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Original article

Systemic toll-like receptor and interleukin-18 pathway activation in patients with acute ST elevation myocardial infarction

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ABSTRACT

Acute myocardial infarction (AMI) is accompanied by increased expression of Toll like receptors (TLR)-2 and TLR4 on circulating monocytes. In animal models, blocking TLR2/4 signaling reduces inflammatory cell influx and infarct size. The clinical consequences of TLR activation during AMI in humans are unknown, including its role in long-term cardiac functional outcome Therefore, we analyzed gene expression in whole blood samples from 28 patients with an acute ST elevation myocardial infarction (STEMI), enrolled in the EXenatide trial for AMI patients (EXAMI), both at admission and after 4-month follow-up, by whole genome expression profiling and real-time PCR. Cardiac function was determined by cardiac magnetic resonance (CMR) imaging at baseline and after 4-month follow-up. TLR pathway activation was shown by increased expression of TLR4 and its downstream genes, including IL-18R1, IL-18R2, IL-8, MMP9, HIF1A, and NFKBIA. In contrast, expression of the classical TLR-induced genes, TNF, was reduced. Bioinformatics analysis and in vitro experiments explained this noncanonical TLR response by identification of a pivotal role for HIF-1α. The extent of TLR activation and IL-18R1/2 expression in circulating cells preceded massive troponin-T release and correlated with the CMR-measured ischemic area ($R = 0.48$, $p = 0.01$). In conclusion, we identified a novel HIF-1-dependent noncanonical TLR activation pathway in circulating leukocytes leading to enhanced IL-18R expression which correlated with the magnitude of the ischemic area. This knowledge may contribute to our mechanistic understanding of the involvement of the innate immune system during STEMI and may yield diagnostic and prognostic value for patients with myocardial infarction.

1. Introduction

Although effective treatment of acute myocardial infarction (AMI) by primary percutaneous coronary intervention (pPCI) and thrombolysis has greatly improved survival and cardiac function, AMI is still a major cause of death and morbidity worldwide. Reperfusion restores tissue oxygenation, but contradictory, also induces adverse effects by reperfusion injury, in which inflammation plays an important role [1,2]. The innate immune response elicited during AMI through activation of Toll-like receptor (TLR)2 and TLR4 on circulating blood cells negatively influences the course of disease in experimental models, i.e. increases infarct size and worsens ventricular remodeling, reviewed in [3,4]. TLR4 deficiency, pharmacological inhibition of TLR4 or TLR2 reduces monocyte- and granulocyte influx into the infarcted area, decreases infarct size and ameliorates cardiac remodeling in myocardial infarction models [5–7]. Transfer of TLR2-deficient bone marrow cells into wild type animals further indicated that the deleterious effect of TLR2 is mediated by circulating immune cells [8]. TLR activation of monocytes results in Myeloid Differentiation primary response gene 88 (MyD88)-dependent nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) signaling, leading to release of proinflammatory cytokines, and matrix degrading metalloproteases. During AMI in mice, transfer of NFkB-p50-deficient bone marrow-derived cells indicated that NFkB activation in these cells is responsible for remodeling and dysfunction of the myocardium [9]. The mean

Abbreviations: AAR, Area at risk; CK-MB, Phosphocreatine kinase isoenzymes CKM and CKB; HIF1, Hypoxia induced factor 1; IL-R, Interleukin receptor; IL-18BP, IL-18 binding protein; LVEF, Left ventricular ejection fraction; EDV, End diastolic volume; MMP, Matrix metalloproteinase; NFKBIA, Nuclear factor kappa-light-chain-enhancer of activated B cells inhibitor alpha; PCR, Polymerase chain reaction; TLR, Toll-like receptor; TNF, Tumor necrosis factor alpha.

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expression of TLR2 and TLR4 (mRNA and surface protein expression) by circulating monocytes and granulocytes is increased during AMI, and unstable angina, compared to controls and patients with stable angina [10–13], indicating that not only leucocyte counts, but also their increased expression of TLR2 and −4 is related to AMI. Several endogenous TLR2 and TLR4 ligands (HSP60, HSP70, HSP90, HMGB1, Fibronectin EDA, the calgranulins S100A8 and S100A9) are expressed in ischemic tissue and also released in the circulation, causing increased TLR expression and TLR activation of circulating cells [14,15]. Next to TLR ligands, hypoxia may also induce TLR2 expression on monocytes [12]. Taken together, although numerous animal studies indicate a harmful effect of the innate inflammatory response on infarct size and cardiac function after AMI, in clinical studies however, the detrimental role of the innate immune system on AMI recovery proves more difficult to unravel [1,16,17]. Treatment with immunosuppressive corticosteroids [18], anti-CD18 (Integrin beta-2) [19] and anti-CD11/CD18 integrin [20] all failed to reduce infarct size and clinical events in STEMI patients, despite the positive effects in animal studies. The functional impact of TLR activation on circulating cells after AMI is largely unexplored in patients. In the present study we aimed at exploring the impact of enhanced TLR expression on circulating leukocytes after STEMI, by analyzing full genome gene expression in circulating cells after STEMI. We hypothesized that this analysis would provide more insight in the contribution of the innate immune system on cardiac function during STEMI.

2. Material and methods

2.1. Patients

Patients with an acute ST-segment elevation myocardial infarction (STEMI) were enrolled in the EXAMI trial of which the study design, procedures and main results have been reported previously [21,22]. The study was approved by the institutional ethical committee on human research and is carried out in compliance with the Helsinki Declaration. This study demonstrated that administering high-dose exenatide intravenously in patients undergoing pPCI is safe and feasible. Briefly, informed consent was obtained from patients with STEMI, selected for pPCI, aged between 18 and 80 years. Forty patients were randomized to 5 μg intravenous exenatide or placebo treatment, just prior to PCI, administered intravenously (iv) in 30 min. Treatment was followed by a continuous infusion of 20 μg/24 h for the duration of 72 h. Patients with multivessel disease, Thrombolysis In Myocardial Infarction (TIMI) 3 flow prior to PCI or without a definite culprit lesion were excluded after CAG. Whole blood samples (2.5 ml) were obtained using PAXgene tubes (PreAnalytix, GmbH, Germany) from 33 patients, at the time of admission at the coronary care unit and before administration of exenatide or placebo (T1), directly after the first 5 μg iv bolus of exenatide or placebo (T2, n = 32) and after 4 months (T4, n = 28) for background expression levels. To establish a relation between gene expression with cardiac function, we used samples from 28 patients, for which all time points were available. Peripheral blood cells were counted in whole blood EDTA samples using an automated hematology analyzer (Cell-Dyn Sapphire, Abbott Diagnostics, Santa Clara, CA, USA). Simultaneously drawn EDTA blood plasma was stored at −20 °C until assayed.

2.2. Cardiac Magnetic Resonance (CMR)

Three to 7 days and 4 months after primary PCI, cardiac MRI with delayed contrast enhancement (DCE) was performed to assess left ventricular function and infarction size. The first MRI is performed to visualize myocardial edema, i.e. the area at risk. The second MRI is performed to measure myocardial fibrosis, i.e. infarct area. Doing so, infarct size can be measured as a percentage of the area at risk. Patients were studied on a clinical 1,5 Tesla scanner with a cardiac phased array receiver surface coil placed on the thorax. ECG-gated cine MR images are obtained during repeated breath-holds in the 3 standard long axis views (4-, 3- and 2-chamber view). Additional short axis slices are acquired covering the entire left ventricle to examine regional and global left ventricular function. T2-weighted imaging, using a Short-Tau Inversion Recovery (STIR) sequence, was performed in long and short axis orientations to estimate the area at risk [23]. First-pass perfusion images were acquired during intravenous injection of a 0.1 mmol/kg bolus of Gadoteric acid (Dotarem, Guerbet, Villepinte, France) at a rate of 3.0 ml/s, using a single-shot saturation recovery gradient-echo pulse sequence. Three short-axis slices were obtained per heartbeat, covering the infarcted area as identified with cine and T2-weighted imaging. After the perfusion sequence, an additional dose of 0.1 mmol/kg Dotarem was intravenously injected as a bolus (cumulative dose 0.2 mmol/kg). DCE images were acquired 10 min post-contrast injection to identify the location and extent of myocardial infarction. All images were stored in a database for blinded analysis. The MRI data were analyzed using the Mass software package (Mass, v.5.1 2010-EXP beta Medis, Leiden, The Netherlands). On the short axis cine slices, the endocardial and epicardial borders are outlined manually in end-diastolic and end-systolic images, excluding trabeculae and papillary muscles. Assessment of global left ventricular function is obtained by calculating left ventricular volumes, mass, and ejection fraction using the summation of slice method multiplied by slice distance. For analysis of segmental myocardial function, each short axis slice is divided in 12 equiangular segments, starting at the posterior septal insertion of the right ventricle. Segmental wall thickening is expressed in absolute values (end-diastolic wall thickness subtracted from end-systolic wall thickness [in millimeters]) and relative values (absolute wall thickening divided by end-diastolic wall thickness [%]). Areas of hyperenhancement are outlined, including central dark zones of microvascular obstruction, allowing the calculation of total infarct size by summation of all slice volumes of hyperenhancement. Infarct size and salvage (area at risk – infarct size) were expressed as percentage of the left ventricle. Salvage index was calculated as the ratio of myocardial salvage and area at risk. Both STIR and DCE images were analyzed in consensus by 2 experienced observers (combined experience in Cardiac MRI more than 10 years (FB, AMB), who were blinded to other patient data at the time of the analysis.

2.3. In vitro hypoxia experiments

Monocytes were isolated from six healthy age- and sex-matched donors (3 males, 3 females, average age 59 year) by density centrifugation using ficoll-paque and percoll and were cultured at a concentration of 1 * 10E6/ml in IMDM (Gibco Life Technologies) supplemented with 1000 Units/ml penicillin/streptomycin (Lonza), and 5% heat inactivated human AB serum, in the absence or presence of graded doses of LPS (LPS-EB Ultrapure from E. Coli 0111:B4 strain, InvivoGen, San Diego, CA, USA), under hypoxic (1% oxygen) or normoxic (20% oxygen) conditions for 2 or 18 h. Culture medium was equilibrated for 3 h in normoxic or hypoxic conditions before monocyte exposure. Total RNA was isolated using the Rneasy mini kit (Qiagen, Venlo, The Netherlands) for gene quantification by real-time PCR.

2.4. mRNA isolation, real-time PCR and whole genome transcriptome analysis

Total RNA was isolated from peripheral blood using the PAXgene RNA isolation kit according to the manufacturers' instructions including a DNAse (Qiagen) step to remove genomic DNA.

For specific gene mRNA quantification, real-time reverse transcriptase-polymerase chain reaction (real-time PCR) measurements were performed on T1, T2 and T4 samples. In brief, 250 ng RNA was reversed transcribed into cDNA, using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). For transcript quantification we used 4% of the generated cDNA for each gene using TaqMan Universal PCR Master Mix (Applied Biosystems) with SYBR