Macrophage 11β-HSD-1 deficiency promotes inflammatory angiogenesis

Zhenguang Zhang1, Agnes E Coutinho1, Tak Yung Man1, Tiina M J Kipari2, Patrick W F Hadoke1, Donald M Salter2, Jonathan R Seckl1 and Karen E Chapman1

1University/BHF Centre for Cardiovascular Science, The Queen’s Medical Research Institute, The University of Edinburgh, Edinburgh, UK
2Centre for Genomic and Experimental Medicine, MRC Institute of Genetic and Molecular Medicine, The University of Edinburgh, Western General Hospital, Edinburgh, UK
*(Z Zhang is now at AV Hill Building, Faculty of Life Science, University of Manchester, Manchester, UK)
†(A E Coutinho is now at Humber College Institute of Technology and Advanced Learning, Toronto, Canada)

Abstract

11β-Hydroxysteroid dehydrogenase-1 (11β-HSD1) predominantly converts inert glucocorticoids into active forms, thereby contributing to intracellular glucocorticoid levels. 11β-HSD1 is dynamically regulated during inflammation, including in macrophages where it regulates phagocytic capacity. The resolution of inflammation in some disease models including inflammatory arthritis is impaired by 11β-HSD1 deficiency or inhibition. However, 11β-HSD1 deficiency/inhibition also promotes angiogenesis, which is beneficial in mouse models of surgical wound healing, myocardial infarction or obesity. The cell types responsible for the anti-inflammatory and anti-angiogenic roles of 11β-HSD1 have not been characterised. Here, we generated Hsd11b1 MKO mice with LysM-Cre mediated deletion of Hsd11b1 to investigate whether 11β-HSD1 deficiency in myeloid phagocytes is pro-angiogenic and/or affects the resolution of inflammation. Resolution of inflammatory K/BxN-induced arthritis was impaired in Hsd11b1 MKO mice to a similar extent as in mice globally deficient in 11β-HSD1. This was associated with >2-fold elevation in levels of the endothelial marker Cdh5 mRNA, suggesting increased angiogenesis in joints of Hsd11b1 MKO mice following arthritis. A pro-angiogenic phenotype was confirmed by measuring angiogenesis in subcutaneously implanted polyurethane sponges, in which Hsd11b1 MKO mice showed 20% greater vessel density than their littermate controls, associated with higher expression of Cdh5. Thus, 11β-HSD1 deficiency in myeloid phagocytes promotes angiogenesis. Targeting 11β-HSD1 in macrophages may be beneficial in tissue repair.

Introduction

Glucocorticoids exert anti-inflammatory effects both in vivo and in vitro. Whilst the potent effects of synthetic glucocorticoids have been widely exploited clinically to treat inflammatory disease, including rheumatoid arthritis, the role of endogenous corticosteroids in regulating inflammation is less well understood (Coutinho & Chapman 2011). Nevertheless, macrophages have emerged as a key target for the anti-inflammatory and immunomodulatory effects of endogenous glucocorticoids (Bhattacharyya et al. 2007, Tuckermann et al. 2007). Macrophages orchestrate much of the tissue remodelling that follows injury (Minutti et al. 2016) and play a key role in the angiogenesis that is
important for tissue repair (Nucera et al. 2011). Macrophages are also important in the excessive or dysregulated angiogenesis that contributes to the pathogenesis of many chronic inflammatory diseases, a maladaptive response in rheumatoid arthritis (Jackson et al. 1997, Koch 1998).

Macrophages express 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), a glucocorticoid metabolising enzyme that, in intact cells, converts the intrinsically inert cortisone and 11-dehydrocorticosterone into the active glucocorticoids, cortisol and corticosterone, respectively (Chapman et al. 2013b). 11β-HSD1 thus amplifies glucocorticoid action. 11β-HSD1 deficiency or inhibition alters inflammatory responses (Chapman et al. 2013a). 11β-HSD1-deficient mice show more severe acute inflammation in thioglycollate-induced sterile peritonitis (Gilmour et al. 2006), following lipopolysaccharide (Zhang & Daynes 2007), in carrageenan-induced pleurisy (Coutinho et al. 2012), in the K/BxN serum-induced model of inflammatory arthritis (Coutinho et al. 2012) and following coronary artery ligation in a model of myocardial infarction (McSweeney et al. 2010). Macrophages are implicated in the altered inflammatory responses of 11β-HSD1-deficient mice. In vitro, peritoneal or splenic macrophages from 11β-HSD1-deficient mice overproduce inflammatory cytokines following lipopolysaccharide stimulation (Zhang & Daynes 2007). During thioglycollate-induced peritonitis, 11β-HSD1-deficient mice show a delay in the acquisition of phagocytic capacity by macrophages, though the inflammation resolves at a similar time to that in control mice (Gilmour et al. 2006). Reduced cholesterol accumulation in macrophages plays a role in the atheroprotective and anti-inflammatory effects of global 11β-HSD1 deficiency or inhibition in atherosclerosis-prone ApoE−/− mice (Garcia et al. 2013, Kipari et al. 2013, Luo et al. 2013). Following myocardial infarction, macrophages accumulate around the infarct zone more rapidly in 11β-HSD1-deficient mice (McSweeney et al. 2010). They switch to a pro-reparative, pro-angiogenic ‘M2’ phenotype earlier than in control mice, resulting in greater angiogenesis in the healing infarct and better recovery of heart function post-myocardial infarction (Small et al. 2005, McSweeney et al. 2010, Michailidou et al. 2012). Global 11β-HSD1 deficiency is also pro-angiogenic in other contexts: in the sponge implantation assay (Small et al. 2005); in adipose tissue of obese mice where it is associated with reduced adipose tissue hypoxia and inflammation (Michailidou et al. 2012) and in cutaneous surgical wounds, where it is associated with improved wound repair (Small et al. 2005).

Here, we have generated mice deficient in 11β-HSD1 in myeloid phagocytes (Hsd11b1MKO) to investigate the role of 11β-HSD1 activity in macrophages. We have used models of inflammation associated with a strong angiogenic response – the K/BxN serum transfer model of inflammatory arthritis and the sponge implantation assay, in which the role of 11β-HSD1 activity in macrophages is examined.

Materials and methods

Animals

All experiments on animals were carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act of 1986 and European Directive 2010/63/EU, following approval by the University of Edinburgh Animal Welfare and Ethical Review Body. Mice were housed in groups (2–5 per cage) under controlled conditions: 12h light/darkness cycle at 21°C with free access to standard rodent chow and water. Hsd11b1f/f mice, with LoxP sites flanking exon 3 of the Hsd11b1 gene, were generated by TaconicArtemis (Cologne, Germany) onto a C57BL/6 background (Verma M, Kipari TMJ, Zhang Z, Man TY, Forster T, Homer NZM, Seckl JR, Holmes MC & Chapman KE, unpublished observations). Hsd11b1MKO mice with myeloid cell 11β-HSD1 deficiency were generated by crossing LysM-Cre (backcrossed to a C57BL/6 background (Cramer et al. 2003)) with Hsd11b1f/f mice. Experimental (Hsd11b1MKO) and littermate control (Hsd11b1f/f) mice were the offspring of male Hsd11b1MKO mice bred with female Hsd11b1f/f mice. Hsd11b1f/fDel1/Del1 mice (Verma M, Kipari TMJ, Zhang Z, Man TY, Forster T, Homer NZM, Seckl JR, Holmes MC & Chapman KE, unpublished observations) were generated from Hsd11b1f/f mice and are homozygous for a germline deletion of exon 3 of the Hsd11b1 gene. All experiments used male mice aged between 8 and 16 weeks.

Peritoneal myeloid cell isolation and 11β-HSD1 activity assay

Mice were killed by CO2 asphyxiation. Resident cells were harvested from the peritoneum by lavage with 5 mL ice-cold PBS as previously described (Gilmour et al. 2006). Inflammatory cells elicited to the peritoneum 24h, 72h or 96h following intra-peritoneal injection of thioglycollate (0.2mL, 10%) were collected by lavage with 5mL ice-cold PBS (Coutinho et al. 2016). At 24h, cells comprise mainly neutrophils and monocytes, whereas the population is predominantly macrophages at 72h and 96h (Melnicoff et al. 1989, Zhang et al. 2008).
Coutinho et al. 2016). Where appropriate, neutrophils were isolated with anti-Ly6G antibody (clone RB6-8C5, Thermo Fisher Scientific) coupled to anti-rat IgG microbeads (Miltenyi Biotec Ltd., Bisley, Surrey, UK) by magnetic-activated cell sorting as described previously (Coutinho et al. 2016). Macrophages were purified by adherence to tissue culture plates using an established procedure that gives >90% purity (Zhang et al. 2008). 11β-HSD1 activity in cultured cells was measured by addition of 5nM [3H]-11-dehydrocorticosterone to the medium, as described previously (Coutinho et al. 2016).

K/BxN serum transfer-induced arthritis

K/BxN serum was generated in-house as described previously (Coutinho et al. 2012). To induce arthritis in mice, 100μL serum was injected intraperitoneally. Inflammation was scored daily for 21 days by an investigator blind to genotype. Scoring was carried out by visual examination according to a clinical index in which each joint was ascribed a score of 0–3, as described previously (Coutinho et al. 2012). The combined scores of all 4 limbs were calculated for each mouse. For histology, joints were fixed in 10% formalin for 1 day and then decalcified in 10% EDTA in neutral buffered formalin and paraffin embedded. Joint sections (4μm) were deparaffinised, hydrated and stained with haematoxylin and eosin for histopathological examination. The severity of pathological changes in the ankle joint was quantified on a scale of 0–3 for each mouse, based on a scoring system modified from a previous protocol (Ruiz-Heiland et al. 2012). Briefly, the main pathological feature, synovium thickening, is assessed and assigned a score of 0 if there is no change compared to untreated, 1 if there is mild proliferation, 2 if there is extended proliferation and 3 if there is severe proliferation with tenosynovitis. RNA was extracted from the hind ankle joints using Trizol (Thermo Fisher Scientific) following pulverisation using a mortar and pestle, under liquid nitrogen.

Sponge implant assay of angiogenesis

Mice were anaesthetised with isoflurane, and a sterilized sponge cube measuring 1x1x1cm (Caligen Foam, Accrington, Lancashire, UK) was implanted subcutaneously on each flank, as described previously (Small et al. 2005). After 21 days, mice were killed and sponges were removed. The sponge from the right flank was frozen at 80°C for later RNA extraction using Trizol (Thermo Fisher Scientific). The sponge from the left flank was fixed in 10% formalin and embedded in paraffin. Sections were stained with haematoxylin and eosin for angiogenesis scoring. Vessel density was determined by Chalkley counting, as described previously (Small et al. 2005) by an investigator blind to experimental group. Briefly, a 25-point Chalkley eyepiece graticule (Graticules Ltd, Edenbridge, Kent, UK) was used to count blood vessels at ×250 magnification. The graticule was placed so that the maximum number of graticule dots overlay with blood vessels. The average count values (3 random areas/section, 2 sections/sample) were recorded as vessel numbers.

Immunohistochemistry

Paraffin-embedded sections (4μm) were deparaffinised, blocked with 10% goat serum after antigen retrieval with citrate buffer (pH 6) and incubated overnight with primary antibodies against isolectin IB4 (Alexa Fluor 488-conjugated isolectin B4, 1:100; I21411, Thermo Fisher Scientific) and α-smooth muscle actin (anti-actin, α-smooth muscle-Cy3 antibody, mouse monoclonal clone 1A4; C6198-100UL, Sigma-Aldrich) to detect endothelial cells and perivascular mural cells, respectively.

Western blotting

Peritoneal cells or purified Ly6G+ cells were homogenised in RIPA buffer (R0278, Sigma-Aldrich) supplemented with proteinase inhibitor cocktail (1:100 dilution; P8340, Sigma-Aldrich) and phosphatase inhibitor (1:100 dilution; P2850, Sigma-Aldrich). Proteins (20μg/sample) were separated by electrophoresis on a 4–12% Bis-Tris gel (NP0323BOX, Thermo Fisher Scientific) and then transferred to a 0.4μm nitrocellulose membrane. Antibodies used for Western blotting recognise 11β-HSD1 (raised in sheep and kindly provided by Dr Scott Webster, the University of Edinburgh (De Sousa Peixoto et al. 2008, Coutinho et al. 2016)), β-tubulin (MAB3408, Merck Millipore) and GAPDH (ab9485, Abcam).

RNA analysis

RNA (1μg) was reverse transcribed into cDNA using a SuperScript III Reverse transcriptase system kit (Thermo Fisher Scientific). Levels of specific cDNAs were measured by quantitative (q)PCR in triplicate, using a LightCycler 480 (Roche) and Universal Probe Library (UPL; Roche)-based assays. Primer and probe information is shown in Supplementary Table 1 (see section on supplementary data given at the end of this article). A standard curve
Measurement of 11β-HSD1 activity in myeloid cell populations from Hsd11b1<sup>MKO</sup> mice showed an 89% decrease in 11β-HSD1 activity in peritoneal resident cells, compared to Hsd11b1<sup>f/f</sup> littermate controls (Fig. 1A), consistent with the disruption of Hsd11b1 in this predominantly macrophage population (Clausen et al. 1999). In contrast, there was no significant reduction in 11β-HSD1 activity in thioglycollate-elicited peritoneal cells from Hsd11b1<sup>MKO</sup> mice collected 24 h (monocytes/macrophages and neutrophils (Melnicoff et al. 1989, Coutinho et al. 2016)) or 96 h following thioglycollate injection (predominantly macrophages (Melnicoff et al. 1989, Zhang et al. 2008)) (Fig. 1B and C). Similarly, 11β-HSD1 activity was not significantly altered in purified neutrophils (Ly6G<sup>+</sup> cells) from the peritoneum of thioglycollate-injected Hsd11b1<sup>MKO</sup> mice, compared to littermate controls (Fig. 1D). 11β-HSD1 activity was maintained in these myeloid cell populations despite the efficient reduction in Hsd11b1 mRNA levels (Supplementary Fig. 2). Western blotting confirmed a marked reduction in 11β-HSD1 protein levels in the resident peritoneal cell population of Hsd11b1<sup>MKO</sup> mice, but only a modest reduction in peritoneal cells collected 24 h or 96 h following thioglycollate injection and in purified neutrophils (Supplementary Fig. 3). These data suggest that the protein half-life of 11β-HSD1 exceeds the half-life of the thioglycollate-elicited monocyte/macrophage and neutrophil populations, but that 11β-HSD1 activity is markedly reduced in the longer-lived resident macrophages.

**Results**

**Disruption of 11β-HSD1 in resident macrophages**

To generate Hsd11b1<sup>MKO</sup> mice with disruption of 11β-HSD1 expression in myeloid phagocytes, LysM-Cre transgenic mice (Cramer et al. 2003) were crossed with mice in which exon 3 of the Hsd11b1 gene is flanked by LoxP sites (Hsd11b1<sup>f/f</sup> mice) (recombination strategy shown in Supplementary Fig. 1). LysM-Cre transgenic mice are reported to efficiently delete LoxP-flanked target genes in granulocytes and mature macrophages, with lower efficiencies in other myeloid cells (Clausen et al. 1999).

**Statistics**

Values are means±standard error of means (s.e.m.). Data were analysed using GraphPad Prism 5.0. Unpaired t-tests (with Welch’s corrections when variance was unequal), and one-way and two-way analysis of variance (ANOVA) were used, as appropriate. Significance was set at P<0.05.

**Figure 1**

11β-HSD1 activity is markedly reduced in resident peritoneal macrophages from Hsd11b1<sup>MKO</sup> mice. Resident peritoneal cells, or cells elicited to the peritoneum by i.p. injection of 0.2 mL 10% thioglycollate (TG), were harvested from Hsd11b1<sup>MKO</sup> (MKO: white bars) and control Hsd11b1<sup>f/f</sup> mice (Con: black bars). 11β-HSD1 activity in peritoneal cells was measured by conversion of [3H]-11-dehydrocorticosterone to corticosterone in resident peritoneal cells (A) and in cells elicited to the peritoneum 24 h (B) or 96 h (C) following thioglycollate injection. Activity was also measured in purified neutrophils (Ly6G<sup>+</sup> cells) isolated from the peritoneum 24 h after thioglycollate injection (D). 11β-HSD1 activity is expressed as pmol corticosterone/h/10<sup>6</sup> cells. Values are means±s.e.m. and were analysed by unpaired t-test (n=3–8, *P<0.05).
macrophage population. Given this, we sought to test the role of macrophage 11β-HSD1 in models of inflammation in which the role of resident macrophages may be evident.

Resolution of experimental arthritis is impaired in Hsd11b1\textsuperscript{MKO} mice

Tissue-resident macrophages have been implicated in the development and resolution of inflammatory arthritis in mice induced by injection of K/BxN serum (Misharin \textit{et al}. 2014). We therefore investigated whether macrophage 11β-HSD1 deficiency contributes to the more severe arthritis that develops in mice globally deficient in 11β-HSD1 following injection of arthritogenic K/BxN serum (Coutinho \textit{et al}. 2012). Hsd11b1\textsuperscript{MKO} mice did not replicate the earlier onset of arthritis seen previously with global 11β-HSD1 deficiency (Coutinho \textit{et al}. 2012). However, resolution of arthritis was impaired in Hsd11b1\textsuperscript{MKO} mice, compared to Hsd11b1\textsuperscript{f/f} controls (Fig. 2A and B). The course of the resolution phase in Hsd11b1\textsuperscript{MKO} mice was very similar to that of Hsd11b1\textsuperscript{Del1/Del1} mice globally deficient in 11β-HSD1 (Fig. 2A and B). Histopathological examination of the joints showed bone erosion and marked fibroproliferation in the synovium (Fig. 2C). Quantification of histopathology using a scoring index showed more marked histopathological changes in Hsd11b1\textsuperscript{MKO} mice, compared to littermate controls (Fig. 2D).

Rheumatoid arthritis is associated with neoangiogenesis within the affected joint (Jackson \textit{et al}. 1997, Koch 1998). Consistent with this, immunofluorescent staining of joints for the endothelial cell marker, isolecitin IB4, and α-smooth muscle actin (α-SMA), a marker of perivascular mural cells, revealed a high degree of vascularisation of the soft tissues of joints of both Hsd11b1\textsuperscript{MKO} and control mice following K/BxN serum transfer arthritis (Fig. 3A). However, qPCR measurement of Cdhs mRNA encoding the endothelial marker, vascular endothelial (VE)-cadherin, showed >2-fold higher levels of Cdhs mRNA in joints of Hsd11b1\textsuperscript{MKO} mice, compared to littermate controls (Fig. 3B).

Greater inflammatory angiogenesis in Hsd11b1\textsuperscript{MKO} mice following subcutaneous sponge implantation

Increased levels of Cdhs mRNA in joints of Hsd11b1\textsuperscript{MKO} mice suggest an increase in neovascularisation of the joint
during inflammatory arthritis. Mice with global 11β-HSD1-deficiency show greater inflammatory angiogenesis induced by subcutaneous sponge implantation (Small et al. 2005). To test whether macrophage 11β-HSD1 influences inflammatory angiogenesis, sponges were implanted subcutaneously in Hsd11b1MKO and littermate control mice, with angiogenesis assessed 21 days later (Figure 4A). Quantification of blood vessel density in sponges showed
20% greater vessel density in Hsd11b1MKO mice (Fig. 4A and B) associated with almost 2-fold higher levels of Cdhh5 mRNA (Fig. 4C). Levels of mRNA encoding IL-1β, a pro-angiogenic cytokine (Voronov et al. 2003), were >3-fold increased in sponges from Hsd11b1MKO mice (Fig. 4D) and there was also a trend for increased expression of mRNA encoding pro-angiogenic angiopoietins (Fig. 4E). Consistent with previous data from mice globally deficient in 11β-HSD1 (McSweeney et al. 2010), increased angiogenesis in Hsd11b1MKO mice was not associated with a change in Vegfa mRNA levels, nor were levels of mRNA encoding several other cytokines altered (Supplementary Fig. 4). These data support a role for macrophage 11β-HSD1 in controlling angiogenesis and thus promoting the resolution of inflammatory arthritis.

Discussion

Our data in Hsd11b1MKO mice demonstrate that macrophages are a key cell type responsible for the pro-angiogenic phenotype conferred by 11β-HSD1 deficiency. This suggests that 11β-HSD1-mediated glucocorticoid regeneration within macrophages alters their phenotype to control an ongoing inflammatory response. In Hsd11b1MKO mice, this happens without the perturbation in the systemic ratio of active to inactive glucocorticoid levels that occurs with global 11β-HSD1 deficiency (Harris et al. 2001; Verma M, Kipari TMJ, Zhang Z, Man TY, Forster T, Homer NZM, Seckl JR, Holmes MC & Chapman KE, unpublished observations). Whether this is mediated by alterations in macrophage polarisation is currently unclear. However, in vitro, bone marrow-derived macrophages from Hsd11b1−/− mice behave similar to wild type (Gilmour et al. 2006, Zhang & Daynes 2007), suggesting the in vivo environment (and possibly substrate availability) is crucial to the physiological outcomes with 11β-HSD1 deficiency. This is suggested by evidence of an earlier macrophage polarisation to a pro-angiogenic ‘M2’ phenotype after myocardial infarction (McSweeney et al. 2010) and by higher expression of the M2 marker, SHIP1, in macrophages of Hsd11b1−/− mice than wild-type controls (Zhang & Daynes 2007).

Whilst resident peritoneal macrophages from Hsd11b1MKO mice showed the predicted reduction in 11β-HSD1 activity and Hsd11b1 mRNA, our finding of near-normal 11β-HSD1 protein and activity in myeloid cells recruited to the peritoneum of thioglycollate-injected Hsd11b1MKO mice, despite a marked reduction in the encoding mRNA, was unexpected. We have previously reported a discrepancy between 11β-HSD1 protein/activity and levels of the encoding Hsd11b1 mRNA in mouse neutrophils, with 11β-HSD1 protein present despite little encoding mRNA (Coutinho et al. 2016). Other studies have noted similar discrepancies in other cell types (Bujalska et al. 2005, Chinetti-Gbaguidi et al. 2012). This suggests that in most myeloid phagocytes 11β-HSD1 protein has a long half-life and persists without renewal from ongoing mRNA translation, at least when inflammatory conditions prevail. The reduction in 11β-HSD1 activity in resident peritoneal macrophages but not in thioglycollate-elicited macrophages may reflect the longer lifespan of the resident macrophage population compared to recruited bone marrow-derived macrophages (Davies et al. 2013). Alternatively, the LysM-Cre transgene may be more highly expressed in resident macrophages. Expression of LysM/Lyz2, encoding LYSM, is known to be heterogeneous in macrophage populations (Faust et al. 2000, Hume 2011). In peritoneal cells, expression of LYSM is reportedly highest in large peritoneal macrophages that are highly phagocytic (Lichanska et al. 1999) and correspond to the resident population (Cain et al. 2013). This implicates resident macrophages in the pro-angiogenic phenotype seen with 11β-HSD1 deficiency. Resident macrophages are important in tissue repair. They efficiently clear apoptotic cells and actively promote revascularisation (Uderhardt et al. 2012, Wang & Kubes 2016). Tissue-resident macrophages promote vessel anastomosis and thus vascular network complexity, acting downstream of vascular endothelial growth factor (VEGF)-α (Fantin et al. 2010). Our finding of unchanged expression of Vegfa in Hsd11b1MKO compared to control mice (also unchanged in global 11β-HSD1-deficient mice in a model of myocardial infarction (McSweeney et al. 2010)) is consistent with 11β-HSD1 acting in tissue-resident macrophages to restrain angiogenesis, downstream of VEGF-α. However, VEGF signalling is regulated at the post-translational level and further investigation is required to establish whether (or how) VEGF signalling is involved in the increased angiogenesis that occurs in Hsd11b1MKO mice. Our data also suggest resident macrophages are an important target of glucocorticoid action in the resolution of inflammation. However, it remains possible that, in addition to macrophages, other cell types contribute to the pro-angiogenic phenotype observed with global 11β-HSD1 deficiency.

Thus, Hsd11b1MKO mice support a role for 11β-HSD1 in restraining and shaping the healing response orchestrated by resident macrophages in ischaemic or injured tissue. These mice will be a useful tool with which to dissect the contribution of macrophages to beneficial (or otherwise...
effects of 11β-HSD1 in other models of tissue repair and/or chronic inflammation.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-17-0223.

Declaration of interest
J R S holds patents on selective 11β-HSD1 inhibitors. None of the other authors has a conflict of interest.

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