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Inhibition of Mitogen Activated Protein Kinase Activated Protein Kinase II with MMI-0100 reduces intimal hyperplasia ex vivo and in vivo

Akihito Muto [a,b], Alyssa Panitch [d], Namho Kim [d], Kinam Park [d], Padmini Komalavilas [e,f], Colleen M. Brophy [e,f], Alan Dardik [a,b,c,*]

[a] Department of Surgery, Yale University School of Medicine, New Haven, CT, United States
[b] Department of Vascular Biology and Therapeutics Program, Yale University School of Medicine, New Haven, CT, United States
[c] VA Connecticut Healthcare System, West Haven, CT, United States
[d] Weldon School of Biomedical Engineering, Purdue University, 206 S. Martin Jischke Drive, West Lafayette, IN 47907, United States
[e] Department of Surgery, Vanderbilt University, Nashville, TN 37232, United States
[f] VA Tennessee Valley Healthcare System, Nashville TN, United States

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ABSTRACT

Vein graft intimal hyperplasia remains the leading cause of graft failure, despite many pharmacological approaches that have failed to translate to human therapy. We investigated whether local suppression of inflammation and fibrosis with MMI-0100, a novel peptide inhibitor of Mitogen Activated Protein Kinase Activated Protein Kinase II (MK2), would be an alternative strategy to reduce cell proliferation and intimal hyperplasia. The cell permeant peptide MMI-0100 was synthesized using standard Fmoc chemistry. Pharmacological doses of MMI-0100 induced minimal human endothelial and smooth muscle cell proliferation (30% and 12% respectively). MMI-0100 suppressed IL-6 expression to control levels, without effect on IL-8 expression. MMI-0100 caused sodium nitroprusside induced smooth muscle cell relaxation and inhibited intimal thickening in human saphenous vein rings in a dose-dependent fashion. In a murine aortic bypass model, MMI-0100 reduced intimal thickness in vein grafts by 72%, and there were fewer F4/80-reactive cells in vein grafts treated with MMI-0100. MMI-0100 prevents vein graft intimal thickening ex vivo and in vivo. These results suggest that inhibition of MK2 with the cell-permeant peptide MMI-0100 may be a novel strategy to suppress fibrotic processes such as vein graft disease.

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1. Introduction

There are >1 million coronary bypass procedures a year worldwide, with human greater saphenous vein remaining the most commonly used conduit. However, less than half of these grafts remain patent after 12 years (Motwani and Topol, 1998), with more recent data from the PREVENT IV trial demonstrating 42% graft occlusion within 18 months (Alexander et al., 2005). Graft failure typically leads to myocardial infarction and death, the need for repeated coronary bypass procedures and, consequently, substantial costs to the healthcare system. Thus, approaches to decrease vein graft failure rates would improve outcomes after arterial bypass procedures, yielding significant clinical and health economic benefits.

The leading cause of bypass graft failure is intimal hyperplasia of the vein conduit (Clowes and Reidy, 1991). While its causes are as yet incompletely understood, intimal hyperplasia likely results from a cascade of events triggered by the tissue response to mechanical injury associated with surgical vein harvest and conduit preparation; in addition, the damage induced by mechanical dilation used to "break" vessel spasm is refractory to current vasodilators and other pharmacologic approaches (Dashwood et al., 2004;Dashwood and Loesch, 2007).

On a cellular–molecular level, intimal hyperplasia is mediated by a sequence of events, including inflammatory processes in response to vessel trauma, resulting in vascular smooth muscle proliferation, migration, and extracellular matrix production (Allaire and Clowes, 1997). This is associated with a phenotypic modulation of smooth muscle cells from a contractile to a synthetic phenotype, with "synthetic" cells secreting extracellular matrix proteins (Mosse et al., 1985). Graft functional responses are also impaired, leading to abnormal vasorelaxation (Klyachkin et al., 1993;Lorusso et al., 2007). All of

Abbreviations: CaMKI, calcium/calmodulin-dependent protein kinase I; EC, endothelial cell; GA, gentamycin/amphotericin; HAEC, human aortic endothelial cells; HASMC, human aortic smooth muscle cells; HCAEC, human coronary artery endothelial cells; hnRNPA0, heterogeneous nuclear ribonucleoprotein A0; HSP27, heat shock protein 27; HSV, human saphenous vein; IL, interleukin; I:M, intima:media; MAPK, mitogen activated protein kinase; MK, mitogen activated protein kinase activated protein kinase; MLEC, mouse lung endothelial cells; PBS, phosphate-buffered saline; PE, phenylephrine; SMC, smooth muscle cell; SNP, sodium nitroprusside; TTP, tristetraprolin.

* Corresponding author at: Yale University School of Medicine, Vascular Biology and Therapeutics, 10 Amistad Street, Room 437D, PO Box 208089, New Haven, CT 06520-8089, USA. Tel.: +1 203 785 7991; fax: +1 203 737 2290.
E-mail address: alan.dardik@yale.edu (A. Dardik).

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these processes lead to pathologic narrowing of the vessel lumen, graft stenosis, and ultimately graft failure (LoGerfo et al., 1983).

Although a number of drugs aiming to reduce development of intimal hyperplasia have been tested in clinical trials, these products have failed. Antithrombotic and antiplatelet agents such as warfarin, clopidogrel and aspirin have little or no effect on intimal hyperplasia (Kent and Liu, 2004). Two large clinical trials for the prevention of coronary and peripheral vascular vein graft failure using an E2F decoy to prevent smooth muscle proliferation also failed in their primary endpoint (Alexander et al., 2005; Conte et al., 2006). Accordingly, availability of novel therapeutic approaches to improve graft patency remains an unmet need.

Recently, Epstein, et al. demonstrated that suppression of the innate immune response in the context of vascular injury dramatically down-regulated the degree of intimal hyperplasia (Danenberg et al., 2002;Epstein et al., 2008). These results suggest that inflammation plays a major role in intimal thickening and that peri-procedural suppression of inflammation could decrease intimal hyperplasia by a clinically meaningful degree. However, immune suppression on a systemic level during surgical procedures and the post-operative recovery period can increase infection risk, and as such is not clinically feasible. Therefore, we investigated whether local suppression of inflammation, via ex vivo vein graft treatment with MMI-0100, a peptide inhibitor of MAPKAP kinase II (MK2), would be a novel alternative strategy to reduce intimal thickening following vein bypass surgery.

Mitogen Activated Protein Kinase Activated Protein Kinase II (MAPKAP Kinase II, MK2) is an intracellular kinase activated by the p38 Mitogen Activated Protein Kinase (MAPK) (Rouse et al., 1994) that, in turn, phosphorylates transcription factors tristetraprolin (TTP) (Sandler and Stoecklin, 2008) and hnRNPA0 (Rousseau et al., 2002). TTP and hnRNPA0 are known to interact with AU-rich regions of mRNA to control mRNA stability and expression. Importantly, studies show that suppression of MK2 activity results in down-regulation of inflammatory cytokine expression, including TNF-α, IL-1β, and IL-6 (Kotlyarov et al., 1999;Winzen et al., 1999;Lehner et al., 2002;Neininger et al., 2002;Wang et al., 2002; Thomas et al., 2008;Funding et al., 2009).

We recently developed a cell-permeant MK2 inhibitor peptide (Lopes et al., 2009) that was based on a peptide designed by Hayess and Bendorff (Hayess and Benndorf, 1997). However, further work with this peptide demonstrated that it was relatively nonselective and toxic, which led to development of significantly more specific inhibitor peptides, including MMI-0100 (Ward et al., 2009). In an animal model of abdominal adhesions, i.e. rat bowel anastomosis, we reported that a single dose of MMI-0100 applied locally at the time of surgery reduces both number and severity of abdominal adhesions without impairing normal intestinal healing, as determined by hydroxyproline content and burst pressure of the colonic anastomosis (Ward et al., 2011).

These results suggest that inhibition of MK2 with MMI-0100 inhibits inflammatory responses leading to excess extracellular matrix deposition and formation of scars and adhesions. Given the role of inflammation in the development of intimal hyperplasia, we investigated whether MMI-0100 could similarly reduce this clinically relevant vascular process and perhaps ultimately vein graft failure. Therefore, we tested whether MMI-0100 affected vascular cell proliferation and reduced intimal hyperplasia ex vivo and in vivo.

2. Material and methods

2.1. Cell culture

Primary human aortic endothelial cells (HAEC) were obtained from Invitrogen; HAECs were cultured in Medium 200 supplemented with LSGS (Low Serum Growth Supplement), containing FBS (2% v/v), hydrocortisone (1 μg/ml), human epidermal growth factor (EGF, 10 ng/ml), Basic Fibroblast Growth Factor (bFGF, 3 ng/ml), gentamycin/amphotericin (GA) and heparin (10 μg/ml). Primary human aortic smooth muscle cells (HASMC) were obtained from Invitrogen; HASMC were cultured in EGM Bullet Kit — EBM-2 Endothelial Basal Medium 2 supplemented with hEGF (10 ng/ml), hydrocortisone (1.0 μg/ml), GA (50 μg/ml), FBS (5%), VEGF, hFGF-B, R^3-IGF-1, and ascorbic acid. Primary human coronary artery endothelial cells (HCAEC) were obtained from Lonza; HCAECs were cultured in Medium 231 supplemented with SMGS (Smooth Muscle Growth Supplement), containing FBS (4.9% v/v), bFGF (2 ng/ml), hEGF (0.5 ng/ml), heparin (5 ng/ml), insulin (5 μg/ml), BSA (0.2 μg/ml), and GA.

All cultures were maintained in 25 cm^2 polystyrene tissue culture flasks in a 37 °C, 5% CO_2/95% air environment, with cell culture media refreshed every other day. All cells were seeded at a density of 20,000–30,000 cells/cm^2, as required by the specific experiment, and allowed to grow to 80–90% confluence before being harvested/passaged. Only cells from early passages (numbers 2–8) were utilized in experiments.

Primary cultures of mouse lung endothelial cells (MLEC) were isolated as previously described (Ackah et al., 2005;Muto et al., 2011). After immunoselection with magnetic beads, endothelial cells were immortalized with polyoma middle T-antigen. Isolated MLECs were maintained with EBM-2/EGM-2 MV SingleQuot Kit Supplement & Growth Factors (Lonza) containing 15% fetal bovine serum. Cell proliferation in MLEC was measured at 24 and 72 h after MMI-0100 treatment by direct cell counting after trypsin treatment.

2.2. MMI-0100 reconstitution/dilution

MMI-0100 was synthesized using standard Fmoc chemistry as previously described, with the peptide sequence YARAAARQARAKALARQLGVAA (Ward et al., 2009). 114 mg of MMI-0100 (MW = 2283.67 g/mol; Moerae Matrix, Inc.) was dissolved in 5 ml of phosphate-buffered saline (PBS) to yield a 0.01 M stock solution, which was divided into 500 μl aliquots and stored at −20 °C. Serial dilutions of stock solution were made to achieve appropriate drug concentrations for each study.

2.3. MTS cell proliferation assay

The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega) was used to assess drug effects on cell proliferation according to the manufacturer's instructions. Briefly, HAECs and HASMCs from early passages were grown to 80–90% confluence in 25 cm^2 tissue culture flasks in a 37 °C/5% CO_2 incubator prior to harvest. 200 μl of each type of cell suspension (at 20,000 cells/cm^2) was seeded onto separate 96-well plates to yield an approximate 60% confluence per well. Cells were allowed to adhere to the plate surface overnight, followed by addition of 20 ng/ml of TNF-α to stimulate production of inflammatory agents. After a 4–6 h incubation period, MMI-0100 peptide drug was added and cells were incubated for another 20–24 h. Each well was then supplemented with 100 μl of fresh medium and 20 μl of CellTiter 96® AQ_{ueous} One Solution Reagent and incubated for an additional 1.5–2 h prior to measuring absorbance of each well at 490 nm with a SoftMax-equipped plate reader.

2.4. Cell apoptosis analysis

The apoptotic effect of MMI-0100 on MLEC was measured at 24 h after MMI-0100 treatment. MLECs were removed from the tissue culture plate by trypsin, and re-suspended at 1.0×10^6/ml concentration. Apoptotic cells were detected by AlexaFluor 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen) using flow cytometry sorting analysis.

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