgG or saline. Thus, administration of anti-LOX-1 antibody reduces myocardial infarction size following ischemia-reperfusion in rats.

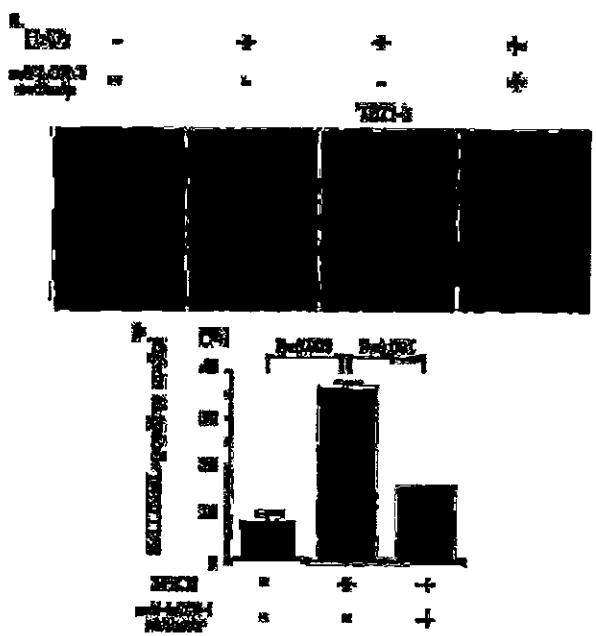

Fig. 2. The increase of TUNEL-positivity in hydrogen peroxide-stimulated cardiac myocytes requires LOX-1-dependent pathways. Cultured cardiac myocytes were stimulated with hydrogen peroxide (10^{-5} M; the right two panels) or saline (the most left panel) for 48 h in the presence of 3.9×10^{-2} mg/mL anti-LOX-1 antibody or normal mouse IgG as indicated. These cells were subjected to quantitative analysis by TUNEL staining as described in Materials and methods. The results are means ± SE of three independent experiments.

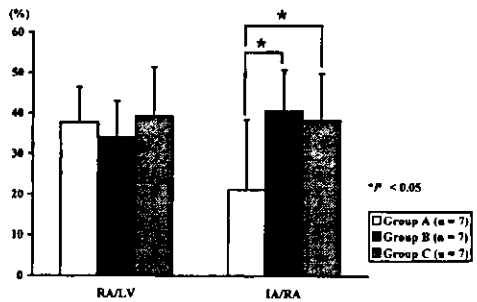

Fig. 3. Effect of anti-LOX-1 monoclonal antibody on myocardial infarction size. RA/LV represents the percentage of risk area relative to total left ventricular weight. Infarct size (IA/RA) is calculated from (infarct area/risk area) ×100%. The data are expressed as means ± SD. Treatment with anti-LOX-1 monoclonal antibody (Group A) significantly reduced the infarct size compared with normal IgG-treated (Group B) or saline-treated (Group C) rats. *$P < 0.05$ vs. Groups B and C.