平成 16-18 年度　厚生労働科学研究費補助金
（臨床研究基盤整備推進研究）
主任研究報告書

各種高脂血症治療薬の糖尿病性血管病予防効果の総合的研究
（若手医師・協力者活用に要する研究）

平成 19 年 4 月

主任研究者　川嶋成乃亮
神戸大学大学院医学系研究科　助教授

はじめに

厚生省労働科学研究費補助金『臨床研究基盤整備推進研究事業』、課題名『各種高脂血症治療薬の糖尿病性心血管病進展予防効果の総合的検討（若手医師・協力者活用に要する研究)』に関して、平成１６年度、１７年度、１８年度の３年間にわたり行いました研究の研究成果をとりまとめ、研究成果報告書を作成いたしました。

専門分野の関係者各位のご参考に供すると共に、さらに当該研究領域での今後の研究に寄与するものと期待しております。

研究組織

研究代表者　　川嶋成乃亮　（神戸大学大学院医学系研究科・助教授）

若手医師及び臨床研究協力者に対する指導者

志手淳也

若手医師　　平山園子

白木里織

篠原正和

臨床研究協力者　　小嶋稔子

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各種高脂血症治療薬の糖尿病性血管病予防効果の総合的研究（若手医師・協力者活用に要する研究）

主任研究者　川嶋成乃亮　　神戸大学大学院医学系研究科　助教授

要旨：薬剤溶出性ステント(DES)挿入後の新生内膜肥厚は糖尿病患者で増強しており、糖尿病は広範囲に血管病変の悪化要因になることが示唆された。早期に血管病のリスクを把握する事が重要で、そのための血清マーカーとしてTLR4発現量やBH4/BH2比が有用であると考えられる。又スタチンは内皮機能を改善し、糖尿病性血管病変を予防する可能性が示唆された。

A．研究目的

糖尿病性血管障害の病態を把握すると共に、その早期検出のための血清マーカーを開発する。

B．研究方法

1．シロリムス溶出ステント(SES)挿入後の新生内膜形成に及ぼす糖尿病の影響、並びにスタチンによる修飾を、OCTカテーテルを用いて検討した。

2．糖尿病をはじめとした高リスク患者、冠動脈疾患患者において、末梢単核球表面のToll-like受容体(TLRs)発現などの種々の血清マーカー、pteridineおよび前腕の内皮機能を測定した。

倫理面への配慮

すべての患者よりあらかじめインフォームドコンセントを文書にて受領した。

C．研究成果

糖尿病の存在はSES上の内膜肥厚を増強させた。スタチンの投与は有意な影響を与えなかった。

冠動脈疾患患者、特に急性冠症候群では、単核球TLR4の発現が増加していた。

また、血清BH4/BH2比は糖尿病患者の内皮機能の良い指標となり、スタチンの投与により、内皮機能改善と共に上昇した。

冠動脈危険因子の数が増加するのに従い血清BH4/BH2比は減弱した。

D．考察

テントにおける内膜肥厚をも増強させる。そのため、糖尿病患者では動脈硬化をはじめとする血管障害の初期段階における病態の把握が重要である。

末梢血TLRsはリスクの同定に、BH4/BH2比は内皮機能障害の判定に有用であり

スタチンは糖尿病患者における内皮障害を改善し、これらのマーカーを改善することが判明した。

E．結論

糖尿病患者においては早期からのリス評価が重要であり、種々の新しい血清クマーカーの開発はスタチンをはじめとした種々の薬剤がどのようにリスクを軽減するかの判定に有用である。

特許・実用新案登録　なし

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An anti-proliferative gene BTG1 regulates angiogenesis in vitro

Kenji Iwai, Ken-ichi Hirata,* Tatsuro Ishida, Shigeto Takeuchi, Tetsuaki Hirase, Yoshiyuki Rikitake, Yoko Kojima, Nobutaka Inoue, Seinosuke Kawashima, and Mitsuhiro Yokoyama

Division of Cardiovascular and Respiratory Medicine, Kobe University Graduate School of Medicine, Kobe, Japan

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Abstract

B-cell translocation gene 1 (BTG1) is a member of the anti-proliferative gene family that regulates cell growth and differentiation. To clarify the role of BTG1 in angiogenesis, we examined the regulation of BTG1 expression in cultured endothelial cells and characterized its function in in vitro models of angiogenesis. BTG1 mRNA was abundantly expressed in quiescent endothelial cells. Addition of serum and angiogenic growth factors decreased BTG1 mRNA levels in endothelial cells. In contrast, BTG1 mRNA was up-regulated in tube-forming endothelial cells on Matrigel. This up-regulation was partially blocked by neutralizing antibody against transforming growth factor-β (TGF-β), and TGF-β increased BTG1 mRNA levels. Inhibition of endogenous BTG1 by overexpression of antisense BTG1 resulted in inhibited network formation, and overexpression of sense BTG1 augmented tube formation in these cell lines. BTG1-overexpressing endothelial cells displayed increased cell migration. These findings suggest that BTG1 may play an important role in the process of angiogenesis.
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The integrity of blood vessels in normal tissues is maintained through negative and positive growth controls of vascular cells, which affect the proliferation and differentiation cellular genetic programs [1]. An alteration of this subtle balance can result in a variety of vascular diseases such as developmental anomalies, tumor growth, and diabetic retinopathy [2]. The mechanisms for the regulation of blood vessel formation are complex. It is generally accepted that new blood vessel formation is achieved by a series of several critical processes: removal of pericytes and smooth muscle cells, degradation of extracellular matrix (ECM), endothelial proliferation and migration, reconstruction of intercellular structures, and morphogenesis of endothelial cells into a capillary-like vascular network. Throughout these angiogenic processes, the control of the cell cycle plays an essential role in cell growth and in the activation of important cellular processes including differentiation, migration, and apoptosis. In particular, the angiogenic properties of the endothelial cell require coordinated changes in endothelial cell morphology, function, and gene expressions. Recent studies have provided insights into the phenotypic changes of endothelial cells through the modulation of gene expression during angiogenesis [3,4]. Although a number of signaling molecules have been identified as candidates that mediate extracellular signals to the control of cell cycle the precise mechanisms governing endothelial cell fate have not been fully elucidated.

B-cell translocation gene 1 (BTG1/APRO2) is a member of the anti-proliferative gene (APRO) family that was identified through the molecular characterization of a chromosomal translocation in a lymphoid malignancy [5–7]. It has been reported that BTG1 is expressed by several types of non-endothelial cells including fibroblasts and T-lymphocytes, and the expression of BTG1 is highly regulated during cell growth and proliferation. BTG1 is strongly expressed in the G0/G1 phases of the cell cycle, and then down-regulated during the G1 phase [6]. Accumulating evidence has demonstrated that BTG1 regulates cell-growth through

* Corresponding author. Fax: +81-78-382-5859.
E-mail address: hiratak@med.kobe-u.ac.jp (K.-i. Hirata).

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interaction with transcription factors. Overexpression of this gene results in a retardation of cell proliferation in the fibroblast cell line [6]. A negative correlation between BTG1 mRNA expression and cell proliferation was also observed in T lymphocytes, macrophages, and testis development [8,9]. Moreover, expression of BTG1 is directly associated with the rate of cancer cell growth and invasion [10]. In addition to the anti-proliferative properties, previous studies have indicated that BTG1 is also involved in cell differentiation and organogenesis both in vitro and in vivo [11,12]. In a myoblast cell line, for instance, BTG1 mediates triiodothyronine- and cAMP-induced myoblast differentiation into myocytes [11,13]. Given the potential role of BTG1 in regulation of cell proliferation and differentiation, it was postulated that BTG1 regulates cell cycle in endothelial cells as well and plays a fundamental role in blood vessel formation. However, little is known regarding expression and function of BTG1 in vascular endothelial cells. In this study, we have sought to explore the expression of BTG1 gene in endothelial cells and to establish the role of BTG1 in angiogenic processes. In vitro angiogenesis assays were performed using endothelial cell lines transfected with sense or antisense BTG1 cDNA. We have demonstrated that BTG1 regulates endothelial cell migration and tube formation. Thus, the findings may provide a novel function of BTG1 in endothelial cells during angiogenesis.

Materials and methods

Materials. All standard culture reagents were obtained from Gibco-BRL/Invitrogen (Grand Island, NY). Matrigel and growth factor-reduced Matrigel were purchased from Becton–Dickinson (Bedford, MA). Recombinant vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), and anti-TGF-β neutralizing antibody were purchased from R&D systems (Minneapolis, MN). Fibronectin, laminin, and collagen were purchased from Asahi Techno Glass (Tokyo, Japan).

Cell cultures. Bovine aortic endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs) were purchased from Cell Systems/Clonetics (Walkersville, MD). A human vascular endothelial cell line, EA.hy926, was kindly provided by Dr. Edgell (University of North Carolina, Chapel Hill, NC). BAECs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 μg/mL sulbenicillin, and 15 μg/mL gentamicin and used between passages 5th and 11th. HUVECs were maintained in RPMI-1640 medium supplemented with 20% FBS, 15 μg/mL endothelial cell growth supplement, 10 U/mL heparin, 10 μg/mL sulbenicillin, and 15 μg/mL gentamicin and used between passages 3th and 5th. EA.hy926 cells were cultured in DMEM supplemented with 5% FBS, 10 μg/mL sulbenicillin, and 15 μg/mL gentamicin.

Stable transfectants of sense and antisense BTG1. Total RNA (1 μg), extracted from HUVECs, was reverse transcribed with oligo(dT) primer and M-MLV reverse transcriptase (Promega, Madison, WI). To amplify human BTG1 cDNA, RT-PCR was employed using *Taq* DNA polymerase (Takara, Kusatsu, Japan) and a primer pair specific to human BTG1 (5′-gtgaattccatggcaccaatttctct-3′, 3′-gtgaattcacttcacgggtg actctg-5′). The PCR product was ligated into the pcDNA3 vector with *Eco*RI–*Xho*I sites to generate sense BTG1, or with *Kpn*I–*Eco*RI sites to generate antisense BTG1. Full-length sequences were determined by dideoxy sequencing. The sense and antisense BTG1-pcDNA3 plasmids were introduced into EA.hy926 cells using the Lipofectamin method (Gibco-BRL, Rockville, MO) and transfectants were selected in the presence of 1 mg/mL G418 sulfate. Exogenous BTG1 mRNA expression of clones was evaluated by Northern blotting using a probe specific to human growth hormone poly(A) of pcDNA3, and endogenous BTG1 mRNA expressions were evaluated by a probe specific to BTG1 cDNA. Total 6 clones (sense) and 4 clones (antisense) were obtained and used for experiments. The mock (pcDNA3 vector)-transfected clone was used as a control.

Northern blot analysis. Total RNA was extracted from cultured cells using the ISOGEN reagent (Nippongene, Tokyo). Aliquots of total RNA (15 μg) were size-fractionated on 1% agarose gel containing 5% formaldehyde and transferred to a Hybond-N membrane (Amersham, Piscataway, NJ). Blots were hybridized at 65 °C in the PerfectHyb buffer (TOYOBO, Osaka, Japan). Human BTG1 cDNA probes were labeled with [α-^{32}P]dCTP by random priming. After washing twice at 65 °C in 0.2% SSC and 0.1% SDS for 30 min, membranes were exposed to imaging plates for 45 min and processed for imaging analysis. RNA loading was determined by ethidium bromide staining of 18S and 28S ribosomal RNA.

In vitro angiogenesis assay. Matrigel and growth factor-reduced Matrigel were dispensed into 24-well tissue culture plates according to the manufacturer's instructions. HUVECs or EA.hy926 cells were dispersed by trypsinization and counted. Then, cells were suspended in RPMI medium with 20% FBS or DMEM with 5% FBS and plated on the surface of the Matrigel at a density of 4×10^4 cells/well (24-well plate). The cells were then placed in a 5% CO_2 incubator at 37 °C and periodically observed. Pictures were taken at 40× magnification using a Digital Output Camera (Olympus DP11) attached to an inverted phase contrast microscope (Olympus IX70) and total network length was measured using the NIH image software [14].

Endothelial cell migration assay. In vitro endothelial cell migration assay was performed using the modified Boyden chamber system [15]. Polycarbonate filters with a pore size of 8 μm (Nucleopore, Cabin John, MD) were coated with 0.1% gelatin for 6 h at room temperature and washed with PBS. Subconfluent EA.hy926 were washed and trypsinized for the minimum time required to induce cell detachment. After the filter was placed between the lower and upper chambers, 4.0×10^5 cells suspended in 1 mL DMEM containing 5% FBS were seeded in the upper compartment. The apparatus was then incubated for 4 h at 37 °C in a humidified incubator with 5% CO_2. After the incubation period, filters were removed, and the upper side of the filters with the cells that did not migrate was scraped off with a rubber cell lifter. The filters were then fixed with methanol and stained with a Giemsa solution. Cell migration was quantified by counting cells of three random microscopic fields (100×) in each well, and all experiments were performed in triplicate and expressed as percentages of the number of total cells counted per well.

Endothelial cell adhesion to extracellular matrix assay. To examine the role of BTG1 in cell adhesion to ECM, 96-well microplates (IWAKI, Tokyo, Japan) were coated with different ECMs, 10 μg/mL collagen type I from porcine tendon, 10 μg/mL collagen type IV from bovine lens, 10 μg/mL fibronectin, and vitronectin from human plasma, and 10 μg/mL laminin from mouse EHS tumor (IWAKI) at room temperature for 1 h, and then rinsed three times with PBS. EA.hy926 cells were seeded at 4×10^4 cells/well in 100 μL DMEM containing 5% FBS onto the coated plates and incubated for 1 h. Medium and non-adhesive cells were removed by rinsing twice with PBS. Endothelial cell numbers were quantified by measurement of alkaline phosphatase activity. Alkaline phosphatase activity per well was evaluated spectrophotometrically by addition of 50 μL/well of alkaline phosphatase yellow liquid substrate (Sigma, St. Louis, MO) and measurement of absorbance at 470 nm with the microplate fluorescence reader