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TLR4 expression in mouse embryonic stem cells and in stem cell-derived vascular cells is regulated by epigenetic modifications ☆

Anna Zampetaki *, Qingzhong Xiao, Lingfang Zeng, Yanhua Hu, Qingbo Xu

Department of Cardiac and Vascular Sciences, St. George's, University of London, London SW17 0RE, UK

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Abstract

Embryonic stem (ES) cells and ES cell-derived differentiated cells can be used in tissue regeneration approaches. However, inflammation may pose a major hurdle. To define the inflammatory response of ES and ES cell-derived vascular cells, we exposed these cells to LPS. With the exception of MIF no significant cytokine mRNA levels were observed either at baseline or after stimulation. Further experiments revealed that these cells do not express TLR4. Analysis of the DNA methylation status of the TLR4 upstream region showed increased methylation. Moreover, in vitro methylation suppressed TLR4 promoter activity in reporter gene assays. ChIP assays showed that in this region histones H3 and H4 are hypoacetylated in ES cells. Interestingly, 5-aza-dC or TSA partially relieves this gene repression. Finally, the increased levels of TLR4 observed in ES cells after treatment with 5-aza-dC or TSA confer responsiveness to LPS, as induction of IL-6 and TNFα mRNA was detected in endotoxin stimulated ES cells.

Keywords: Endotoxin challenge; DNA methylation; Histone hypoacetylation

Embryonic stem (ES) cells are totipotent cells derived from the inner cell mass of blastocysts. Their ability of unlimited self-renewal and differentiation to a variety of somatic cell types makes these cells an attractive target for new therapeutic approaches that involve tissue regeneration. In tissue engineering, the vasculogenic potential of human ES-derived endothelial cells has been explored. Differentiated ES cells grown on a biodegradable polymer scaffolds has been shown to form vascular networks within the engineered-tissue like structures [1–3]. ES cells have also been used in attempts to improve cardiac function. Cardiac committed-ES cells when transplanted into infarcted myocardium differentiate into cardiomyocytes and improve left ventricular function [4]. Transfusion of progenitor cells may improve organ function by various ways, e.g., improving neovascularization, inhibiting apoptosis, enhancing endogenous repair mechanisms or differentiating to specific cell types. Although it is still not known whether repopulation of the injured tissue is required or whether transient secretion of paracrine mediators by the transplanted cells can rescue organ function, stem cells represent a promising potential in cardiovascular, chronic and degenerative diseases [5,6].

Inflammation plays a central role in most of the pathological conditions where cell transplantation could be applied [7,8]. Complex interactions occur between inflammatory cells and the damaged tissue and expression of adhesion molecules, cytokines, matrix metalloproteinases and growth factors is extremely elevated. The use of stem cell-derived cells or progenitor cells that do not elicit an inflammatory response and thus do not further augment the damage in the injured area could have beneficial effects on the recovery of organ function. The study of the potential inflammatory response of the transplanted cells could provide valuable insights into the efficiency of such

☆ *Abbreviations:* LPS, lipopolysaccharide; SMC, smooth muscle cells; 5-aza-dC, 5-aza-2-deoxycytidine; TSA, trichostatin A; SAM, *S*-adenosylmethionine; ChIP, chromatin immunoprecipitation; AcH3, acetylated histone 3; AcH4, acetylated histone 4.

* Corresponding author. Fax: +44 20 8725 2812.
E-mail address: azampeta@sgul.ac.uk (A. Zampetaki).

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therapeutic interventions. However, it is unknown whether ES and ES-derived cells have the ability to respond to inflammatory stimuli.

The innate immune system responds to infection through a panel of pattern recognition receptors (PRR) which recognize conserved sequences, such as bacterial lipoproteins and endotoxin (LPS) [9]. One of the main families of PRR is the family of Toll-like receptors (TLR). To date, 11 mammalian Toll homologues have been identified and designated, TLR1-11. Following ligation, TLRs signal through adapter molecules to activate the NF-κB pathway. This results in an immune response characterized by the production of cytokines, antimicrobial products and the regulation of costimulatory molecules. Interestingly, little is known about TLR expression and their functions in stem cells.

In the present study, we used the endotoxin model to investigate the inflammatory response of mouse ES, ES-derived endothelial cells (esEC) and ES-derived smooth muscle cells (esSMC). Our experiments showed that these cells do not upregulate cytokine expression and are unresponsive to LPS stimulation due to diminished levels of TLR4, the transmembrane receptor that recognizes LPS [10]. This repression of gene expression is mediated by epigenetic events that include methylation of the TLR4 promoter region and hypoacetylation of histones H3 and H4. These modifications have a profound effect on the promoter activity. Inhibition of DNA methylation or histone deacetylase activity can partially restore TLR4 expression in ES cells and confer responsiveness to endotoxin.

Methods

Cell culture. SMCs were isolated by enzymatic digestion of mouse aortas as described elsewhere [11] and were cultured in DMEM supplemented with 15% FCS, 2 mM L-glutamine and 100 mg/l gentamicin. Mouse ES cells (D3) were obtained from ATCC (Manassas, VA, USA) and grown on gelatin-coated flasks. Cell passages 3–15 were used for experiments. To maintain the ES cells in an undifferentiated state leukaemia inhibitory factor (LIF, 1000 U/mL) was added to the culture medium (DMEM, ATCC) supplemented with 10% FCS, 2 mM L-glutamine, 100 mg/l gentamicin and 10^{-4} M 2-mercaptoethanol (2-ME).

To obtain esEC cells, ES were plated on collagen IV slides and cultured in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/l gentamicin and 5×10^{-5} M 2-ME for 4 days. The cells were subsequently subjected to shear stress at 12 dyn/cm^2 for 24 h. Expression of EC markers was detected by RT-PCR and confirmed by FACS.

To obtain esSMC, ES cells were cultured on type IV mouse collagen coated flasks in α-minimal essential medium (αMEM; Gibco), supplemented with 10% FCS, 2 mM L-glutamine, 100 mg/l gentamicin and 5×10^{-5} M 2-ME for 3 to 4 days. Sca-1$^+$ cells were sorted from the cell culture by magnetic labelling cell sorting (MACS) with anti-Sca-1 microbeads (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany). Sca-1$^+$ cells were resuspended and cultured in fresh ES cell growth medium. For SMC differentiation, Sca-1$^+$ cells were plated on collagen IV-coated dishes or flasks, and cultured in αMEM supplemented with 10% FCS and 5×10^{-5} M 2-ME and 10 ng/ml PDGF-BB (Sigma). esSMC passage 5 was used for all experiments. Expression of SMC markers was detected by RT-PCR and confirmed by FACS.

RNase protection assay (RPA). Total RNA was extracted using the Qiagen kit according to the manufacturer's instructions. To estimate the expression of cytokines, RNase protection assay was performed, using mCK2b and mCK5c multi-probe template sets (RiboQuant, Pharmingen, San Diego, CA) and [α-^{32}P]UTP (Amersham Biosciences) according to the manufacturer's recommendations. The "RNase-protected" fragments were purified and resolved on a 5% sequencing gel and autoradiographed. For quantification, signals for each sample of the blot were normalized to the housekeeping gene L32.

Reverse transcription-polymerase chain reaction. Total RNA was extracted using the Qiagen kit according to the manufacturer's instructions and any potential contaminating chromosomal DNA was digested using the DNA-free kit (Ambion). The procedure used for RT-PCR was similar to that described elsewhere [12]. In brief, 2 μg of RNA was converted to cDNA using Promega Reverse Transcription System (Promega, Madison, WI). cDNA products were amplified by PCR using gene-specific primers. The primers used were: TLR4 forward "GCT TTC ACC TCT GCC TTC AC," TLR4 reverse "AGG CGA TAC AAT TCC ACC TG," TNFα forward "AGC CCC CAG TCT GTA TCC TT," TNFα reverse "CTC CCT TTG CAG AAC TCA GG" IL-6 forward "CGA TGA TGC ACT TGC AGA AA," IL-6 reverse "GGA AAT TGG GGT AGG AAG GA," and GAPDH forward "CGG AGT CAA CGG ATT TGG TCG TAT" and GAPDH reverse "AGC CTT CTC CAT GGT GGT GAA GAC." PCR conditions were as follows: 94 °C for 3 min and then 40 cycles for TLR4, TNFα and IL-6 or 26 cycles for GAPDH at 94 °C for 30 s, 58 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 10 min. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Real-time polymerase chain reaction experiments were performed with the Brilliant SYBR Green QPCR core reagent kit (Stratagene), using the Mx4000 (Stratagene) real-time thermocycler according to the company's instructions. Amplification was performed with 40 cycles and an annealing temperature of 58 °C. Copy numbers were calculated by the instrument's software from standard curves. The specificity of the amplification reaction was determined by a melting curve analysis. For quantification TLR4 and IL-6 and TNFα mRNA expression was normalized to the housekeeping gene GAPDH.

Western blot analysis. The procedure used was similar to that described previously [12]. Antibodies against TLR4 or β-actin were from Santa Cruz Biotechnology. Specific antibody–antigen complexes were detected by using the ECL Western Blot Detection Kit (Amersham Pharmacia Biotech, UK).

Plasmid construct. An expression vector harboring sequences of the mouse TLR4 promoter was created using genomic DNA from SMCs. A 0.7 kb fragment (−667/+29) from the TLR4 promoter was generated by PCR using TLR4 forward (−667) TCA GTC CTC GAG ACG AGC CTG CTC CTA TCT and TLR4 reverse (+29) TCA GTC AAG CTT GAA GTG AGA GTG CCA ACC primers. The PCR product was then digested with *Xho*I and *Hin*dIII (Promega), gel purified and cloned into the *Xho*I/*Hin*dIII site of the pGL3-basic vector to create plasmid pTLR4-Luc(−667). The construct was verified by sequencing.

Transient transfection. For transfection experiments, 5×10^4 cells per well were seeded in 12-well plates and left to adhere overnight. The cells were then transfected with the TLR4 expression and pCMV-β-galactosidase vectors using Fugene-6-Reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. After 24 h, fresh medium was added to the transfected cells and the plates were returned to the incubator. In experiments where TSA was used, ES cells were exposed to 50 nM TSA for 24 h. The cells were then washed twice with ice-cold PBS and lysed in the Reporter Lysis Buffer (Promega, Madison, WI). The luciferase and the β-galactosidase activities were determined using luciferase and β-galactosidase enzyme assay systems, respectively (Promega, Madison, WI). The latter was used to calculate transfection efficiency in each experiment. At least three independent transfections were performed in triplicate.

In vitro DNA methylation. TLR4 promoter vectors were methylated with M.SssI methylase (New England Biolabs) in the presence of 160 μM SAM at 37 °C overnight. The completion of methylation was confirmed by resistance to *Bsa*AI digestion. Mock methylated controls were obtained by omitting the M.SssI methylase from the assays.

Region-specific methylation was performed by excision of the TLR4 promoter by *Xho*I and *Hin*dIII restriction digestion as described