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Inhibition of intimal hyperplasia in murine aortic allografts by the oral administration of the transforming growth factor-beta receptor I kinase inhibitor SD-208

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BACKGROUND: Transforming growth factor-beta (TGF-β) plays a significant role in the pathogenesis of the intimal hyperplasia of transplant arteriosclerosis (TA). The aim of this study was to evaluate the efficacy of an oral inhibitor of TGF-β receptor I kinase (SD-208) on the development of TA.

METHODS: BALB/c ($H\text{-}2^d$) donor aortas were transplanted into C57BL/6 ($H\text{-}2^b$) recipients, and the mice then received different doses (40 or 60 mg/kg) of SD-208 or control vehicle by daily gavage for 8 weeks. The grafts were analyzed by histology and morphometry at 1, 2, 4, 6 and 8 weeks after transplantation. The effects of TGF-β and SD-208 on neointimal smooth muscle-like cell (SMLC) and vascular smooth muscle cell (VSMC) proliferation and migration were evaluated, and the expression levels of Smad3, P-Smad3, connective tissue growth factor (CTGF) and collagen I were determined by in vitro experiments.

RESULTS: The intimal hyperplasia of the SD-208–treated group was significantly reduced compared with the vehicle-treated control group (32% and 48% reduction for 40 mg/kg and 60 mg/kg SD-208 compared with the controls, respectively [$n = 5$], $p < 0.05$). SD-208 reduced SMLC proliferation and the production of intimal collagen by 21% and 75%, respectively, in the grafts. SD-208 also abolished the promoting effect of TGF-β on SMLC proliferation and migration but did not affect TGF-β inhibition of VSMCs in vitro. CTGF, a protein downstream of TGF-β, was downregulated with the inhibition of Smad3 phosphorylation by SD-208, both in vitro and in vivo. Moreover, we found that the endogenous Smad3 in SMLCs was upregulated from 2 weeks after transplantation and was 64% higher than in VSMCs at 8 weeks.

CONCLUSION: These results demonstrate that SD-208 can effectively reduce the formation of intimal hyperplasia of TA in the murine aortic allograft model.

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Transplant arteriosclerosis (TA) is the leading cause of late allograft dysfunction after the first year of transplant; this condition is characterized by a diffuse concentric vascular intimal hyperplastic lesion composed of smooth

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muscle-like cells (SMLCs) and associated extracellular matrix (ECM).[1,2] The smooth muscle actin–positive cells in the neointima are labeled SMLCs, which differ from medial vascular smooth muscle cells (VSMCs) in their function and phenotype.[3,4] The activation and proliferation of SMLCs is the central feature of intimal hyperplasia, but the molecular mechanisms that trigger SMLC proliferation are poorly elucidated.[5]

Increasing evidence has demonstrated that there is a strong association between transforming growth factor-beta (TGF-β) expression and TA progression.[6,7] TGF-β likely enhances TA by regulating SMLC proliferation and promoting the production of ECM proteins.[7,8] In addition, more recent evidence has indicated that activation of the Smad3 signaling pathway is mainly responsible for this stimulatory effect of TGF-β.[9,10]

It is hypothesized that a blockade of the TGF-β pathway could lessen the severity of TA. Given the complex and contradictory effects of TGF-β on VSMC function, the underlying mechanisms are difficult to define. TGF-β has a dual effect on cell growth in cultured VSMCs, as it promotes growth at low concentrations (<0.1 ng/ml) but inhibits growth at higher concentrations.[11] Nevertheless, TGF-β expression is significantly increased in TA lesions.[7] Thus, a more complete understanding of the TGF-β signaling pathway in SMLCs is necessary to clarify the conflicting functions of TGF-β in intimal hyperplasia.

The inhibitors of the TGF-β signaling pathway that have been reported include anti-sense oligonucleotides and anti-sense RNA, ligand traps and peptides and small molecule inhibitors.[12] Specific small molecules designed to antagonize the actions of TGF-β at the level of receptor-dependent intracellular signal transduction are particularly promising, as small molecules are much cheaper and more easily administered. In this study we used SD-208, a potent and selective orally active inhibitor of TGF-β receptor I kinase, in an experimental model of aorta transplantation, and we attempted to determine the mechanisms of its inhibitory effects on TA progression and SMLC regulation.

Methods

Mice

Adult male BALB/c (*H-2*^{d}) and C57BL/6 (*H-2*^{b}) mice were purchased from HFK Bioscience Co., Ltd. (Beijing, China). All studies were performed in compliance with the guidelines of the animal care and use committee of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

Reagents

The TGF-β receptor I kinase inhibitor SD-208 was synthesized by Scios, Inc. (Fremont, CA). Recombinant TGF-β1 was purchased from R&D Systems (Minneapolis, MN). The Smad2/3 and P-Smad2/3 antibodies were from Cell Signaling Technology (Fremont, CA); the α-smooth muscle actin (α-SMA) and Ki-67 antibodies were from Abcam (Cambridge, MA), and the connective tissue growth factor (CTGF) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The Cell Counting Kit-8 (CCK-8) was purchased from Beyotime Institute (Biotech, China). Other reagents were purchased from Sigma (St. Louis, MO), unless specified otherwise.

Aorta transplantation and animal treatment

BALB/c and C57BL/6 male mice (8 to 9 weeks old) served as donors and recipients in the allotransplantations, respectively, and C57BL/6 mice served as both donors and recipients in the isotransplantations. Aortic transplantation was performed as described previously.[4] Recipients did not receive immunosuppression therapy after the operation. SD-208 (40 or 60 mg/kg) in 1% methylcellulose was administered from Days 1 to 56 after surgery by gavage once daily for the treatment group; the control group received an equal volume of 1% methylcellulose. The allografts and isografts were harvested at 1, 2, 4, 6 and 8 weeks after transplantation and were then processed for histologic analysis as described in what follows.

Tissue staining and morphometric analysis

Morphometric analysis was performed on elastin-stained arteries, and Masson's trichrome staining was used to identify collagen deposition. Paraffin sections of the harvested grafts were cut at a 5-μm thickness. Six sections from each graft were selected for analysis using the IMAGE-PRO PLUS 6.0 software (Media Cybernetics, Inc., Silver Spring, MD). Three sections were taken from the center of each graft, and another three were cut at least 100 μm away from the center. A digitized image of each section was captured, and the neointimal and medial areas were circumscribed manually and measured as previously described.[13] The ratio between the neointimal (I) and medial (M) areas was calculated. The results of the neointimal/medial area ratio (I/M) represent the mean value of each group. The error bars represent the standard errors of the mean.

Immunohistochemical analysis

Paraffin-embedded grafts were cut into 5-μm sections for analysis. Immunostaining was performed with the following antibodies: rabbit anti-Smad2/3; rabbit anti–P-Smad2/3; rabbit anti–α-smooth muscle actin (α-SMA); rabbit anti–Ki-67; or rabbit anti-CTGF. Immunohistochemistry was performed as described previously.[14] For quantification, P-Smad2/3–positive and Ki-67–positive cells and the total number of cells were counted in eight high-power fields (magnification 400×) selected from each entire section using IMAGE-PRO PLUS software. The mean optical densities of the Smad2/3, α-SMA and CTGF staining were also determined using IMAGE-PRO PLUS. The means were averaged, and the standard error of the mean was calculated for each group.

VSMC and SMLC proliferation assay

Medial VSMCs from the descending thoracic aortas and SMLCs from the allografts were isolated using enzymatic digestion as previously described.[15] The arteries were harvested from the recipient mice 8 weeks after transplantation. The cells were used at passages 3 to 5 for the study.

The cell proliferation assay was performed using a CCK-8 according to the manufacturer's instructions. Cells were seeded in 96-well plates at 1.0×10^4 cells per well, cultured for 24 hours, and then serum-starved for 24 hours in medium containing 0.5% fetal bovine serum (FBS). Subsequently, the cells were stimulated for 24 hours with 5 ng/ml TGF-β1 or solvent (phosphate-buffered saline [PBS]) in the presence of

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