Microvascular Research 89 (2013) 80–85

Contents lists available at ScienceDirect

Microvascular Research

journal homepage: www.elsevier.com/locate/ymvre

Notch activation promotes endothelial survival through a PI3K-Slug axis

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ARTICLE INFO

Article history:
Accepted 25 May 2013
Available online 3 June 2013

ABSTRACT

Rationale: Loss of endothelial viability correlates with initiation and progress of vascular pathology. However, much remains to be learned about pathways required to maintain the balance between cell viability and apoptosis. Notch activation can enhance or inhibit apoptosis but its role in maintaining the endothelium needs further delineation.
Objective: This study aims to identify the mechanisms by which Notch activation regulates endothelial viability.
Methods and results: Endothelial cells transduced with active Notch were treated with lipopolysaccharide (LPS) or homocysteine to induce endothelial apoptosis. Notch protected against LPS-induced cell death but exacerbated homocysteine-induced apoptosis. Inhibition of PI3K revealed that ligand-induced activation of endogenous Notch initiates parallel death and survival pathways and exhibits a differential effect on endothelial survival depending on the apoptotic stimulus. PI3K activity regulated the expression of Slug, which was required for survival in Notch-activated endothelial cells. Homocysteine, but not LPS, blocked both PI3K activity and Slug expression in Notch-activated cells, leading to increased endothelial apoptosis.
Conclusions: Notch signaling leads to activation of parallel survival and apoptotic pathways in endothelial cells. The interaction of Notch with other signaling pathways plays an important contextual role in regulating endothelial viability.

Introduction

Vascular homeostasis in the adult requires active maintenance by a balance of signals that leads to a quiescent and viable endothelial monolayer. Endothelial apoptosis, which can occur when harmful stimuli are present in the blood, plays a role in the pathophysiology associated with atherosclerosis and sepsis (Bannerman and Goldblum, 2003; Tricot et al., 2000). Induction of endothelial survival signaling, therefore, may protect against the effect of injurious chemicals in the bloodstream and halt the progression of some cardiovascular diseases. Several signaling pathways have been implicated in the balance of survival and apoptosis in endothelial cells (EC), and Notch signaling has been suggested to play a critical role in regulating vascular homeostasis (Dou et al., 2008; MacKenzie et al., 2004; Noseda et al., 2004).

There is conflicting evidence regarding the role of Notch signaling on endothelial viability. Multiple studies have shown that Notch has an anti-apoptotic effect on cultured human EC (MacKenzie et al., 2004; Patel et al., 2005; Quillard et al., 2008; Takeshita et al., 2007). However, tumor necrosis factor-induced EC apoptosis is mediated by activation of Notch2, suggesting that the effect of Notch on EC apoptosis may be context-dependent (Quillard et al., 2009). There is a lack of detailed work regarding the contextual role of Notch in cell death and survival in the same cell type.

We have previously shown that activation of Notch1 and Notch4 can protect EC against lipopolysaccharide (LPS)-induced apoptosis (MacKenzie et al., 2004). Here we present evidence that ligand-induced Notch activation induces parallel survival and death pathways. Notch provides protective activities against EC apoptosis through the induction of Slug via a Notch-activated PI3K-dependent pathway. Notch-induced apoptosis is only unveiled when an apoptotic trigger, such as homocysteine, is able to inhibit the concomitant survival pathway.

Materials and methods

Cell culture

The human dermal microvascular endothelial cell line HMEC (provided by the Centers for Disease Control and Prevention in Atlanta, GA) were cultured as previously described (Noseda et al., 2004). Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as previously described (Karsan et al., 1997). The retroviral producer cell line AmphoPhoenix was obtained from Dr. Gary Nolan (Stanford University, Pal Alto, CA) and cultured in DMEM (Sigma-Aldrich, St. Louis,

Abbreviations: LPS, lipopolysaccharides; EC, endothelial cells; HMEC, human microvascular endothelial cells.

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http://dx.doi.org/10.1016/j.mvr.2013.05.007

MO) supplemented with 10% heat-inactivated calf serum, 2 mmol/L glutamine and 100 U each of penicillin and streptomycin. All cells were maintained at 37 °C in 5% CO_2.

Immunofluorescence staining

For immunofluorescent staining of cultured cells, retroviral-transduced HMEC cells were plated at a density of 1.5×10^5 cells on a 4-well chamber slide (BD Biosciences, San Jose, CA). The cells were serum-starved overnight and treated with DMSO (Sigma-Aldrich, St. Louis, MO) or LY294992 (Cell Signaling Technology, Danvers, MA) for the specified time, then fixed and blocked/permeablized. Rabbit anti-Activated caspase 3 (BD Pharmingen, Franklin Lakes, NJ) was used at a dilution of 1:100 and the goat anti-rabbit Alexa594 conjugated secondary antibody was used at 1:200 (Molecular Probes, Invitrogen, Carlsbad, CA). The cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/mL) (Sigma-Aldrich, St. Louis, MO). Immunofluorescent staining was detected with an imaging microscope (Axioplan II; Carl Zeiss, Inc.), and images were captured with a digital camera (1350EX; QImaging, Surrey, BC, Canada).

Apoptosis/survival assays

HMEC were serum-starved overnight with MCDB + 2% calf serum and HUVEC were serum-starved with MCDB + 5% calf serum. The cells were treated with 100 ng/mL LPS from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO) and 25 μmol/L of ALLN (N-Acetyl-L-leucyl-L-leucyl-L-norleucinal) (EMD Chemicals, Gibbstown, NJ) to inhibit the parallel protective effect of LPS, or 7.5 mmol/L homocysteine (Sigma-Aldrich, St. Louis, MO) for the specified time, unless otherwise stated. Untreated cells were used as control for homocysteine treatment, while cells treated with 25 μmol/L ALLN were used as controls for the LPS + ALLN treatment to examine the effect of LPS. For γ-secretase inhibitor (GSI) treatment, HUVEC were pre-treated with 100 nmol/L of *N*-[*N*-(3,5-difluorophenacetyl)]-l-alanyl-3-amino-1-methyl-5phenyl-1,3-dihydro-benzo[e](1,4)diazepin-2-one (DFP-AA) for 16 h before induction of apoptosis with LPS and homocysteine.

Annexin V binding assay

Suspended and adherent cells trypsinized from the plate were pooled. The cells were resuspended in binding buffer (10 mmol/L HEPES (Sigma-Aldrich, St. Louis, MO), pH 7.4; 140 mmol/L NaCl (Sigma-Aldrich, St. Louis, MO); 2.5 mmol/L $CaCl_2$ (Sigma-Aldrich, St. Louis, MO)) at a concentration of ~1×10^6 cells/ml. Two and a half micro-liters of PE-conjugated Annexin V (Invitrogen, Carlsbad, CA) was added to 1×10^5 cells followed by incubation for 15 min at room temperature. 400 μL of ice-cold binding buffer with propidium iodide was added to the cells and the percentage of AnnexinV positive cells was determined by flow cytometry and analyzed by FCS Express (De Novo Software, Los Angeles, CA).

Reverse-transcription quantitative PCR (RT-qPCR)

Total RNA was isolated from cells using TriZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (2.5 μg) was treated with DNase I (Invitrogen, Carlsbad, CA) and reverse transcribed using the Superscript II kit with random primers (Invitrogen, Carlsbad, CA). For each PCR reaction 2.5 μL of the cDNA was used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control. Real-time qPCR was performed on The Applied Biosystems 7900HT Fast Real-Time PCR System by using the Power SYBR® Green PCR kit (Applied Biosystems, Foster City, CA). Primers used were 5′-AGA TGC ATA TTC GGA CCC AC-3′ and 5′-CCT CAT GTT TGT GCA GGA GA-3′ for human Slug; 5′-AGA GTG CGG ACG AGA ATG GAA ACT-3′ and 5′-CGT CGG CGC TTC TCA ATT ATT CCT-3′ for human Hey1; and 5′-GCA AAT TCC ATG GCA CCG T-3′ and 5′-TCG CCC CAC TTG ATT TTG G-3′ for human GAPDH.

Statistical analysis

Results were expressed as means ± standard error of mean (SEM). Data were analyzed using a two-tailed Student's t-test or a paired Student's t-test using the GraphPad Prism statistical program (GraphPad Software, La Jolla, CA).

Results

Notch protects EC against LPS-induced apoptosis, but exacerbates the apoptotic effect of homocysteine

To examine the effect of Notch activation on EC viability, human microvascular EC (HMEC) were treated with two different apoptotic stimuli known to affect EC function in the context of vascular disease. HMEC transduced with the constitutively-active Notch1 construct (HMEC-Notch1IC) showed increased viability compared to the vector control (HMEC-vector) when stimulated with LPS, but showed decreased viability when stimulated with homocysteine (Fig. 1A). To examine whether the increased cell survival was due to a decrease in apoptosis in HMEC-Notch1IC, LPS- or homocysteine-treated cells were examined by Annexin V binding. Upon LPS treatment, HMEC-Notch1IC showed a marked decrease in the apoptotic population compared to HMEC-vector (Fig. 1B, Supplemental fig. 1), while Notch activation exacerbated the apoptotic effect of homocysteine (Fig. 1C, Supplemental fig. 1). To confirm that the differential effects on apoptosis are not an artifact of the human microvascular endothelial cell line or the over-expression system, primary human umbilical vein EC (HUVEC) were pretreated with γ-secretase inhibitor (GSI) to block endogenous Notch signaling and then challenged with LPS or homocysteine. GSI-treated HUVEC showed increase in LPS-induced cell death, but does not affect homocysteine-induced death (Fig. 1D). Thus, in EC, Notch activation can act to either inhibit or promote apoptosis, depending on the stimulus.

Notch activates the PI3K pathway in a cell non-autonomous manner, which is essential for survival of Notch-activated EC

In EC, homocysteine and LPS have the ability to activate some of the same apoptotic pathways (Munshi et al., 2002; Tyagi et al., 2006; Zhang et al., 2001). However, while LPS can also stimulate anti-apoptotic signaling through activation of the PI3K pathway (Wong et al., 2004), homocysteine interferes with PI3K signaling in EC (Suhara et al., 2004). Both HMEC and HUVEC expressing Notch1IC exhibited higher PI3K activity as shown by increased phosphorylation of its downstream effector Akt (Fig. 2A). Since PI3K signaling is downstream of many growth factor receptors, we examined whether Notch activates PI3K through a cell-autonomous or non-autonomous pathway. Conditioned medium from HMEC-Notch1IC, but not HMEC-vector, activated PI3K in parental HMEC (Figs. 2B and C), suggesting the presence of a secreted PI3K-activating factor induced by Notch.

To examine the role of PI3K activation in Notch-activated EC survival, HMEC-Notch1IC was treated with an inhibitor of PI3K, LY294002, at a dose sufficient to completely block Akt phosphorylation (40 μmol/L) (Supplemental fig. 2). PI3K inhibition led to significant increase in apoptosis in HMEC-Notch1IC, but not in HMEC-vector as quantified by activation of Caspase 3 (Fig. 2D, Supplemental fig. 3). Increased apoptosis in HMEC-Notch1IC with PI3K inhibition was verified with another PI3K inhibitor, wortmannin, by Annexin V binding (Fig. 2E). When endogenous Notch signaling was activated by co-culturing parental HMEC and Notch ligand (Jagged1 and Dll4)-expressing HMEC, PI3K inhibition also increased apoptosis (Fig. 2F), indicating that physiological