Geranyl-Barnes Facilitates TNF-α mediated MCP-1 Expression and Release by Rat Peripheral Nerve Microvascular Endothelial Cells

K.A. Langert, 1,2 C.L. Von Zee, 1,3 and E.B. Stubbs, Jr, 1,3
1 Research Service, Edward Hines Jr. VA Hospital, Hines, IL; 2 Program of Neuroscience, 3 Ophthalmology, Loyola University of Chicago, Maywood, IL.

INTRODUCTION

Guillain-Barré syndrome (GBS) is a leading cause of autoimmune neuronomuscular paralysis. Proinflammatory cytokines, particularly TNF-α, play a pivotal role in the pathogenesis of GBS. TNF-α, acting through NF-kB, induces the transcription of many inflammatory mediators, including chemokines. These chemokines facilitate the transendothelial migration of autoreactive leukocytes into the sciatic nerve, which is a hallmark feature of the pathogenesis of GBS.1

Inhibition of HMG-CoA reductase with statins has been shown to markedly attenuate the course of experimental autoimmune neuritis (EAN), 2, an established animal model of GBS. In vivo, statins restrict transendothelial migration of autoreactive leukocytes into peripheral nerves.3 The mechanism by which this occurs remains unknown, but may involve limiting production of key downstream metabolic intermediates of cholesteryl biosynthesis, including the isoprenoid farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Isoprenylation is a required step in the functional activation of small GTP-binding proteins, including the TNF Receptor Activation of RhoA in particular has been shown to regulate cytokine-mediated upregulation of the chemokine monocyte chemoattractant protein-1 (MCP-1), a mediator of leukocyte recruitment that is implicated in the onset and progression on GBS.4

In this study, we sought to determine the mechanism by which statins disrupt transendothelial migration using SV40-transformed peripheral nerve microvascular endothelial cells (PNMECs).

METHODS

PNMEC Cell Culture: Rat primary PNMECs were purged from sciatic nerves of naive male Lewis rats and cultured in T10 flasks supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin, 50 µg/ml endothelial cell growth supplement, and 0.1% heparin at 37°C under an atmosphere of 5% CO2/95% air. Primary PNMECs were immortalized by SV40 transduction using an SV40LT15 retroviral cell line expressing a replication-defective SV40 virus with a neo-resistance gene (Dr. P. Johnston, University College of London, London, UK). Following transduction, PNMECs were plated into complete culture media containing G418 (200 µg/ml). G418-resistant cell lines were isolated by single colony selection. A single clone (4.3) demonstrating morphological and immunophenotypical characteristics of primary PNMEC cultures was expanded and analyzed.

Treatment: Confluent cultures were treated with media alone (vehicle control) or with TNF-α as indicated. In some cases, cultures were pretreated 1 h, as indicated, with vehicle (0.01% ethanol or 0.5% DMSO), lovastatin, simvastatin, or pravastatin, with a direct inhibitor of geranylgeranylation-1 (GGTI-298) or farnesyltransferase (F370 OVC), or with selective GTPase inhibitors (NSC227686, ML141, or C3 exosome) as specified.

Immunocytochemistry: Cells were cultured on collagen-coated coverslips, fixed, blocked, and incubated overnight at 4°C in the presence of mouse anti-Van Wildende factor (WVF, 1:150 dilution), mouse anti-PECAM-1 (C321, 1:100 dilution), or rabbit anti-MCP-1 (1:200 dilution) primary antibody. Immunostained cells were washed in PBS and incubated for 1 h at 25°C in the presence of AlexaFlour488 conjugated goat anti-mouse secondary antibody (1:100 dilution) or FITC-conjugated goat anti-rabbit secondary antibody (1:100 dilution), respectively, and counterstained with DAPI.

Real Time RT-PCR: Total RNA was extracted from treated cultures with TRIzol reagent and 5 µg reverse transcription using SuperScript III First-Strand Supermix. MCP-1 and GAPDH specific cDNA sequences were amplified by real-time IQ SYBR Green (Applied Biosystems) RT-PCR cycling conditions with published published primers. GAPDH was used as a reference control. For each sample, the specificity of the product was determined by melt curve analysis. The endogenous expression of GAPDH was unaltered by drug treatment. Relative fold-changes in MCP-1 mRNA content were normalized to expression levels of GAPDH using the 2^(-ΔΔCt) method of Livak.

MCP-1 ELISA: Cells were cultured on collagen-coated 24- or 96-well plates, as indicated, and fixed with buffered (pH 7.4) 4% PFA. For whole-cell MCP-1 analysis, fixed cells were incubated overnight at 4°C in the presence of rabbit anti-MCP-1 antibody. Immunostained cells were washed and incubated for 1 h at 37°C in the presence of mouse anti-Van Wildende factor (WVF, 1:150 dilution). Immunostained cell membrane was fixed in formaldehyde. Endothelial cell membrane content was extracted with 0.125 M solution of sodium chloride and sonicated. Aliquots of the supernatant were used for ELISA analysis.

MCP-1 ELISA: Supernatants were collected from endothelial cells cultured for 24 h in the presence of TNF-α (10 ng/ml) or with or without 10 µM simvastatin (Sim) or 10 µM GGTI-298 (GGTI). Supernatants were also treated with TNF-α (10 ng/ml) or with or without 1 µM Pravastatin (Prav) or 10 µM pravastatin (Prav). Supernatants treated with TNF-α were pretreated with or without 10 µM pravastatin (Prav) or 10 µM pravastatin (Prav).

RESULTS

Figure 1. Characterization of SV40-transformed PNMECs

Figure 2. TNF-α increases MCP-1 mRNA and protein expression

Figure 3. Inhibition of protein secretion does not alter TNF-α mediated increases in MCP-1 mRNA content

Figure 4. Inhibition of protein secretion does not alter TNF-α mediated increases in intracellular MCP-1 protein expression

Figure 5. Simvastatin attenuates TNF-α mediated increases in MCP-1 protein secretion

Figure 6. Inhibition of geranylgeranylation attenuates TNF-α mediated increases in MCP-1 protein secretion

Figure 7. Disruption of geranylgeranylation attenuates transendothelial migration

Figure 8. GGTI-298 disrupts intracellular MCP-1 protein distribution in PNMECs

Figure 9. Cdi-264 facilitates TNF-α mediated increases in MCP-1 protein secretion

SUMMARY/CONCLUSION

SV40-transformed rat peripheral nerve microvascular endothelial cells retain a phenotypic characteristic of primary endothelial cells.

- TNF-α elicited marked increases in MCP-1 mRNA and protein expression and secretion and induces MCP-1 dependent transendothelial migration of THP-1 monocytes in vitro.
- Inhibition of protein geranylgeranylation attenuates TNF-α mediated increases in secreted MCP-1 protein, as well as chemotaxis of THP-1 monocytes, independently of mRNA or intracellular protein expression.
- Intracellular trafficking and vesicle release of MCP-1 is dependent, in part, on Cdi-264 GTPase.

REFERENCES


ACKNOWLEDGEMENTS:

This work was supported, in part, by grants from the Department of Veterans Affairs (I3R313R, B3759F), NIN (I0R350601033), and a VA Pre-Doctoral Associated Health Rehabilitation Research Fellowship (KAL).