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Low shear stress induces M1 macrophage polarization in murine thin-cap atherosclerotic plaques☆

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ABSTRACT

Macrophages, a significant component of atherosclerotic plaques vulnerable to acute complications, can be pro-inflammatory (designated M1), regulatory (M2), lipid- (Mox) or Heme-induced (Mhem). We showed previously that low (LSS) and oscillatory (OSS) shear stress cause thin-cap fibroatheroma and stable smooth muscle cell-rich plaque formation respectively in ApoE-knockout ($ApoE^{-/-}$) mice. Here we investigated whether different shear stress conditions relate to specific changes in macrophage polarization and plaque morphology by applying a shear stress-altering cast to the carotid arteries of high fat-fed $ApoE^{-/-}$ mice. The M1 markers iNOS and IRF5 were highly expressed in macrophage-rich areas of LSS lesions compared to OSS lesions 6 weeks after cast placement, while the M2 marker Arginase-1, and Mox/Mhem markers HO-1 and CD163 were elevated in OSS lesions. Our data indicates shear stress could be an important determinant of macrophage polarization in atherosclerosis, with low shear promoting M1 programming.

1. Introduction

Increased macrophage accumulation is a hallmark of atherosclerotic plaques prone to acute complications. Upon exposure to interferon-γ (IFN-γ) and/or LPS in vitro, macrophages undergo classical "M1" activation mirroring T helper 1 (Th1) lymphocyte polarization, inducing interleukin (IL)-12, tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS), and are regulated by the transcription factor IRF5 [1]. Alternatively activated "M2" macrophages – polarized by the Th2 cytokines IL-4 and/or IL-13 – produce IL-10, express Arginase-1 and the mannose receptor CD206.

In human plaques, transcriptomics and immunohistochemical analysis revealed a mixed representation of macrophage populations. However, some compartmentalization was observed within different plaque regions, with M1 markers being more prevalent in the shoulders of the plaque [2] and symptomatic plaques, while M2 macrophages appear in stable areas of the plaque [3] or in the adventitia [2].

Evidence suggests that the M1/M2 dichotomy is oversimplified and that macrophage plasticity generates a spectrum of macrophage "programming". Oxidized phospholipids induce heme oxygenase-1 (HO-1) expression in murine plaque "Mox" macrophages [4], and intraplaque hemorrhage-derived heme induces the expression of CD163 and HO-1 by human Mhem plaque macrophages [5,6].

Previously we designed a shear stress altering perivascular cast to mimic human pro-atherogenic hemodynamic patterns [7]. We showed that LSS induces vulnerable plaques or thin-cap fibroatheromas (TCFAs) with high macrophage content, while OSS induces smooth muscle cell-rich plaques. Although this model does not reproduce all features of human plaque vulnerability, the hemodynamic patterns are well characterized [7]. Herein, we utilized this model to investigate whether there are differences in macrophage polarization in LSS- compared to OSS-modulated plaques.

2. Material and methods

2.1. Maintenance of animals

$ApoE^{-/-}$ mice on a C57BL/6 background were purchased from Charles River Laboratories, bred in-house, housed under specific-pathogen free conditions and studied according to UK Home Office regulations. Mice undergoing surgical intervention had the normal chow diet replaced at 17 weeks of age by a cholate-free high fat diet from

Abbreviations: $ApoE^{-/-}$, apolipoprotein E knockout; HO-1, heme oxygenase-1; IL-4, interleukin 4; IL-10, interleukin 10; IL-12, interleukin 12; IL-13, interleukin 13; IFN-γ, interferon gamma; iNOS, inducible nitric oxide synthase; IRF5, interferon regulatory factor 5; LSS, low shear stress; 8-HG, 8-hydroxyguanosine; OSS, oscillatory shear stress; TCFA, thin-cap fibroatheroma; Th1, T helper 1; TNF-α, tumor necrosis factor alpha.

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Special Diets Services (Essex, UK) consisting of (w/w) cocoa butter (15%), cholesterol (0.25%), maize starch (10%), casein (20%), sucrose (40.5%), cellulose (5.95%), corn oil (1%), 50% choline chloride (2%), methionine (0.2%) and mineral mixture (5.1%) [7].

2.2. Surgical placement of a perivascular carotid cast

Littermates were randomly assigned to the 6 and 9 week time points. Only female mice were used for the study. Mice were anesthetized with isoflurane and a midline ventral neck incision was made. The right common carotid artery was isolated and a perivascular cast tied around it with ligatures as previously described [7].

2.3. Tissue preparation for histology

At the desired time point, mice were euthanized and perfused in situ with sterile saline via a cannula inserted into the left ventricle. The carotid arteries were snap frozen in OCT (optimum cutting temperature) embedding medium using liquid nitrogen and cryosectioned into 5 μm sections for the entire length of the carotid artery.

2.4. Morphological analysis of the carotid artery

Staining of the elastic lamina was performed using the Accustain kit (Sigma-Aldrich Inc., St. Louis, USA) according to the manufacturer's instructions. Necrotic areas were identified following Hematoxylin and Eosin (H&E) staining; slides were fixed in paraformaldehyde, stained with hematoxylin followed by Eosin, dehydrated in ethanol and cleared in Xylene before mounting. ImageJ software was used to quantify the intima:media ratio and necrotic core areas. Lipid-positive areas were identified following Oil Red-O and Hematoxylin staining, and collagen identified following use of Masson Trichrome stain kit (TCS Biosciences Ltd., Buckingham, UK) according to the manufacturer's instructions. Quantification of lipids and collagen was performed using Clemex software. Images were captured under identical microscope, camera, and light conditions. As serial sections were taken along the entire length of the carotid artery, one in every 12 slides (with ten sections per slide) was used per stain, and up to 20 sections per stain per mouse were quantified.

2.5. Immunohistochemistry

Immunohistochemical staining was performed using standard ABC (avidin-biotinylated complex) protocols. Antibodies used were rat anti-mouse CD68 (clone FA-11, AbD Serotec, UK), and CD206 (clone MR5D3, AbD Serotec), rabbit anti-mouse iNOS, HO-1 and IRF5 (rabbit polyclonals, Abcam, UK) and goat anti-mouse 8-hydroxyguanosine (goat polyclonal, Abcam, UK). Sections were incubated with unconjugated primary antibodies, followed by biotinylated rabbit anti-rat or goat anti-rabbit secondary antibodies, Avidin–Biotin peroxidase complexes (ABC) and DAB solution (3,3′-diaminobenzidine) (Vector Laboratories, US). Cell nuclei were counterstained with Hematoxylin. Images were captured and immunopositivity was quantified in up to 20 sections per marker per mouse, as above.

2.6. Immunofluorescence staining and Confocal Microscopy

Tissue sections were incubated with Alexa Fluor 647-conjugated rat anti-CD68 (clone FA-11, AbD Serotec), biotinylated rat anti-CD206 (clone MR5D3, AbD Serotec) followed by Alexa Fluor 488-conjugated streptavidin, and/or rabbit anti-iNOS or HO-1 (both polyclonal, Abcam) followed by Alexa Fluor 568-conjugated goat anti-IgG secondary antibody, and DAPI nuclear stain. For smooth muscle cell staining, sections were incubated with Cy3-conjugated mouse anti-α-smooth muscle cell actin (clone 1A4, Sigma-Aldrich Inc.). Stained sections were imaged using a Leica SP5 Inverted Confocal microscope.

2.7. Real time quantitative PCR

Total RNA was isolated from the LSS and OSS segments carefully isolated from whole murine carotid arteries under a dissecting microscope, using the Qiagen RNeasy kit and reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Life Technologies). cDNA was pre-amplified with custom TaqMan pre-amplification primers and TaqMan pre-amplification master mix (Life Technologies). qRT-PCR was performed using TaqMan low density array cards (Life Technologies) containing TaqMan gene expression assays, and TaqMan gene expression master mix on a 7900HT Fast Real-Time PCR system (Applied Biosystems). 18S rRNA was used as a housekeeping gene and the 2-ΔΔCt method was used to analyze relative changes in gene expression.

2.8. Statistical analysis

Analysis of immunohistochemistry data (with the exception of IRF5) was performed using a series of type III Two-way ANOVAs, taking into account the pairing of LSS and OSS observations within but not between the two time points. Where significant effects were observed from the ANOVA, post-hoc testing was performed using two-tailed t-tests (paired or unpaired as appropriate) for all possible contrasts and Bonferroni corrected p-values were calculated. Because of heteroscedasticity and non-normality we performed a single Mann–Whitney-U test for IRF5 at the time point of interest. Statistical analysis of qRT-PCR data was performed using paired two-tailed t-tests.

3. Results

Examination of lesions formed 6 and 9 weeks after cast placement revealed morphological differences (Fig. 1). We assessed the significance of these differences using a series of two-way ANOVAs. For SMC α-actin we saw significant main effects for both shear stress type (LSS: 17.8 ± 5.4% versus OSS: 28.1 ± 6.1%, $F = 52.1$, $p = 2 \times 10^{-7}$) and time ($F = 12.6$, $p = 0.002$) demonstrating higher expression in OSS than LSS and a reduction in expression at 9 weeks compared to 6 weeks. However, not all of the characteristics normally associated with TCFAs had developed at this time point (Fig. 1A–C and Supplementary Table 1). Instead, after cast placement for 9 weeks, the full TCFA plaque morphology was evident (Fig. 1B–C and Supplementary Table 1), including a significant reduction in SMCs (LSS: 6.4 ± 4.4% versus OSS: 21.8 ± 12.4%; as reported above), lipid (LSS: 26.0 ± 7.4% versus OSS: 16.5 ± 6.5%, post-hoc test $p = 0.041$) and collagen content (LSS: 29.0 ± 10.9% versus OSS: 42.9 ± 12.6%, post-hoc test $p = 0.03$). The two-way ANOVA for the effect of shear stress type on necrotic core area at 6 and 9 weeks revealed a significant interaction ($F = 22.9$, $p = 8 \times 10^{-5}$) and a main effect for stress type ($F = 45.5$, $p = 7 \times 10^{-7}$). Post-hoc testing demonstrated significantly larger necrotic areas for 9 week LSS lesions than 9 week OSS lesions (LSS: 33.4 ± 8.1% versus OSS: 13.8 ± 5.3%, $p = 0.00014$) (Fig. 1C). A two-way ANOVA for the effect of shear stress type on oxidative stress (8-hydroxyguanosine) from 8-HG staining at 6 and 9 weeks revealed a dominant interaction effect ($F = 47.1$, $p = 7 \times 10^{-7}$). Post-hoc testing revealed that OSS scored significantly lower than LSS for 8-HG (8-hydroxyguanosine) staining at 6 weeks ($p = 0.00176$) but significantly higher at 9 weeks ($p = 0.00124$, Fig. 1C). All ANOVA results are reported in Supplementary Table 1.

Two-way ANOVAs for the effect of shear stress type on macrophage markers at 6 and 9 weeks revealed interaction effects for CD68 ($F = 9.2$, $p = 0.0059$) and iNOS ($F = 18.3$, $p = 0.00028$). Post-hoc testing revealed that at 6 weeks following cast placement, the antibody against the macrophage marker CD68 stained a larger area in LSS regions than in OSS lesions (35.4 ± 11.9% versus 18.0 ± 12.1% lesion area, $p = 0.013$, Fig. 2A and C) and that iNOS expression was similarly elevated (24.2 ± 5.1% versus 6.2 ± 7.3%, $p = 0.02$, Fig. 2A and C). IRF5 was also