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Selective suppression of endothelial cytokine production by progesterone receptor

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ABSTRACT

Steroid hormones are well-recognized suppressors of the inflammatory response, however, their cell- and tissue-specific effects in the regulation of inflammation are far less understood, particularly for the sex-related steroids. To determine the contribution of progesterone in the endothelium, we have characterized and validated an in vitro culture system in which human umbilical vein endothelial cells constitutively express human progesterone receptor (PR). Using next generation RNA-sequencing, we identified a selective group of cytokines that are suppressed by progesterone both under physiological conditions and during pathological activation by lipopolysaccharide. In particular, IL-6, IL-8, CXCL2/3, and CXCL1 were found to be direct targets of PR, as determined by ChIP-sequencing. Regulation of these cytokines by progesterone was also confirmed by bead-based multiplex cytokine assays and quantitative PCR. These findings provide a novel role for PR in the direct regulation of cytokine levels secreted by the endothelium. They also suggest that progesterone-PR signaling in the endothelium directly impacts leukocyte trafficking in PR-expressing tissues.

1. Introduction

Inflammation contributes to the susceptibility and progression of many diseases that exhibit gender based differences in prevalence. These include, but are not limited to, autoimmune disease, cardiovascular disease and sexually transmitted infections (Kaushic et al., 2011; McCombe et al., 2009; Meyer et al., 2006). The prevailing hypothesis is that endocrine–immune interactions drive this sexual dimorphism by affecting the sensitivity to various inflammatory stimuli. Evidence for this emanates from studies demonstrating the requirement for the immune system in hormonally controlled processes including implantation, cycling, and pregnancy (Challis et al., 2009; Gilliver, 2010; Jones, 2004; King and Critchley, 2010; Red-Horse and Drake, 2004; van Mourik et al., 2009). For example, symptoms of rheumatoid arthritis and multiple sclerosis are reduced during pregnancy, suggesting that hormones not only modulate local inflammatory reactions, but also can affect systemic immune responses as well (Adams Waldorf and Nelson, 2008; Hughes, 2012; Martocchia et al., 2011). While much is known of the cellular and molecular control of the immune system by estrogen, glucocorticoids, and androgen signaling, the action of progesterone and its downstream targets are far less understood.

Progesterone has been generally assumed to play an anti-inflammatory role in immune regulation. In fact, the physiological reduction of progesterone prior to menstruation and preceding labor results in a marked influx of inflammatory cells (macrophages, neutrophils, and T cells) into the decidua resembling a local inflammatory response (Hamilton et al., 2012, 2013; Jones, 2004; Shynlova et al., 2008). Moreover, mice with complete deletion of PR (PRKO) were found to have increased immune cell infiltration into the uterus and impaired thymic function (Tibbetts et al., 1999a, 1999b). At the cellular level, PR expression has been demonstrated in a variety of immune cell types indicative of a direct regulation by progesterone (Butts et al., 2008; Gilliver, 2010; Hughes, 2012). However, these findings do not explain progesterone control of other leukocyte populations that do not express PR in vivo, such as natural killer cells and granulocytes. Therefore, it is likely that paracrine factors such as cytokines and chemokines act as effectors of steroid hormones, thus enabling systemic immune modulation in the absence of leukocyte steroid receptors. In fact, there is ample evidence in the literature for regulation of immune function by progesterone through its effect on smooth muscle, stromal, and perivascular cells (Gotkin et al., 2006; Hardy et al., 2006; Luk et al., 2010; Shields et al., 2005; Shynlova et al., 2008). Due to its multiple cellular targets, a comprehensive dissection of cell specific signaling, as well as direct downstream targets of PR, is necessary to understand the multiple immune-modulatory functions of progesterone.

Abbreviations: PR, progesterone receptor; LPS, lipopolysaccharide; HUVEC, human umbilical vein endothelial cell; PRKO, progesterone receptor knockout; hPR, human progesterone receptor; DAPI, 4′,6-diamidino-2-phenylindole.

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The endothelium is an active participant in immune cell trafficking and is an important barrier in the regulation of leukocyte extravasation into tissues (Ley et al., 2007; Pober and Sessa, 2007). Upon activation by an inflammatory stimulus, endothelial cells acquire new capabilities including cytokines/chemokine secretion and the expression of endothelial–leukocyte adhesion molecules (Pober and Sessa, 2007). Several reports have demonstrated expression of PR within different human vascular beds (Ingegno et al., 1988; Iruela-Arispe et al., 1999; Krikun et al., 2005; Maybin and Duncan, 2004; Perrot-Applanat et al., 1995; Rodríguez-Manzaneque et al., 2000), including endothelial cells of human atherosclerotic vessels (Vázquez et al., 1999). Functionally, progesterone has been found to mediate endothelial cell proliferation, transcriptional repression of endothelial–leukocyte adhesion molecules, as well as MMP secretion (Otsuki et al., 2001; Rodríguez-Manzaneque et al., 2000; Vázquez et al., 1999) implicating a direct function of progesterone in the endothelium. Therefore, we hypothesized that progesterone signaling may modulate the immune system by transcriptionally altering endothelial cell activation and expression of immunomodulatory factors.

Here we provide evidence that PR signaling in the endothelium directly regulates cytokine expression both under physiological conditions, as well as following an acute inflammatory stimulus. PR is able to selectively and directly target a cohort of endothelial cytokines resulting in transcriptional repression and reduction in protein levels by the endothelium. These findings expand our understanding of the cell specific function of progesterone in the endothelium and its potential role in immune regulation through direct mediation of cytokine production.

2. Materials and methods

2.1. Virus production and transduction

Human PR cDNA was PCR amplified and cloned into a lentiviral vector using the following primers with attached restriction site sequences: 5′-PR-Xbal (GCTATCTAGAATGACTGAGCTGAAGGCA) and 3′-PR-STOP-EcoRI (GCTAGAATTCCTACTTTTTATGAAAGAGAAG). Lentivirus-based vectors encoding PR cDNA were generated by transient cotransfection of 293 T cells with a three-plasmid combination, as described previously, with slight modifications (Naldini et al., 1996). The construct pMD.G was used for the production of the VSV-G viral envelope in combination with the packaging constructs pMDLg/pRRE and pRSV-REV, whereas the pRRL constructions correspond to the different transfer vectors. Briefly, 100 mm dishes of nonconfluent 293 T cells were co-transfected with 6.5 μg of pMDLg/pRRE, 3.5 μg of pMDG (encoding the VSV-G envelope), 2.5 μg of pRSV-REV and 10 μg of pRRL-hPR, by the CaPi-DNA coprecipitation method (Chen and Okayama, 1987; Sakoda et al., 1992). The plasmid vectors were provided by Dr Luigi Naldini (University of Torino, Italy). Next day, the medium was adjusted to make a final concentration of 10 mM sodium butyrate and the cells were incubated for 8 h to obtain high-titer virus production as previously described (Sakoda et al., 1999). Conditioned medium was harvested 16 h later and passed through 0.45 mm filters. Viral titer was determined by assessing viral p24 antigen concentration by ELISA (the Alliance® HIV-I p24 ELISA Kit, Perkin Elmer) and hereafter expressed as μg of p24 equivalent units per milliliter.

2.2. Cell culture

Human umbilical vein endothelial cells were cultured in MCDB-131 media (VEC Technologies, Rensselaer, NY) supplemented with charcoal stripped fetal bovine serum (Omega Scientific, Tarzana, CA). For bead-based multiplex cytokine arrays, HUVECs were grown to confluence in 48 well plates and treated with LPS (1 μM; 0111: B4; Sigma, St. Louis, MO) and/or progesterone (100 nM; Sigma, St. Louis, MO) for 4, 8, and 24 h. Media without serum were collected, and run in triplicate on a 42-plex array analyzed by Eve Technologies. For immunocytochemistry, HUVECs were seeded onto Lab-Tek II 8-well chamber slides (Thermo Scientific, Rochester, NY) and fixed with 4% paraformaldehyde. Cells were probed with an antibody against PR (1:400; clone SP2, Lab Vision, Kalamazoo, MI) followed by an Alexa Fluor secondary (1:300, Invitrogen, Grand Island, NY). Nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI, 1:1000; Invitrogen, Grand Island, NY). Images were acquired using a Zeiss LSM 520 multiphoton microscope (Zeiss, Germany).

2.3. Immunoblotting

Total HUVEC lysates were resolved by SDS-PAGE, and nitrocellulose membranes (Optitran BA-S 83; Dassel, Germany) were incubated overnight with an anti-PR antibody (1:2000; clone SP2, Lab Vision, Kalamazoo, MI) and anti-GAPDH antibody (1:1000, Millipore, Billerica, MA). Blots were incubated with HRP-conjugated secondary (1:5000; Bio-Rad Laboratories, Hercules, CA) and developed using Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, Kalamazoo, MI). A Bio-Rad ChemiDoc XRS + and accompanying Image Lab software was used for detection (Bio-Rad Laboratories, Hercules, CA).

2.4. RNA isolation, qPCR, and library preparation

Total RNA was extracted using RNeasy Kit (Qiagen, Valencia, CA). RNA was reverse transcribed using SuperScript First-strand Synthesis System (Invitrogen, Grand Island, NY). qPCR was performed using SYBR Green reagent (Qiagen, Valencia, CA) and PCR products were run on an Opticon2 PCR machine (MJ Research; BioRad, Hercules, CA). Libraries for RNA-sequencing were generated using an Illumina Multiplex System (Illumina, San Diego, CA) and sequenced using HIseq-2000 (Illumina, San Diego, CA). RNA-seq datasets have been deposited in the NCBI Gene Expression Omnibus with the accession number GSE46502.

2.5. RNA-seq analysis

Multiplex runs were debarcoded by in house Unix shell script. Reads were aligned to the human genome (hg19) using TopHat v2.0.4 (Trapnell et al., 2009) and processed with Cufflinks v2.0.1 (Trapnell et al., 2010). Assemblies for all samples were merged using CuffMerge and pairwise differential expression was assessed using Cuffdiff. Genes with a p-value smaller than 0.01 where considered significant. Heatmaps with relative expression were generated by visualizing the log2 values of each gene rpkm divided with the average rpkm of all samples using Java treeview (de Hoon et al., 2004).

2.6. ChIP-sequencing and analysis

HUVECs were infected with hPR lentivirus, grown to confluence, and treated with progesterone for 1 h. For each condition (non-infected-negative control, PR + P, PR only, and IgG control) 10×10^6 cultured HUVECs were used per IP. Cells were crosslinked with 1% formaldehyde, resuspended in 400 μL of lysis buffer (1% SDS, 20 mM EDTA and 50 mM Tris–HCl (pH 8.0)) containing protease inhibitors (Roche, Indianapolis, IN), and sonicated to achieve 200 bp fragments. Samples were immunoprecipitated with 3 μg of anti-PR or IgG antibody. Protein A Dynabeads (Invitrogen, Grand Island, NY) were used to isolate antibody–PR complexes and eluted using 50 mM Tris–HCl, pH 8.0. Crosslinks were reversed by incubation at 65 °C and DNA was purified using Qiagen MinElute Columns. Libraries were generated using Ovation Ultralow IL Multiplex System 1–8 (Nugen, San Carlos, CA) and sequenced using HIseq-2000 (Illumina, San Diego, CA). ChIP-seq data sets have been deposited in the NCBI Gene Expression Omnibus with the accession number GSE43786.