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CLINICAL PERSPECTIVE

Transplantation of stem or progenitor cells has the potential to improve and restore cardiac function. To date, experimenters investigating the possible therapeutic effects of stem cells in the heart have used models of infarction, and little information is available about the therapeutic potential of cell transplantation for heart failure due to dilated cardiomyopathy. In the present study, we demonstrated that transplantation of stem cells improved cardiac function in a model of myocarditis. We found evidence that stem cells may work to improve heart function by both myogenesis and angiogenesis while inhibiting myocardial fibrosis. Based on our data, part of the mechanism for this improvement may occur through the action of stem cells as a source of growth factors and cytokines in the heart. This study supports the overall notion that mesenchymal stem cells transplanted into the failing heart have potential as a new therapeutic strategy for the treatment of dilated cardiomyopathy.

Invited Review

Am J Physiol Regul Integr Comp Physiol 288: R1432–R1437, 2005;
doi:10.1152/ajpregu.00662.2004.

Adrenomedullin: angiogenesis and gene therapy

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Nagaya, Noritoshi, Hidezo Mori, Shinsuke Murakami, Kenji Kangawa, and Soichiro Kitamura. Adrenomedullin: angiogenesis and gene therapy. *Am J Physiol Regul Integr Comp Physiol* 288: R1432–R1437, 2005; doi:10.1152/ajpregu.00662.2004.—Adrenomedullin (AM) is a potent, long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma. AM signaling is of particular significance in endothelial cell biology since the peptide protects cells from apoptosis, promotes angiogenesis, and affects vascular tone and permeability. The angiogenic effect of AM is mediated by activation of Akt, mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2, and focal adhesion kinase in endothelial cells. Both AM and its receptor, calcitonin receptor-like receptor, are upregulated through a hypoxia-inducible factor-1-dependent pathway under hypoxic conditions. Thus AM signaling plays an important role in the regulation of angiogenesis in hypoxic conditions. Recently, we have developed a nonviral vector, gelatin. Positively charged gelatin holds negatively charged plasmid DNA in its lattice structure. DNA-gelatin complexes can delay gene degradation, leading to efficient gene transfer. Administration of AM DNA-gelatin complexes induces potent angiogenic effects in a rabbit model of hindlimb ischemia. Thus gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of tissue ischemia. Endothelial progenitor cells (EPCs) play an important role in endothelial regeneration. Interestingly, EPCs phagocytose ionically linked DNA-gelatin complexes in coculture, which allows nonviral gene transfer into EPCs. AM gene transfer into EPCs inhibits cell apoptosis and induces proliferation and migration, suggesting that AM gene transfer strengthens the therapeutic potential of EPCs. Intravenous administration of AM gene-modified EPCs regenerate pulmonary endothelium, resulting in improvement of pulmonary hypertension. These results suggest that in vivo and in vitro transfer of AM gene using gelatin may be applicable for intractable cardiovascular disease.

regeneration; endothelium; ischemia; pulmonary hypertension

ADRENOMEDULLIN (AM) IS A POTENT, long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma (36). The peptide consists of 52 amino acids with an intramolecular disulfide bond, sharing slight homology with calcitonin gene-related peptide and amylin. Immunoreactive AM is detected in plasma and a variety of tissues including, blood vessels, heart, and lungs (19). Particularly, AM shows a variety of effects on the vasculature that include vasodilatation (23), regulation of permeability (16), inhibition of endothelial apoptosis (31), and promotion of angiogenesis (1, 35, 60). In addition, AM has protective effects against vascular injury, including oxidative stress (33, 69, 84). It is becoming clear that either activation or disruption of AM signaling might contribute to many pathological conditions, including hypertension (22), congestive heart failure (55), pulmonary hypertension (29), neoplastic growth (39), and inflammatory disease (59). To date, the major biological acitivities of AM in vitro and in vivo are *1)* vasodilation, *2)* diuresis and natriuresis, *3)* positive inotropic effect, *4)* inhibition of endothelial cell apoptosis, *5)* induction of angiogenesis, *6)* inhibition of cardiomyocyte apoptosis, *7)* suppression of aldosterone production, *8)* anti-inflammatory activity, and *9)* antioxidant activity. We and others have demonstrated that intravenous administration of AM decreases systemic and pulmonary arterial pressure and induces diuresis and natriuresis (47, 52, 65), suggesting that AM is involved in the regulation of vascular tone and body fluid. Subsequent studies have demonstrated beneficial hemodynamic effects and direct cardioprotective effects of AM infusion in the treatment of congestive heart failure (57, 61–64).

Until recently, only vascular endothelial growth factor (VEGF) (80), fibroblast growth factor (68), platelet-derived growth factor (37), and angiopoietin (74) were known to have profound angiogenic effects. More recently, however, the angiogenic potential of AM has attracted investigators' attention (35, 41, 59, 81). A previous study has shown that vascular abnormalities are present in homozygous AM knockout mice (70), suggesting that AM is essential for vascular morphogenesis. AM activates the PI3K/Akt-dependent pathway in vascular endothelial cells (58), which is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation (27). These findings raise the possibility that AM plays a role in modulating angiogenesis and neovascular-

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ization. This review focused on the angiogenic effects of AM and the therapeutic potential of AM gene transfer for the treatment of intractable cardiovascular disease.

ENDOGENOUS AM PRODUCTION IN ISCHEMIC CONDITIONS

Hypoxia (14, 53) and cytokine production (73) in ischemic heart disease or septic shock, as well as shear stress (7) in hypertension and heart failure induce AM secretion by vascular cells (Fig. 1). We have shown that plasma AM level is increased in patients with acute myocardial infarction (40, 49), peripheral arterial occlusive disease (75), and congestive heart failure (28, 55). Tissue levels of AM peptide and mRNA are also markedly increased in ischemic myocardium (18, 50) and failing heart (8, 56, 78, 82). These findings suggest that expression of AM is upregulated under tissue ischemia and inflammation, both of which are associated with neovascularization. An in vitro study has demonstrated that AM is upregulated through a hypoxia-inducible factor-1 (HIF-1)-dependent pathway under hypoxic conditions (14). Thus hypoxia/HIF-1 is one of the most potent regulators of AM production (Fig. 1). A recent study has demonstrated that heterozygous AM knockout mice [AM(+/−)] show significantly less blood flow recovery with less collateral capillary development than their wild-type mice (20). Administration of AM promotes blood flow recovery and capillary formation in AM(+/−) mice. These findings suggest that endogenous AM may play an important role in the regulation of angiogenesis under ischemic conditions. Considering the angiogenic potency of AM, increased endogenous AM represents a compensatory mechanism as an angiogenic factor promoting neovascularization under hypoxic conditions.

ANGIOGENIC EFFECTS OF AM AND ITS SIGNALING PATHWAY

AM signaling is of particular significance in endothelial cell biology since the peptide protects cells from apoptosis (31), promotes angiogenesis (35, 60), and affects vascular tone (23). Angiogenesis is a multistep process that involves migration and proliferation of endothelial cells, functional maturation of the newly assembled vessels, and remodeling of the extracellular matrix (26). Akt, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2), and focal adhesion kinase (p125FAK) play an important role in angiogenesis in endothelial cells. Kim et al. (35) demonstrated that AM activated Akt, MAPK/ERK1/2, and p125FAK in human umbilical vein endothelial cells (HUVECs), and produced increases in their DNA synthesis and migration. AM induced tube formation in HUVECs, and its effect was inhibited by pretreatment with a phosphatidylinositol 3′-kinase (PI3K) inhibitor or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)1/2 inhibitor. These findings suggest that AM exerts angiogenic activities through activation of Akt, MAPK, and p125FAK in endothelial cells (Fig. 1). In vivo, overexpression of AM augments collateral flow in ischemic tissues partly through activation of endothelial nitric oxide synthase (eNOS) (1). Earlier studies have shown that the vasodilatory effects of AM are mediated by cAMP/protein kinase in smooth muscle cells (SMCs) (23) and by the eNOS/NO pathway in endothelial cells (17). Thus AM-induced angiogenesis and vasodilation may synergistically improve blood perfusion in ischemic tissues.

Recently, a seven-transmembrane G-protein-coupled receptor, calcitonin receptor-like receptor (CRLR), and receptor activity modifying proteins (RAMPs) have been recognized as integral components of the AM signaling system (38, 43). CRLR has demonstrated the expression of the transcript predominantly in microvascular endothelial cells. This finding supports the view that CRLR is potentially a major mediator of the effects of AM on the vasculature. The effect of AM on CRLR is modified by RAMP2 and RAMP3. The angiogenic effect of AM is mediated by CRLR/RAMP2 and CRLR/RAMP3 receptors (Fig. 1). VEGF and AM act synergistically to induce angiogenic-related effects on endothelial cells in vitro (11). However, blocking antibodies to VEGF cannot significantly inhibit AM-induced capillary tube formation by

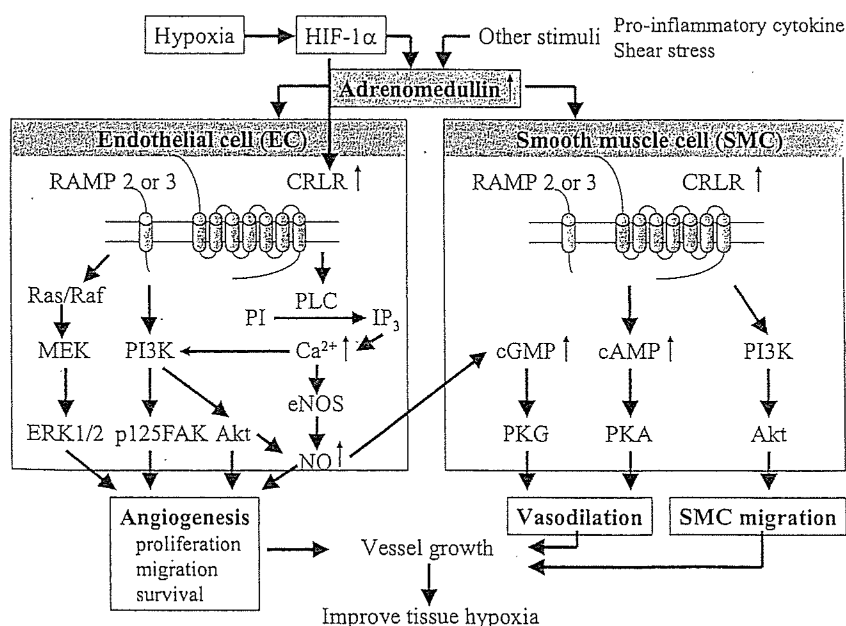

Fig. 1. Signaling pathway of adrenomedullin (AM) in vascular endothelial cells and smooth muscle cells. Both AM and calcitonin-receptor-like receptor (CRLR) are upregulated through a, hypoxia-inducible factor-1 (HIF-1)-dependent pathway under hypoxic conditions. AM binds to CRLR modified by receptor-activity-modifying protein 2 (RAMP2) and RAMP3. AM induces angiogenesis through activation of Akt, MAPK, and p125FAK in endothelial cells. AM also induces SMC migration and vasodilation. These activities synergistically improve tissue ischemia. MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; p125FAK, focal adhesion kinase; PLC, phospholipase C; PI, phosphatidylinositol; IP_3, inositol triphosphate; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; cGMP, guanosine 3′,5′-cyclic monophosphate; PKG, protein kinase G; PKA, protein kinase A.

HUVECs, indicating that AM does not function indirectly through upregulation of VEGF. Interestingly, AM and CRLR are both upregulated under hypoxic conditions in microvascular endothelial cells, although expression of RAMPs is not activated by hypoxia in microvascular cells (54). The activity of the CRLR promoter under hypoxic conditions is regulated at least in part through hypoxia-responsive regulatory element binding transcription factor HIF-1. Thus the simultaneous transcriptional upregulation of CRLR and its ligand AM in endothelial cells might play a significant role in the vascular responses to hypoxia and ischemia by creating a potent survival loop.

SMCs are essential for the generation of functional and mature blood vessels (26). We demonstrated in vivo that intramuscular administration of AM increased the number of αSMA-positive cells involved in the formation of vascular structures (25). In vitro, AM enhanced SMC migration, which was inhibited by wortmannin, a PI3K inhibitor. Recent studies using homozygous AM knockout mice have suggested that AM is essential for vascular morphogenesis (6, 21, 70). Taking these findings together, it is possible that AM contributes to vessel maturation through enhancement of SMC migration via a PI3K/Akt-dependent pathway (Fig. 1). This feature of AM-induced angiogenesis is different from VEGF-induced angiogenesis, which is not associated with vessel muturation.

In tumor cells, inflammation and hypoxia increase AM expression, and the elevated expression of AM is associated with tumor neovascularization in xenografted endometrial tumors and renal cell carcinoma (12, 86). AM also acts as a tumor cell survival factor underlying human carcinogenesis. Thus hypoxia-induced AM plays a part in tumor angiogenesis in conjunction with VEGF, and facilitates tumor growth under hypoxic conditions. As angiogenesis is an essential process in tumor-host interactions for tumor growth, maintenance, and metastasis, finding ways to regulate the action of AM may provide a new avenue for developing anticancer therapy (16).

THERAPEUTIC ANGIOGENESIS

A variety of studies have demonstrated that AM gene delivery serves as therapeutic tool to protect the cardiovascular system, including the heart (9, 32, 85), kidney (83), and vasculature (2, 84). In this section, we describe the angiogenic potential of AM gene transfer using novel gene delivery systems.

Nonviral gene transfer. Peripheral vascular disease is a crucial health issue affecting an estimated 27 million people (5). Despite recent advances in medical interventions, the symptoms of some patients with critical limb ischemia fail to be controlled. Although gene therapy has been shown to be an effective approach for angiogenesis (10, 24, 72), it is still unsatisfactory because of the biohazard of viral vectors, low transfection efficiency, and premature tissue-targeting. Therefore, highly efficient and safe gene transfer is desirable. Recently, we developed a novel nonviral vector, gelatin hydrogel, which allows highly efficient and long-lasting gene transfer (13, 30, 81). Gelatin has been widely used as a carrier of protein because of its capacity to delay protein degradation (76, 77). Plasmid DNA is known to be negatively charged. Thus we used gelatin as a vector for gene therapy. Biodegradable gelatin was prepared from pig skin. The gelatin was characterized by a spheroid shape with a diameter of ~30 μm, water content of 95% and an isoelectric point of 9 after swelling in water (76, 77). After 2-h incubation, positively charged gelatin held negatively charged plasmid DNA in its positively charged lattice structure. DNA particles are released from the gelatin through its degradation. As a result, DNA-gelatin complexes can delay gene degradation, leading to efficient gene transfer (13, 30, 44, 81).

We examined whether nonviral vector gelatin-mediated AM gene transfer induces therapeutic angiogenesis in a rabbit model of hindlimb ischemia (81). Seven days after intramuscular injection of AM DNA-gelatin complexes, there was intense AM immunoreactivity surrounding the gelatin in the skeletal muscles. AM production in the AM-gelatin group was enhanced compared with that in the naked AM DNA group, which received plasmid AM DNA alone. Unlike AM production in the naked AM group, AM overexpression in the AM-gelatin group lasted for longer than 2 wk. Importantly, AM DNA-gelatin complexes induced more potent angiogenic effects in a rabbit model of hindlimb ischemia than naked AM DNA, as evidenced by significant increases in histological capillary density, calf blood pressure ratio, and laser Doppler flow. These results suggest that the use of biodegradable gelatin as a nonviral vector augments AM expression and enhances AM-induced angiogenic effects. AM DNA-gelatin complexes were distributed mainly in connective tissues. It is interesting to speculate that the delay of gene degradation by gelatin may have been responsible for the highly efficient gene transfer. Thus gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of severe peripheral vascular disease.

Cell-based gene transfer. Recently, transplantation of stem cells or progenitor cells has been shown to regenerate a variety of tissues. Endothelial progenitor cells (EPCs) have been discovered in adult peripheral blood (4, 79). EPCs are mobilized from bone marrow into the peripheral blood in response to tissue ischemia or traumatic injury, migrate to sites of injured endothelium, and differentiate into mature endothelial cells in situ (15, 34). Transplantation of EPC induces therapeutic angiogenesis in the ischemic heart or limb (34, 42, 71). However, some patients are refractory to conventional cell therapy because of insufficient cell number, poor survival, or impaired differentiation. Thus a novel therapeutic strategy to enhance the angiogenic properties of EPCs is desirable. Considering the variety of protective effects of AM on vascular endothelial cells, we hypothesized that AM gene transfer into EPCs would strengthen the therapeutic potential of EPCs. Genetically modified EPCs may serve not only as a tissue-engineering tool to reconstruct the vasculature but also as a vehicle for gene delivery to injured endothelium.

Here, we present a new concept for cell-based gene delivery into the vasculature, consisting of three processes (44). First, positively charged gelatin is readily complexed with negatively charged plasmid DNA. Second, EPCs phagocytose ionically linked plasmid DNA-gelatin complexes in coculture, which allows nonviral gene transfer into EPCs with high efficiency. Third, intravenously administered gene-modified EPCs are incorporated into injured vascular beds. This novel gene delivery system has great advantages over conventional gene therapy; it is nonviral and noninvasive, and it provides highly efficient gene targeting into the vasculature. These benefits

may be achieved mainly by the capability of EPCs to phagocytose DNA-gelatin complexes and to migrate to sites of injured endothelium. Genetically modified EPCs markedly secreted AM into the culture medium, and AM overproduction lasted for more than 2 wk. The proliferative activity of AM DNA-transduced EPCs exceeded that of nontransduced EPCs. Furthermore, AM gene transfer inhibited apoptosis of EPCs in vivo and in vitro. Thus ex vivo AM gene transfer strengthened the therapeutic potential of EPCs.

Primary pulmonary hypertension (PPH) is a rare, but life-threatening disease characterized by progressive pulmonary hypertension, ultimately producing right ventricular failure and death (67). Median survival in patients with PPH is considered to be 2.8 years from the time of diagnosis. Thus novel and effective therapy is needed for the treatment of pulmonary hypertension. Because endothelial dysfunction may play a role in the pathogenesis of pulmonary hypertension such as PPH (3), pulmonary endothelial cells may be a therapeutic target for the treatment of pulmonary hypertension. We have demonstrated that administration of AM peptide decreases pulmonary vascular resistance in patients with PPH (45, 46, 48, 51). Thus we investigated the effects of AM gene-modified EPCs on pulmonary hypertension in rats (44). AM gene-transduced EPCs were similarly incorporated into the pulmonary vasculature. Immunohistochemical analyses demonstrated that the transplanted EPCs were of endothelial lineage and formed vascular structures. Intravenous administration of AM-expressing EPCs significantly decreased pulmonary vascular resistance compared with EPCs alone (−39%). Kaplan-Meier survival curves demonstrated that rats with pulmonary hypertension transplanted with AM-expressing EPCs had a significantly higher survival rate than those given culture medium or EPCs alone. These findings suggest that AM gene-modified EPCs using gelatin may serve not only as a tissue-engineering tool to reconstruct the pulmonary vasculature, but also as a vehicle for gene delivery to injured pulmonary endothelium. This hybrid cell-gene therapy may be applicable for intractable cardiovascular disease, including ischemic heart disease. Thus genetic manipulation of stem cells opens new avenues for regenerative medicine.

GRANTS

This work was supported by the Research Grant for Cardiovascular Disease (16C-6) from the Ministry of Health, Labor and Welfare, Industrial Technology Research Grant Program in 2003 from New Energy and Industrial Technology Development Organization of Japan, Health and Labor Sciences Research Grants-Genome 005, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, and the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

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治療の歴史

血管新生療法

竹下　聡

はじめに

血管新生療法（therapeutic angiogenesis）[1]は，血管増殖因子やその遺伝子，あるいは骨髄や末梢血細胞を用いて血管新生を促進させ，組織虚血の改善を図る治療法である。循環器領域における初の遺伝子治療としても知られる vascular endothelial growth factor（VEGF）遺伝子を用いた血管新生療法が，米国の Isner らによって行われたのは 1994 年のことである[2]。現在までにすでに 10 年以上が経過し，遺伝子以外にも増殖因子蛋白，骨髄細胞，末梢血細胞などを用いたさまざまな治療が試みられ，その有効性も検証されつつある。各々の治療法の詳細は他稿に譲り，ここでは血管新生療法がどのように生まれ，どのように育ってきたか，その歴史を概説する。

血管新生療法の臨床応用まで

血管新生療法のコンセプトそのものは決して新しいものではない。80 年代後半には，ネコの虚血肢モデルに対して大網の脂質分画を投与し，虚血を改善させる試みが行われている。大網や脂肪細胞の再生医療への応用は最近のトピックであり，このような研究がすでに 20 年近く前に存在したことは興味に値する。これらの血管新生療法と Isner らが行ったそれとの差異は，後者が VEGF という血管内皮細胞に特異的な増殖因子を用いた点にある。90 年代初頭，Isner らは家兎の虚血肢モデルに VEGF 蛋白を投与し，血管新生療法の臨床応用を検討した。動脈投与，静脈投与，繰り返し投与，ヘパリンの併用などさまざまな投与法を検討し，投与法のいかんに関わらず，側副血行の促進には 100〜1000 μg の VEGF 蛋白が必要なことを明らかにした。しかしながら，大量の VEGF 蛋白を投与すると，投与した蛋白が全身を循環し，非目的部位へと到達するのは避け難い。血管増殖因子の全身への拡散は，糖尿病患者においては網膜症を悪化させ，癌患者では腫瘍血管の発達を促進させる。また，一部の血管増殖因子は NO を介した血管拡張作用を有し，遷延性低血圧を惹起する。事実，VEGF 蛋白を用いた血管新生療法の臨床試験では，低血圧を避けるために投与量が制限された。

大量の蛋白投与に伴う副作用を回避するために行き着いた結論が遺伝子を用いたローカルドラッグデリバリーであった。Isner らはカテーテルを用いて血管細胞へ VEGF 遺伝子を経皮的に導入し，それらの細胞から VEGF 蛋白を分泌させようと考え，表面が親水性ゲルでコーティングされた冠動脈形成術用バルーンカテーテル（ハイドロゲル・バルーンカテーテル）による遺伝子導入を試みた。ハイドロゲルは，狭窄部位におけるバルーン通過性を改善するために施されたコーティングであるが，Isner らはこのゲルにプラスミド DNA の水溶液をしみ込ませ，遺伝子キャリアとして用いた。通常の PTCA テクニックによりバルーンを目的部位へと進め，4〜8 気圧で 1 分間バルーンを拡張させることで遺伝子の血管壁への導入が可能であった。その導入効率はリポゾームによる遺伝子導入に比し 100 倍以上の高効率ではあったが，βガラクトシダーゼ遺伝子を用いた組織的検討では，導入部位のわずか 0.1% 以下の細胞にしか遺伝子発現が認められなかった[3,4]。このわずかな細胞によって血管新生を促進することが可能なのかが問題となるのだが，遺伝子の導入効率（transfection efficiency）と治療効率（therapeutic efficiency）とは同義ではない。遺伝子産物である増殖因子が細胞外へと分泌されれば，たとえ導入効率は低くとも，パラクリン効果が期待できる[5]。この仮説は動物実験によって検証された。すなわち，ハイドロゲル・バルーンカテーテルを用いて家兎虚血肢モデルに VEGF 遺伝子の導入を行うと，約 3 週間にわたりその発現が認められ，VEGF 蛋白の動脈内投与と同等以上の側副路発達効果が得られたのである。一方，末梢血中の VEGF 蛋白の濃度は ELISA による測定限界付近にあり，きわめて低値であった。つまり遺伝子の導入効率は低くとも，治療効果を得るに十分な VEGF の局所濃度が維持可能であり，逆に末梢血中濃度は希釈効果によって低く抑えられたのであ

たけした さとし：国立循環器病センター心臓血管内科

る。ここで忘れてならないのは，本法がプラスミドDNA以外には何らベクターを用いない遺伝子導入法（naked DNA アプローチ）である点で，臨床応用における高い安全性が期待された。

末梢動脈閉塞症に対する VEGF を用いた血管新生療法

1994年，Isnerらは血管新生療法の臨床試験を開始した[2]。この臨床試験は，循環器領域における初の遺伝子治療としても知られており，内科治療や外科治療不応性の重症末梢動脈閉塞症患者を対象として行われた。遺伝子治療から1〜2ヵ月で，血管造影上の新生血管出現が得られ，下肢疼痛や難治性潰瘍が消失した。副作用は下腿浮腫や良性血管腫など，一過性の軽微なものであった。しかしながら，バルーンカテーテルを用いた遺伝子導入は，動脈穿刺が不可能な例，下肢の動脈硬化が高度でカテーテルによるアプローチが困難な例，遺伝子導入に際し解離などの血管損傷リスクが高い例などには施行できない。そこで考案されたのが，虚血筋への遺伝子導入である[6]。Baumgartnerらは，VEGFプラスミドの筋注により，7〜8割の症例で血管造影上の側副路発達や臨床症状の改善を得ることに成功した[7]。この遺伝子導入法の単純化により，カテーテルでは治療困難であった症例にも血管新生療法が可能となり，その適応は大きく拡大することとなる。また，本法は心筋へも応用可能であり，虚血性心疾患に対する血管新生療法の臨床応用への契機となった。

虚血性心疾患に対する VEGF を用いた血管新生療法

90年代後半，Losordoらは胸部小切開法により重症狭心症患者の左室にVEGFプラスミドを筋注し，狭心症状の著明な改善と心筋シンチによる虚血所見の改善を得ることに成功した[8]。さらにLosordoらは，NOGAと呼ばれる心筋マッピングのシステムを用いて，心内膜側から経皮的にVEGF遺伝子の導入を行い，良好な治療効果を得た[9]。現在，このNOGAシステムを用いた経皮的遺伝子治療は，二重盲検試験によって検証中である。最近，類似のプロトコールを用いた臨床試験の結果がKastrupらにより報告されたが，VEGF治療群において左室壁運動の改善は認められたものの，自覚症状や心筋シンチ所見の改善は得られていない[10]。本法の有効性については，さらなる検討が必要である。

血管内皮前駆細胞の発見と細胞治療

遺伝子を用いた血管新生療法の臨床応用が進むなか，1997年，Asaharaらはヒト末梢血中のCD34陽性細胞の分画中に成熟内皮細胞へと分化しうる血管内皮前駆細胞（endothelial progenitor cell：EPC）が存在することを明らかにした[11]。これを契機として，遺伝子や蛋白を中心とした血管新生療法に，細胞移植を用いた血管新生療法の新しい流れが加わった。

EPCは血球血管芽細胞（ヘマンジオブラスト）と呼ばれる幹細胞より分化するが，成人では通常骨髄中にあり，末梢血中にはきわめてわずかしか存在しない。Kalkaらはヒト末梢血単核球からEPCを分離培養し，マウスの虚血肢モデルに投与することで下肢虚血の改善を得た[12]。一方，Shintaniらは自己骨髄由来単核球移植によって家兎虚血肢の血管新生が増強することを報告した[13]。移植された自己骨髄単核球が虚血組織における血管形成に参加，もしくは血管増殖因子を放出することで局所の血管新生を刺激したものと思われ，自己骨髄単核球細胞移植による血管新生療法の臨床応用への契機となった。

自己骨髄単核球細胞移植による血管新生療法

末梢動脈閉塞症に対する自己骨髄細胞移植の有用性は，2000年，国内3施設（久留米大学，関西医科大学，自治医科大学）によるTherapeutic Angiogenesis Using Cell Transplantation（TACT）trialにおいて示された[14]。全身麻酔下で採取した数百ccの骨髄液から単核球を分離後，虚血肢に移植することで，ABI（上肢・下肢血流比）は0.97ポイント増え，トレッドミル歩行距離は2.6倍に改善した。また，下肢疼痛は9割，皮膚潰瘍は8割の症例で改善した。同様のプロトコールを用いた多施設臨床試験がすでに実施されており，少なくとも本法の短期成績に関しては確立された治療法といっても過言ではない。

末梢血細胞を用いた血管新生療法の臨床応用に関しては，顆粒球コロニー刺激因子（granulocyte colony stimulating factor：G-CSF）を用いて末梢血中の単核球からCD34陽性細胞を分離したり，末梢血単核球細胞移植にアドレノメデュリンの局所投与を併用するなどさまざまな試みがなされている。その有効性に関してはまだ不明な点が多いものの，侵襲性の低さや細胞採取の容易さなど末梢血細胞移植の

メリットは大きく，今後の発展が期待される。

一方，虚血性心疾患に対する細胞治療に関しても，骨髄細胞の冠動脈内注入やNOGAシステムを用いた心筋内移植など，さまざまな臨床試験が進行中である。急性心筋梗塞患者の冠動脈内に骨髄単核球細胞を投与した初期の臨床試験では，梗塞サイズの減少や左室機能の改善，心筋バイアビリティーの改善が報告されているが，その治療効果については否定的な報告も少なくない。また，左室機能改善などの治療効果が血管新生によって得られたものなのか，あるいは心筋細胞の再生によるものなのか，その機序についても不明な点は多い。虚血下肢に対する細胞移植ほど確立された治療にはまだ至っていないというのが現状である。

おわりに

血管新生療法は血管増殖因子を用いた遺伝子治療として幕を開けた。しかしながら，遺伝子のパテント問題や倫理的ハードルの高さから，現在では細胞移植による血管新生療法が主流となりつつある。

虚血下肢に対する細胞移植の治療成績は良好であるが，臨床症状の改善にもかかわらず血管造影での改善を認めないことも少なくない。果たして細胞移植により血管新生が本当に促進されたのか？ 単に潰瘍の創傷治癒機転が促進されただけではないのか？ その治療機序に関してはいまだ不明な点が少なくはなく，今後の研究成果が期待される。

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Atherosclerosis 177 (2004) 353–360

ATHEROSCLEROSIS

www.elsevier.com/locate/atherosclerosis

Application of real-time RT-PCR to quantifying gene expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human abdominal aortic aneurysm☆

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Received 23 October 2003; received in revised form 13 May 2004; accepted 8 July 2004
Available online 11 September 2004

Abstract

Backgound: The relative expression levels of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), key regulators in remodeling of extracellular matrix, are considered to play a pivotal role in the development of abdominal aortic aneurysm (AAA). However, few data exist regarding quantitative assessment of their expression in clinical settings. *Methods:* In 22 patients with AAA who underwent graft replacement, tissue samples of the AAA and non-dilated aorta were obtained. Using a real-time RT-PCR method that enabled quantitative measurement of mRNA levels in small tissue samples, we determined gene expression levels of MMPs and TIMPs relative to that of glutaraldehyde 3-phosphate dehydrogenase in each sample. *Results:* The expression levels of the MMP-1 and -3 genes were significantly augmented in AAA compared with non-dilated regions (4.48 ± 2.01 versus 0.26 ± 0.12, $P < 0.01$ and 1.89 ± 1.00 versus 5.01 ± 0.97, $P < 0.05$, respectively). Although genes for TIMP-1, -2 and -3 tended to be upregulated in AAA, relative expression levels of MMP-1 to TIMP-1, MMP-1 to TIMP-2, MMP-1 to TIMP-3, and MMP-3 to TIMP-2 were still higher in AAA than in non-dilated regions (1.12 ± 0.63 versus 0.10 ± 0.03, 4.13 ± 1.12 versus 0.43 ± 0.11, 1.61 ± 0.59 versus 0.14 ± 0.03, and 7.81 ± 1.60 versus 2.56 ± 0.76, respectively, $P < 0.05$). *Conclusion:* These results demonstrate that the present real-time RT-PCR method is reliable for the determination of mRNA levels in small samples of vascular tissue and that disproportional expression of both MMP-1 and MMP-3 relative to TIMPs relates pathologically to the evolution of AAA.
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Keywords: Abdominal aortic aneurysm; Matrix metalloproteinase; Tissue inhibitor of metalloproteinase; Gene expression; Real-time RT-PCR

1. Introduction

The initiation and development of abdominal aortic aneurysm (AAA), a disease characterized by progressive degeneration of the abdominal aorta and a life-threatening prognosis, involve definite alterations of the structural components of the aortic wall, in which unregulated turnover

☆ Apart of this work was presented at the 52nd Annual Scientific Session, American College of Cardiology, in Chicago, and at the XIIIth International Symposium on Atherosclerosis in Kyoto, 2003.

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0021-9150/$ – see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2004.07.013

of the extracellular matrix is commonly demonstrated [1–4]. Matrix metalloproteinases (MMPs), which degrade extracellular structural proteins like elastin and collagen, and regulate tissue remodeling in a variety of pathophysiological conditions [5], could be implicated in the formation of AAA [6,7].

The activities of MMPs are regulated on multiple levels: transcription and translation of the inactive precursors (zymogens), posttranslational activation of zymogens by proteolysis, and interactions of mature MMPs with tissue inhibitors of metalloproteinases (TIMPs) [8]. Each of the four TIMPs known to date binds to and inactivates most of the MMPs [9], and expression of TIMP-1, -2, and -3 has been reported in aortic tissue [10–13]. Thus, the balance between MMP and TIMP expression is considered to regulate the net degeneration of extracellular matrix [14]. Carrell et al. [12] demonstrated that using competitive reverse transcription PCR (RT-PCR) method MMP-3 and TIMP-3 genes were characteristically overexpressed in AAA when compared with those in aortic occlusive tissues obtained from different patient groups. However, few data exist regarding the relative gene expression of MMPs and TIMPs in human samples of AAA, probably due to technical difficulties in measuring mRNA levels in relatively small samples of AAA and adjacent non-dilated tissue. In this study, we overcame this problem using real-time RT-PCR and analyzed gene expression levels of MMPs and TIMPs in the wall of AAA and non-dilated aorta. Tissue and cellular localization of MMPs and TIMPs was also examined by immunohistochemical staining.

2. Subjects and methods

2.1. Subjects

The protocol of this study was approved by the institutional ethical committee. Written informed consent was obtained from all 22 patients who underwent elective graft replacement for AAA (20 males and 2 females; mean age, 71.3 ± 1.6 years). None of the AAA patients suffered from clinically unstable state such as rupture before surgery. The diameter of AAA measured by computed tomography ranged from 50 to 68 mm (mean, 56 ± 19 mm). The prevalence of risk factors for AAA was as follows: hypertension in 18, hyperlipidemia in 14, smoking in 12, and diabetes mellitus in 5 out of 22 patients.

2.2. Aortic samples

During graft replacement for AAA, a strip of aortic wall that contained the dilated region and lacked mural thrombus was carefully excised. An infra-renal aortic strip without dilation was also obtained from seven patients as control. Mean wet weight of the AAA and non-dilated specimens was 297.9 and 83.2 mg, respectively. All the samples were quickly frozen in liquid nitrogen and stored at −80 °C until extraction of RNA. A part of the tissue was placed in tissue fixative (Histochoice, Hedwin, Baltimore) for immunohistochemical evaluation.

2.3. RNA preparation and cDNA synthesis

The samples were homogenized in 1.0 ml ISOGEN™ reagent (Nippon Gene, Tokyo, Japan), thoroughly mixed with 0.2 ml chloroform, and centrifuged at 15,000 × g for 15 min at 4 °C.

The aqueous supernatant was transferred into a micro test tube, mixed with 0.6 ml isopropanol, and centrifuged at 15,000 × g for 15 min at 4 °C. The precipitated total RNA was rinsed with 70% ethanol, and then resuspended in RNase-free water. The concentration and integrity of the extracted total RNA were assessed using spectrophotometry and formaldehyde/agarose gel electrophoresis. Then, 10 μg total RNA was treated with DNase Free™ reagent (Ambion, Austin, TX) for 60 min, and then reverse-transcribed with Superscript II™ (Invitrogen, Carlsbad, CA) at 37 °C for 60 min using random primers (TaKaRa, Tokyo, Japan). The volume of the resultant cDNA mixture was adjusted to 100 μl by adding double-distilled water, and stored in small aliquots at −20 °C until further use. The integrity of each cDNA mixture was checked by amplification of glutaraldehyde 3-phosphate dehydrogenase (GAPDH) (Fig. 1A) with *ExTaq* (TaKaRa, Tokyo, Japan), using the primer set 5′-ACCACAGTCCATGCCATCAC-3′/5′-TCCACCACCCTGTTGCTGTA-3′.

2.4. Primers and probes for real-time RT-PCR

Using Primer Express™ software (Applied Biosystems, Foster, CA), several sets of primers were designed for each of the genes for MMP-1, -2, -3 and -9 and TIMP-1, -2 and -3. The primer set amplifying a target cDNA most effectively, which was estimated by electrophoresis and staining with ethidium bromide, was selected for final use (Fig. 1). Subsequently, the TaqMan probe inherent to each primer set was prepared, which was an oligonucleotide labeled with a reporter dye (FAM) at the 5′-end and a quencher dye (TAMRA) at the 3′-end. The sequences of the primers and TaqMan probes used in this study are summarized in Table 1.

2.5. Real-time RT-PCR

Real-time RT-PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The reaction solution was assembled in a volume of 25 μl, which comprised TaqMan Universal PCR Master Mix (Applied Biosystems), forward and reverse primers (final concentration 300 nM each), TaqMan probe (final concentration 200 nM) and cDNA mixture (25 ng). The conditions for real-time RT-PCR were preheating at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles of shuttle heating at 95 °C for 15 s and at 60 °C for 1 min. Throughout this study, the

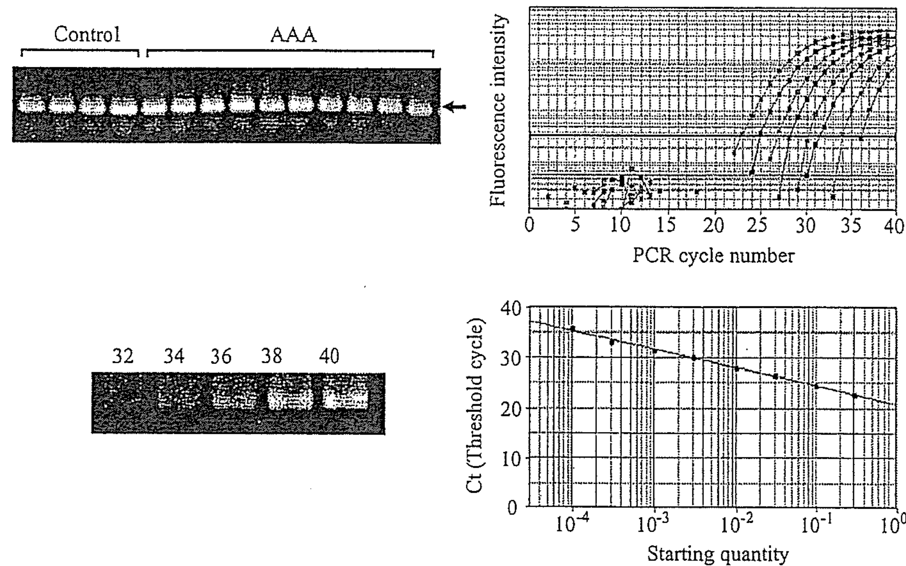

Fig. 1. Validation of cDNA synthesis and real-time RT-PCR. (Left) In electrophoretic analysis of RT-PCR for GAPDH, the amount of PCR product for GAPDH, indicated by an arrow, was equivalent among 12 representative AAA and 4 non-dilated (control) cDNAs. As for cycle-dependent amplification of MMP-1 cDNA, the numbers of cycles of PCR are shown above the electrophoretic gel. Note that the PCR product of MMP-1 was exponentially amplified according to the number of cycles. (Right) For the amplification curve of real-time RT-PCR, eight serially diluted standard cDNAs are shown. The ordinate is the logarithm of the fluorescent signal from the TaqMan probe for MMP-1 cDNA. The threshold, indicated by the horizontal line, was set at the signal intensity where the PCR products for MMP-1 exhibited exponential growth for all cDNAs. The intercept of the threshold and each amplification curve gave Ct (threshold cycle) for MMP-1 of each diluted standard cDNA. In the working standard of real-time RT-PCR for MMP-1, the abscissa is the logarithm of the dilution factor for the standard cDNA. The initial quantity of MMP-1 in a test cDNA mixture was predicted as the intercept of its Ct and this working standard.

cDNA mixture from a particular AAA sample was used to generate the working standard for quantitation of the cDNA of interest, which plots the relationship between the dilution of the standard cDNA and the corresponding Ct value (the number of cycles necessary to obtain a threshold fluorescent signal) (Fig. 1). The initial quantity of the cDNA of interest in a certain cDNA mixture was calculated from the working standard and then normalized to that of GAPDH determined with Pre-developed TaqMan Assay Reagent Endogenous Control^{TM} (Applied Biosystems). The normalized value for each target cDNA reflects the expression level of the corresponding gene in a test sample relative to the standard tissue sample.

The methodological validity of real-time RT-PCR used in the present study was verified by comparing with the conventional RT-PCR using *ExTq* [15]. Using cDNA mixtures of 250 ng, approximately 10 times as much as those used for real-time RT-PCR, we set up a series of RT-PCR with amplification cycles of 27, 30, 33 and 36. After acrylamide gel electrophoresis and SYBR Green staining, the target PCR products were quantified by densitometry (EDAS290, KODAK) and then plotted against PCR cycles.

2.6. Immunohistochemical study

After overnight fixation, the samples were embedded in paraffin and sectioned at 4 μm intervals. Tissue sections were deparaffinized with xylene followed by immersion in graded alcohol. They were washed three times for 5 min each in phosphate-buffered saline (PBS) and blocked with bovine serum albumin for 60 min. Specimens were then incubated with primary antibodies against CD68, MMP-1, -2, -3, -9 and TIMP-1, -2, -3 (Fuji Chemical, Tokyo, Japan) overnight at 4 °C. After they were washed in PBS, specimens were incubated with biotinylated rabbit anti-mouse IgG for 60 min at room temperature. Specimens were then washed with PBS and stained with horseradish peroxidase-conjugated streptavidin. The tissue sections were also stained with hematoxylin-eosin for histologic evaluation.

2.7. Data analysis

The mean and standard error of triplicate data are presented. Statistical analysis was performed by Mann–Whitney test using Stat View 5.0 software on a Macintosh computer. A P value less than 0.05 was considered significant.

3. Results

3.1. Quality of cDNAs and efficiency of real-time RT-PCR

Amplification of GAPDH was equivalent among all the cDNAs synthesized. Each primer set for PCR shown in Table 1 exponentially amplified its target cDNA depending

Table 1
Sequence of primer and probe

MMP-1 SENSE	5′-ACGGATACCCCAAGGACATCT-3′
MMP-1 ANTISENSE	5′-TCAGAAAGAGCATCGATATG-3′
MMP-1 TaqMan probe	5′-FAM-CAGCTCCTTTGGCTTC-CCTAGAACTGTGAA-TAMRA-3′
MMP-2 SENSE	5′-GGACAC ACTAAAGAAGATGC AGAAGT-3′
MMP-2 ANTISENSE	5′-CGCATGGTCTCGATGGTATTC-3′
MMP-2 TaqMan probe	5′-FAM-ACTGCCCCAGACAGGT-GATCTTGACC-TAMRA-3′
MMP-3 SENSE	5′-GAAATGAGGTACGAGCTG-GATACC-3′
MMP-3 ANTISENSE	5′-ATGGCTGC ATCGATTTTCCT-3′
MMP-3 TaqMan probe	5′-FAM-AGAGGCATCCACACCCTA-GGTTTCCCTC-TAMRA-3′
MMP-9 SENSE	5′-CCCGGAGTGAGTTGAACC A-3′
MMP-9 ANTISENSE	5'-GGATTTAC ATGGC ACTGCCA-3'
MMP-9 TaqMan probe	5′-FAM-ATGACATCCTGCAGT-GCCCTGAGGACTA-TAMRA-3′
TIMP-1 SENSE	5′-CTGCGGATACTTCC AC AGGTC-3′
TIMP-1 ANTISENSE	5′-GCAAGAGTCCATCCTGCAGTT-3′
TIMP-1 TaqMan probe	5′-FAM-CACAACCGCAGCGA-GGAGTTTCTCA-TAMRA-3′
TIMP-2 SENSE	5′-ATAAGCAGGCCTCCAACGC-3′
TIMP-2 ANTISENSE	5′-GAGCTGGACCAGTCGAAACC-3′
TIMP-2 TaqMan probe	5′-FAM-CTGTGGCCAACTGCAAAA AAAGCCTC-TAMRA-3′
TIMP-3 SENSE	5′-GC AGATAGACTC AAGGTGTGTGAAA-3′
TIMP-3 ANTISENSE	5′-TCCCTC ACTCTTAC ATGC AGAC A-3′
TIMP-3 TaqMan probe	5′-FAM-CCACTGCATGTCCC-AACCAGACTGTGT-TAMRA-3′

on the cycle number. Representative fluorescent curves of real-time RT-PCR for MMP-1 and the corresponding working standard are shown in Fig. 1. The working standard exhibited a linear relationship with a slope factor of −3.47 and correlation coefficient of >0.99. Similar results were obtained for the other target genes.

3.2. Expression levels of MMP and TIMP genes

Normalized values for MMP and TIMP expression in the AAA and non-dilated region are summarized in Table 2. The genes for MMP-1 and MMP-3 were significantly upregulated in AAA compared to non-dilated regions (Fig. 2). Enhanced expression of MMP-2 and MMP-9 was also observed in AAA, although it was not significant. Expression levels of TIMP-1, TIMP-2 and TIMP-3 genes also tended to be upregulated in AAA in comparison with those in non-dilated regions.

Overexpression of the MMP-1 gene was confirmed by conventional RT-PCR in which less amplification was required in AAA than in non-dilated regions to obtain the same amount of the PCR product, whereas GAPDH was equally obtained in every cycle of amplification (Fig. 3).

Table 2
MMP and TIMP levels in AAA and non-dilated aorta

mRNA	Control	AAA	*P* value
MMP-1	0.26 ± 0.12	4.48 ± 2.01	0.002
MMP-2	0.45 ± 0.12	0.76 ± 0.20	0.351
MMP-3	1.89 ± 1.00	5.01 ± 0.97	0.042
MMP-9	0.63 ± 0.31	1.69 ± 0.54	0.115
TIMP-1	2.22 ± 0.79	4.75 ± 1.52	0.302
TIMP-2	0.82 ± 0.14	1.28 ± 0.56	0.183
TIMP-3	1.66 ± 0.80	4.02 ± 1.35	0.408
MMP-1/TIMP-1	0.10 ± 0.03	1.12 ± 0.63	0.022
MMP-1/TIMP-2	0.43 ± 0.11	4.13 ± 1.12	0.009
MMP-1/TIMP-3	0.14 ± 0.03	1.61 ± 0.59	0.007
MMP-2/TIMP-1	0.29 ± 0.12	0.33 ± 0.16	0.261
MMP-2/TIMP-2	0.54 ± 0.08	0.87 ± 0.19	0.575
MMP-2/TIMP-3	0.37 ± 0.14	0.95 ± 0.45	0.855
MMP-3/TIMP-1	0.84 ± 0.12	1.80 ± 0.35	0.136
MMP-3/TIMP-2	2.56 ± 0.76	7.81 ± 1.60	0.035
MMP-3/TIMP-3	1.52 ± 0.38	2.76 ± 0.53	0.266
MMP-9/TIMP-1	0.43 ± 0.21	0.71 ± 0.22	0.611
MMP-9/TIMP-2	1.38 ± 0.59	1.72 ± 0.41	0.869
MMP-9/TIMP-3	0.65 ± 0.30	0.87 ± 0.29	0.971

Data are mean ± S.E.M.

3.3. Expression ratios of MMPs to TIMPs

All the combination ratios of four MMPs to three TIMPs examined in this study were higher in AAA than in control (Table 2). Among them, the expression ratios of four, MMP-1 to TIMP-1, MMP-1 to TIMP-2, MMP-1 to TIMP-3 and MMP-3 to TIMP-2, were significantly higher in AAA than in non-dilated regions (1.12 ± 0.63 versus 0.10 ± 0.03, 4.13 ± 1.12 versus 0.43 ± 0.11, 1.61 ± 0.59 versus 0.14 ± 0.03 and 7.81 ± 1.60 versus 2.56 ± 0.76, respectively, $P < 0.05$) (Fig. 4). It is of clinical interest to correlate the ratios of MMPs/TIMPs with the severity of AAA. However, there were no correlation between above four ratios of MMPs/TIMPs and size of AAA in the present study.

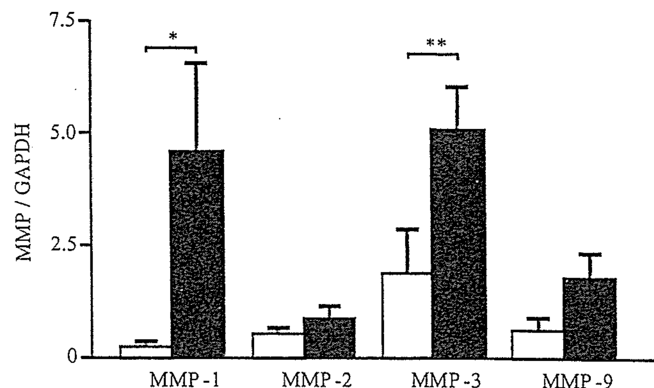

Fig. 2. Gene expression levels of MMPs in AAA and non-dilated regions. The ordinate is the relative cDNA quantity of an MMP normalized to that of GAPDH. Closed and open bars indicate AAA and non-dilated regions, respectively. Data are mean ± S.E.M. * $P < 0.01$, ** $P < 0.05$.

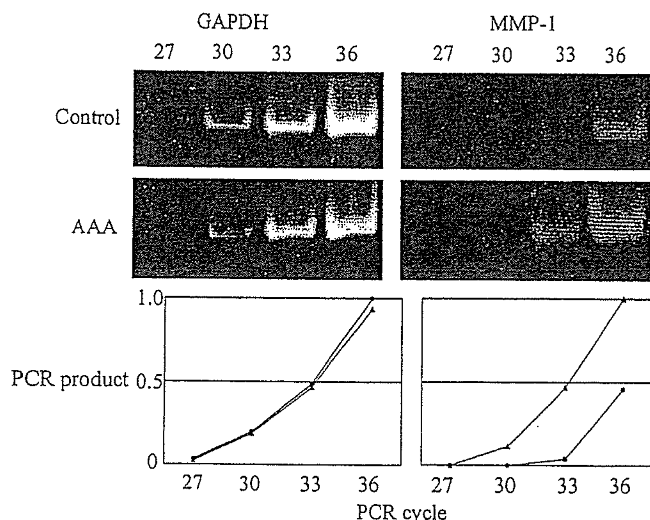

Fig. 3. Conventional RT-PCR for gene expression of GAPDH and MMP-1 in AAA and non-dilated (control) regions. (Upper) The result of gel electrophoresis and DNA staining for RT-PCR. The amplification cycles were presented above the gel. (Lower) The relationship of the amount and cycle of RT-PCR. The data for non-dilated and AAA regionas were presented in circles and triangles, respectively. In this representative case, the amounts of MMP-1 cDNAs for 30 and 33 cycles in AAA were larger than those for 33 and 36 cycles in non-dilated regions. Because PCR theoretically doubles the products through one cycle, the initial amount of cDNA for MMP-1 in this AAA was estimated to be nearly eight times as much as that in non-dilated regions, which was in good agreement with the data obtained by real time RT-PCR for the same sample with upregulation of 7.25.

3.4. Histological findings and immunochemical localization of MMPs and TIMPs

The specimens of AAA consisted of thinned or thickened vascular tissue where typical atheromatous plaques with infiltration of macrophages and lymphocytes were present. Under these conditions, macrophages in the deep layer of the intima and extracellular matrix of the lipid core were positive for MMP-1. Most macrophages were positive for MMP-3, which was also expressed in the extracellular matrix of the lipid core. There was scattered MMP-9 positivity in the macrophages and lymphocytes in the relatively deep layer. It was interesting that most macrophages positive for MMP-1 and MMP-3 were also positive for TIMP-1, which was also positive in the extracellular matrix. TIMP-2 and TIMP-3 were positive mainly in the macrophages in the intima (Fig. 5A). In the non-dilated tissue, there was mild atherosclerotic change and MMPs and TIMPs were scatteredly expressed in macrophages and/or extracelluar matrix (Fig. 5B).

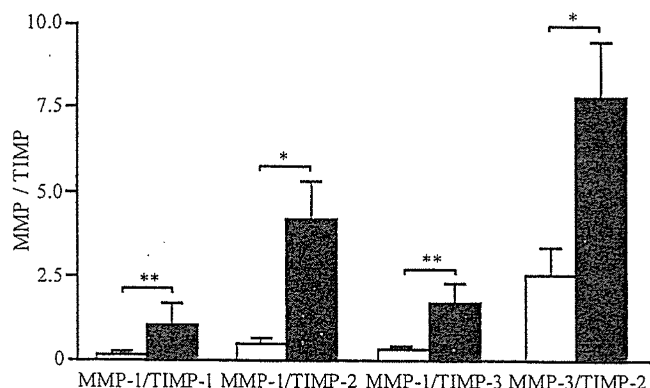

Fig. 4. Relative expression levels of MMPs versus TIMPs in AAA and non-dilated regions. The ordinate is the ratio of the normalized cDNA quantity of an MMP versus that of a TIMP. Closed and open bars indicate AAA and non-dilated regions, respectively. Data are mean ± S.E.M. * $P < 0.01$, ** $P < 0.05$.

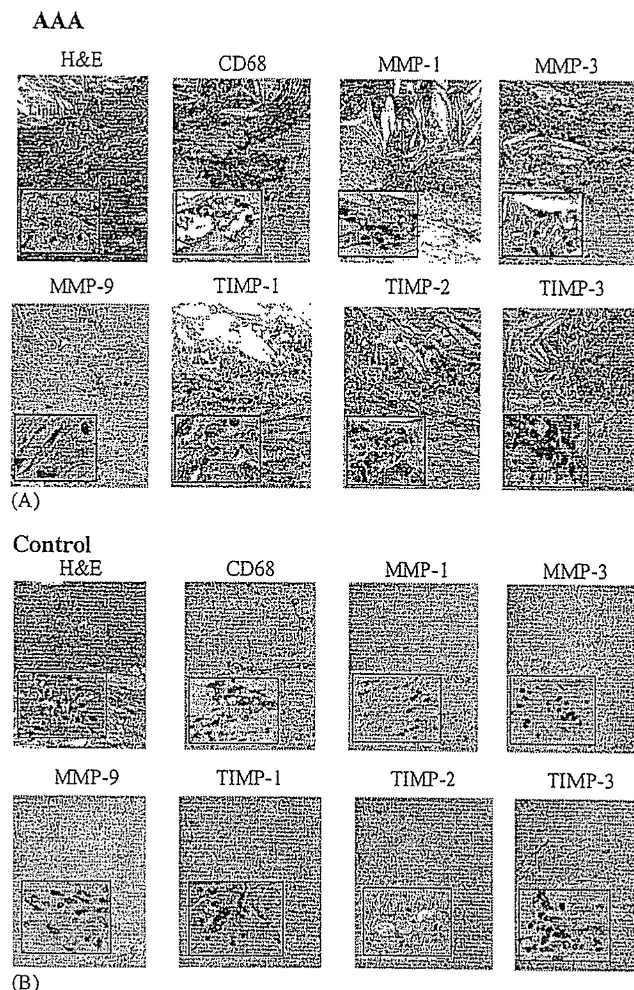

Fig. 5. Histological and immunohistochemical findings of subserial tissue sections from AAA and non-dilated (control) regions. (Upper) Hematoxylin-esosin (H & E) staining showed foam cells and lymphocytes infiltrating around the lipid core of a large atheromatous plaque (100× and 400×, original magnification). Macrophages stained by CD-68 in the deep layer of the intima and extracellular matrix of the lipid core were positive for MMP-1. Most macrophages were positive for MMP-3, which was also expressed in the extracellular matrix of the lipid core. Scattered MMP-9 positivity was observed in macrophages and lymphocytes in the deep layer of the intima. The extracellular matrix and macrophages in the intima were positive for TIMP-1. Most macrophages in the lipid core were also positive for TIMP-2 and TIMP-3. (Lower) In the non-dilated (control) region, there existed mild atherosclerotic change. Under these conditions, MMPs and TIMPs were less pronouncedly expressed in macrophages and/or extracelluar matrix than those in AAA.

4. Discussion

4.1. Methodological advantages

For detection and quantification of the mRNA of interest in AAA, we applied real-time RT-PCR method that had been already established for the quantitative evaluation of gene expression [16]. The principle of this technique is to measure the PCR product at each cycle by means of a fluorescence-labeled oligonucleotide probe, and to predict the initial amount of the cDNA of interest from Ct (the number of PCR cycles necessary to obtain the threshold signal of fluorescence). This technique makes it possible to quickly and accurately estimate the expression levels of many genes in small tissue samples from the aorta examined in the present study as well as even from the carotid and coronary artery tissues [17,18]. There are two critical parameters; the integrity of the cDNA synthesized and the amplification efficiency of the primers used for PCR. In the present study, these two parameters were assessed by conventional electrophoresis with ethidium bromide staining. The amount of PCR product for GAPDH, a representative housekeeping gene, was equivalent in all the samples, and each primer set for PCR exponentially amplified the target cDNA depending on the number of cycles. Therefore, ideal working standards could be generated for all the genes examined. In addition, real-time RT-PCR seems to have the higher sensitivity and equivalent quantitativeness as compared with conventional RT-PCR that requires the larger amount of cDNA for determination than that for the present method.

4.2. Dominant expression of MMPs over TIMPs in AAA

We showed that the gene expression of MMP-1 and MMP-3 was significantly augmented in AAA compared with that in non-dilated control regions. Although upregulation of either MMP-1 or MMP-3 in AAA was shown in different clinical studies [12,19], it is quite interesting that in the present study both MMP-1 and MMP-3 were demonstrated to be upregulated simultaneously in AAA. Carrell et al. [12] showed that expression of MMP-3 gene had highly significant increase in AAA over aortic occlusive tissues, although that of MMP-1 was not different between them. This may be explained by the possibility that MMP-1 gene was upregulated in aortic occlusive tissue [18] as well as that in AAA tissue, obscuring overexpression of MMP-1 in AAA.

MMP-1 specifically cleaves collagen types I, II, and III; which are key components of the extracellular framework of the arterial wall and major constituents of human atherosclerotic lesions and activate other MMPs such as MMP-2 and -9 [8] that degrade denatured collagen and elastin. MMP-3 digests collagen and several other extracellular matrix proteins. It also plays a crucial role in the pericellular MMP activation cascade by cleaving other MMP proenzymes such as MMP-1 and MMP-9 to their active form. Indeed, aneurysm formation in the thoracic and abdominal aorta was less frequent in mice with double knockout of the apoE and MMP-3 genes than in apoE knockout mice [20]. Thus, it is reasonable to consider that simultaneous upregulation of both MMP-1 and MMP-3 could have resulted in aggravation of AAA in the present cases. Tung et al. [21], however, conducted a gene expression survey in AAA using a cDNA macroarray technique, which failed to detect MMP-1 and MMP-3 transcript in either AAA or normal aorta. This apparent inconsistency with our and others' [12,18] results may be due to the sensitivity of signal detection with the cDNA macroarray, which is much lower than that with RT-PCR methods.

In a mouse model, elastase-induced aneurysmal degeneration of the abdominal aorta was suppressed by targeted gene disruption of MMP-9 [22]. Recently, administration of doxycycline, which mainly decreased MMP-9 [23] as well as MMP-1 [24] production, was shown to effectively prevent the formation of angiotensin II-induced AAA, providing indirect evidence for a role of MMP-9 and/or MMP-1 in AAA [25]. We observed 2.7-fold higher gene expression of MMP-9 in AAA compared with non-dilated regions, though this was not significant. Therefore, one might speculate that, in addition to MMP-1 and MMP-3, MMP-9 could be involved in the pathogenesis of AAA, although MMP-9 appears to have a protective role limiting atherosclerotic development [26].

TIMP-1, TIMP-2 and TIMP-3 all exhibited relatively higher expression in AAA than in non-dilated regions, although Carrell et al. [12] indicated that only TIMP-3 gene expression in AAA was significantly higher than that in aortic occlusive tissue. As observed in carotid occlusive tissue [18], TIMP-1 and -2 genes could be upregulated in aortic occlusive tissue which was used for comparison, obscuring the difference in expression of these genes in AAA and aortic occlusive tissues. TIMPs, which are produced by various types of cells including macrophages as observed in the present study, counteract most of the MMPs [9]. TIMPs can interfere with the proteolytic processing of MMP zymogens, as well as block substrate binding to activated MMPs which can perform their biological function after activation, and where local stoichiometric excess over endogenous inhibitors such as TIMPs prevails [27].

In a rat model in which decellularized segments of guinea pig aorta were orthotopically grafted into the abdominal aorta, aneurysmal formation of the grafted aorta was suppressed by TIMP-1 overexpression resulting from seeding of rat smooth muscle cells retrovirally transfected with the TIMP-1 gene [28]. Aneurysm formation in the thoracic and abdominal aorta was more frequent in mice with double knockout of the apoE and TIMP-1 genes than in apoE knockout mice [29]. Taken together, TIMPs seem to exert some inhibitory roles in the development of human AAA.

From this point of view, the most important finding of this study was that all the expression ratios of MMPs versus TIMPs were higher in AAA than in non-dilated regions. Particularly, the expression ratios of MMP-1/TIMPs and MMP-

3/TIMP-2, all of which were expressed in the extracellular matrix as well as in macrophages as also demonstrated by Newman et al. [30], were significantly higher in AAA. Augmentation of TIMP gene expression is considered to be an adaptive phenomenon to cope with rampant destruction of extracellular matrix by MMPs. The dominant expression of MMPs over TIMPs in AAA suggests that compensatory expression of TIMPs is insufficient to counteract the degenerative role of MMPs in the formation of AAA.

4.3. Implications and limitations

Inactivation of MMPs by pharmacological agents [25] or gene-targeting techniques [20] has been shown to effectively inhibit the development and progression of experimental AAA. The results of prolonged administration of doxycycline, a non-selective MMP antagonist, in patients with a small AAA suggested that doxycycline might have effects on AAA expansion through suppression of multiple MMPs [31]. In addition, several factors have been identified as positive regulators of the expression of TIMP genes, such as growth factors, phorbol esters and inflammatory cytokines [9]. Therefore, the effective manipulation to regulate the altered MMP/TIMP ratios as shown in the present study may further result in improving the clinical prognosis of AAA.

Although there was no correlation between MMPs/TIMPs and severity of AAA such as diameter, it is possible that velocity of aneurysm growth or changes in morphology associated with rupture may reflect different MMPs/TIMPs relationship. Examination of unstable patients in whom rapid changes in AAA diameter and/or presence of impending rupture can be monitored before surgery may clarify this issue.

One of the limitations of the present study was that as a control we used adjacent non-dilated tissue which might contain mild atherosclerotic lesions, because it was difficult to obtain normal aortic tissues from patients with AAA. It is interesting, however, to consider that the present study demonstrates the different gene expression of MMPs and TIMPs in AAA and adjacent tissue which may represent the pathophysiological condition of pre-aneurysmal aortic tissue.

In the present study, we used real-time RT-PCR, which gives an estimate of mRNA expression for each enzyme and inhibitor, and did not determine the enzyme activities. It may still be difficult, however, to extract these proteinases bound strongly to connective tissue and to quantitatively assay enzyme activities [32]. Therefore, evaluation of mRNA expression of MMPs and TIMPs by the present real-time RT-PCR method should be reliable for evaluation of the relative production of MMPs and TIMPs in clinical tissue samples. Further investigation of the regulatory mechanism of MMP and TIMP genes in which physiological and pathological stimulation upregulates an everbroader spectrum of MMPs [33] and other factors such as inflammatory cytokines [34] will provide a clue to the pathogenesis and to devising novel therapeutic agents for AAA, even at an established stage.

Acknowledgments

This work was supported in part by grants from the Ministry of Health and Welfare of Japan (to M.Y.), the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR) of Japan (to A.S.), and the Japan Cardiovascular Research Foundation (to A.S.).

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Optimum Collection and Storage Conditions for Ghrelin Measurements: Octanoyl Modification of Ghrelin Is Rapidly Hydrolyzed to Desacyl Ghrelin in Blood Samples, *Hiroshi Hosoda,[1,3] Kentaro Doi,[1] Noritoshi Nagaya,[2] Hiroyuki Okumura,[2] Eiichiro Nakagawa,[2] Mitsunobu Enomoto,[2] Fumiaki Ono,[2] and Kenji Kangawa[1,3*]* ([1] Department of Biochemistry, National Cardiovascular Center Research Institute, and [2] Department of Internal Medicine, National Cardiovascular Center, Osaka 565-8565, Japan; [3] Translational Research Center, Kyoto University Hospital, Kyoto 606-8507, Japan; * address correspondence to this author at: Department of Biochemistry, National Cardiovascular Center Research Institute, National Cardiovascular Center, Osaka 565-8565, Japan; fax 81-6-6835-5402, e-mail kangawa@ri.ncvc.go.jp)

Ghrelin is an acylated peptide with growth-hormone-releasing activity *(1)*. It was first isolated from rat and human stomach during the search for an endogenous ligand to the "orphan" G-protein-coupled receptor, growth hormone secretagogue receptor *(2)*. The peptide contains 28 amino acids, and n-octanoylation of the Ser-3 hydroxyl group is necessary for biological activity. Most studies have focused on the somatotropic and orexigenic roles of ghrelin; therefore, little is known about the kinetics of this peptide. Because the ester bond is both chemically and enzymatically unstable, elimination of the octanoyl modification of ghrelin can occur during storage, handling, and/or dissolution in culture medium *(3)*. Because of increased interest in ghrelin measurements, a standardized method of sample collection is required.

In the present study, which focused on the active form of ghrelin, we investigated the effects of anticoagulants and storage conditions on ghrelin stability. To distinguish the active form of ghrelin, we established two ghrelin-specific RIAs; N-RIA recognizes the N-terminal, octanoyl-modified portion of the peptide, whereas C-RIA recognizes the C-terminal portion. Thus, the value determined by N-RIA specifically measures active ghrelin, whereas the value determined by C-RIA gives the total ghrelin immunoreactivity, including both active and desacyl ghrelin *(4–6)*. The minimum detectable quantities in the N- and C-RIAs were 5.0 and 50 pmol/L, respectively. The respective intra- and interassay CV were 3% and 6% for the N-RIA and 6% and 9% for the C-RIA (n = 8 assays). Data are reported as the mean (SD). Comparisons of the time course of ghrelin concentrations between subgroups were made by two-way ANOVA for repeated measures, followed by the Scheffé test. $P < 0.05$ was considered statistically significant.

All blood samples were taken from three healthy male volunteers who gave written informed consent. Blood was taken from the forearm vein and immediately divided into tubes for serum and plasma preparation using (*a*) disodium EDTA (1 g/L) with aprotinin (500 000 kIU/L), (*b*) disodium EDTA alone, (*c*) heparin sodium, or (*d*) no anticoagulant. Synthetic human ghrelin was added to each blood sample at a final concentration of 40 μg/L; each sample was then sequentially divided into two aliquots for incubation at either 4 or 37 °C. After incubation for 0, 30, and 60 min, blood samples were centrifuged, diluted 1:200 in RIA buffer, and subjected to ghrelin-specific RIAs. A comparison of the effects of different anticoagulants on the detected ghrelin concentrations is shown in Table 1A. Although the serum and three different plasma samples tested gave comparable results for total ghrelin by C-RIA, the N-RIA gave ghrelin concentrations that were significantly decreased at 37 °C. When the ghrelin was measured by N-RIA, serum samples were highly affected by such treatment; samples stored for 60 min at 37 °C lost ~35% of the ghrelin compared with the basal values at 0 min ($P < 0.05$). The ghrelin concentrations in samples containing heparin as an anticoagulant were also significantly decreased ($P < 0.05$). When EDTA–aprotinin was used as the anticoagulant for plasma treatment, the decreases in ghrelin stability were smaller than for other procedures. Storage at 4 °C also improved ghrelin stability.

To explore optimum storage conditions, we examined the effect of plasma pH on ghrelin stability. The EDTA–aprotinin-treated plasma (n = 3) was divided into five samples; the pH was then adjusted to 3, 4, 5, 6, or 7.4 with 1 mol/L HCl. Synthetic human ghrelin was then added to each sample aliquot at a final concentration of 75 μg/L. Each of the five plasma aliquots was then subdivided into two, with one stored at 4 °C and the other stored at 37 °C. The effects of acidification on ghrelin stability in plasma are summarized in Table 1B. When stored at 37 °C, ghrelin concentrations measured by N-RIA gradually decreased at all pH values tested. However, ghrelin was most stable in highly acidified plasma samples (pH 3–4). At pH 3–5 and a storage temperature of 4 °C, the stability of ghrelin in plasma did not change significantly over a 6-h period. By C-RIA, ghrelin concentrations remained stable across the different pH and storage temperature conditions.

We then evaluated the effects of repeated freezing and thawing on the stability of ghrelin. EDTA–aprotinin-treated plasma samples were divided into two pH groups; one was acidified to pH 4, whereas the other was not acidified (pH 7.4). After the addition of synthetic human ghrelin (75 μg/L), we subjected the samples to four freeze–thaw cycles. Repeated freezing and thawing also influenced ghrelin stability (Table 1C). As in the N-RIA, ghrelin concentrations in untreated plasma samples decreased significantly with each successive freeze–thaw cycle, whereas the ghrelin remained relatively stable after acidification. Ghrelin concentrations by C-RIA were unchanged despite repeated freeze–thaw treatments in both acidified and untreated plasma samples.

As well as differences in assay methodologies, differences in sample handling, such as the method of storage, effects of anticoagulants, or previous freezing and thawing of the samples, could influence the reported values *(7–10)*. Instability of peptides and proteins can be divided into two forms: chemical and physical instability *(11, 12)*.

Table 1. Effect of anticoagulants and storage conditions on ghrelin stability.[a]

A. Ghrelin measurements in serum and different plasma samples

		Mean (SD) percentage of baseline					
		C-RIA			N-RIA		
		0 min	30 min	60 min	0 min	30 min	60 min
EDTA–aprotinin	37°C	100.0 (6.2)	101.0 (4.4)	102.9 (10.4)	100.0 (9.1)	102.6 (5.1)	89.6 (1.8)
	4°C		100.4 (4.0)	101.1 (4.5)		97.4 (1.3)	99.9 (12.3)
EDTA	37°C	100.0 (7.4)	98.5 (5.4)	98.4 (3.8)	100.0 (3.7)	83.6 (9.9)	85.3 (3.3)
	4°C		98.2 (2.6)	98.6 (7.6)		96.5 (1.8)	88.3 (6.5)
Heparin	37°C	100.0 (10.0)	104.3 (6.6)	91.1 (13.1)	100.0 (5.0)	88.1 (2.3)	77.4 (2.2)[b]
	4°C		104.4 (6.2)	102.1 (12.2)		92.5 (0.8)	86.9 (4.3)
Serum	37°C	100.0 (9.9)	100.4 (10.3)	98.2 (11.8)	100.0 (10.9)	87.5 (1.3)	65.1 (6.9)[b]
	4°C		96.2 (7.1)	98.4 (8.7)		96.5 (0.8)	94.6 (7.4)

B. Effects of storage pH, duration, and temperature on ghrelin stability

			Mean (SD) percentage of baseline					
		pH	0 h	1 h	2 h	3 h	4 h	6 h
C-RIA	RT	7.5	100.0 (6.6)	95.7 (5.0)	95.5 (6.3)		98.5 (5.4)	102.1 (6.5)
		6	100.0 (5.2)	96.0 (5.3)	91.3 (8.2)		96.6 (6.1)	97.9 (4.6)
		5	100.0 (6.1)	101.9 (8.2)	99.0 (6.0)		100.3 (8.4)	104.1 (5.2)
		4	100.0 (9.2)	96.0 (6.4)	98.0 (3.5)		100.2 (2.9)	97.1 (3.4)
		3	100.0 (2.2)	96.1 (4.9)	95.6 (4.7)		95.2 (2.7)	89.8 (3.9)
	4°C	7.5	100.0 (2.0)			100.9 (8.3)		99.3 (3.3)
		6	100.0 (3.6)			95.1 (4.1)		98.1 (1.8)
		5	100.0 (4.0)			101.2 (7.8)		105.9 (5.1)
		4	100.0 (7.1)			99.1 (3.3)		99.1 (4.1)
		3	100.0 (4.2)			99.4 (1.7)		101.3 (5.0)
N-RIA	RT	7.5	100.0 (5.0)	81.8 (1.0)[c]	72.0 (3.1)[d]		50.6 (2.9)[d]	37.5 (2.6)[d]
		6	100.0 (8.8)	93.3 (5.7)	78.8 (2.7)[c]		54.7 (1.2)[d]	38.8 (2.9)[d]
		5	100.0 (12.8)	92.8 (4.1)	86.4 (2.2)		74.1 (3.8)[b]	59.2 (7.6)[c]
		4	100.0 (12.5)	94.5 (7.4)	90.8 (5.2)		78.8 (3.1)[b]	74.0 (9.8)[c]
		3	100.0 (6.0)	98.4 (1.9)	96.9 (0.9)		82.2 (2.4)[b]	76.4 (6.1)[c]
	4°C	7.5	100.0 (6.9)			66.5 (4.4)		40.4 (3.5)[b]
		6	100.0 (6.1)			96.3 (0.2)		83.0 (1.2)[b]
		5	100.0 (5.5)			103.2 (2.0)		87.7 (11.7)
		4	100.0 (0.8)			102.2 (0.4)		99.6 (5.7)
		3	100.0 (12.8)			105.8 (0.3)		103.5 (1.6)

C. Effects of repeated freeze–thaw cycles on plasma ghrelin stability

		Cycles				
		1	2	3	4	5
C-RIA	HCl (−)	100.0 (8.8)	94.7 (7.2)	92.8 (6.6)	95.5 (5.1)	91.2 (7.3)
	HCl (+)	100.0 (5.2)	96.0 (5.3)	91.3 (8.2)	96.6 (6.1)	97.9 (4.6)
N-RIA	HCl (−)	100.0 (4.0)	89.8 (2.7)[b]	59.9 (6.3)[d]	28.1 (5.2)[d]	14.5 (3.3)[d]
	HCl (+)	100.0 (4.3)	94.1 (4.2)	94.4 (5.7)	95.4 (4.2)	93.8 (7.0)

[a] Results are for triplicate measurements. Values measured at 0 min, 0 h, or zero cycles are the baseline values.
[b–d] Compared with baseline: [b] $P < 0.05$; [c] $P < 0.01$; [d] $P < 0.001$.

The chemical degradation of peptides is influenced by the pH of the aqueous solution; human parathyroid hormone and luteinizing-hormone-releasing hormone derivatives are examples *(13–15)*. We demonstrated that in whole blood and plasma, ghrelin is unstable. The degradation of octanoylated ghrelin was shown to be attributable to hydrolysis to desacyl ghrelin (see Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue6/). Acidification is a simple, reliable procedure that protected against degradation of the acylated modification and dramatically improved stability at pH 4. On the other hand, the stability of the octanoyl modification of ghrelin was markedly decreased in strongly acidic (below pH 2), neutral, and alkaline solutions (data not shown).