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Acidosis differently modulates the inflammatory program in monocytes and macrophages

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ABSTRACT

Inflammation, ischemia or the microenvironment of solid tumors is often accompanied by a reduction of extracellular pH (acidosis) that stresses the cells and acts on cellular signaling and transcription. The effect of acidosis on the expression of various inflammatory markers, on functional parameters (migration, phagocytic activity) and on signaling pathways involved was studied in monocytic cells and macrophages. In monocytic cell lines acidosis led to a reduction in expression of most of the inflammatory mediators, namely IL-1ß, IL-6, TNF-α, MCP-1, COX-2 and osteopontin. In primary human monocytes MCP-1 and TNF-α were reduced but COX-2 and IL-6 were increased. In RAW264.7 macrophage cell line IL-1ß, COX-2 and iNOS expression was increased, whereas MCP-1 was reduced similar to the effect in monocytic cells. For primary human monocyte-derived macrophages the regulation of inflammatory markers by acidosis depended on activation state, except for the acidosis-induced downregulation of MCP-1 and TNF-α. Acidosis affected functional immune cell behavior when looking at phagocytic activity which was increased in a time-dependent manner, but cellular motility was not changed. Neither ERK1/2 nor CREB signaling was stimulated by the reduction of extracellular pH. However, p38 was activated by acidosis in RAW264.7 cells and this activation was critical for the induction of IL-1ß, COX-2 and iNOS expression. In conclusion, acidosis may impede the recruitment of immune cells, but fosters inflammation when macrophages are present by increasing the level of COX-2 and iNOS and by functionally forcing up the phagocytic activity.

1. Introduction

Different pathological conditions like inflammation, ischemia or solid tumors are accompanied by a reduction of the extracellular pH. This is caused by hypoxia, glycolytic cell metabolism or a hampered removal of acidic metabolic byproducts due to impaired blood perfusion [1]. Typical values for interstitial tissue pH found in inflammatory disease [2] or in solid growing tumors [3] are in the range of pH 6.0 and pH 7.0. Since acidosis can regulate cellular responses by affecting enzyme activity, ion transport, protein and DNA synthesis as well as the level of cAMP and calcium, an acidic microenvironment can impair immune cell function. The effect of reduced pH on polymorphonuclear leukocyte and lymphocyte function seems mainly inhibitory, but concerning acidosis and macrophages there are only very few studies (overview see [4]). Since not only macrophages, but also their precursors are exposed to the acidic microenvironment, we analyzed the impact of a reduced extracellular pH on immune cell function and the expression of different inflammatory mediators in monocytes (THP-1, Mono Mac 6, primary human blood monocytes) and macrophages (PMA-differentiated THP-1, RAW264.7, primary human monocyte-derived macrophages). The set of inflammatory markers tested comprises interleukin-1ß (IL-1ß), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1/CCL2), cyclooxygenase-2 (COX-2/PTGS2), inducible isoform of nitric oxide synthase (iNOS/NOS2), and osteopontin (SPP1). IL-1ß is mainly produced by monocytes and macrophages and plays a critical role in inflammation by influencing both innate and adaptive immune response (overview see [5]). IL-1ß is involved in the response to tissue damage and cell death, which is often the result of extracellular acidosis. It leads to the activation of NF-κB and MAPK signaling. IL-6 is promptly produced in response to tissue injuries and infections and has pro- or anti-inflammatory activity depending on the microenvironmental conditions [6]. It can switch the balance between tissue repair and carcinogenesis [7], and it affects immunity for instance by acting on monocytic migration [8] and by regulating the differentiation of B-cells and subsets of T-cells [9]. One of the most important pro-inflammatory cytokines is TNF-α, which is mainly produced by macrophages and T-cells and which contributes to inflammation, cell proliferation, differentiation, leukocyte adhesion and apoptosis [10,11]. TNF-α can act through MAPK signaling, transcription factors or caspases and induces the

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expression of various signaling molecules and reactive oxygen species (ROS) [11]. MCP-1 is expressed by various cell types either constitutively or after induction by growth factors, cytokines or oxidative stress [12]. It is involved in the activation and recruitment of leukocytes. Induction of COX-2 and iNOS promotes inflammation by the production of prostaglandins and reactive nitrogen species, respectively [13,14]. Osteopontin is a secreted matricellular protein that regulates the recruitment of monocytes/macrophages and the secretion of cytokines by leukocytes [15]. It modulates tissue repair and inflammation and might therefore be regulated itself by the pH of the microenvironment [16].

The aim of the present study was to analyze whether extracellular acidosis affects the expression of these inflammatory markers in monocytic or macrophagic cell lines. For comparison all measurements were also performed in primary human monocytes and macrophages (including macrophages which were polarized to M1 or M2 cells). Additionally, the impact of acidosis on functional parameters such as cell migration or phagocytic activity was studied. Finally, the underlying signaling pathways responsible for changes in cytokine expression were investigated.

2. Material and methods

2.1. Cell culture

Experiments were performed on monocytic cell lines (Mono Mac 6, THP-1) and on macrophages (RAW264.7, PMA-differentiated THP-1). RAW264.7 murine macrophages (ATCC TIB-71) were grown in DMEM medium supplemented with 10% fetal calf serum (FCS), THP-1 [17] and Mono Mac 6 [18] monocytic cells in RPMI with 10% FCS. All cells were grown at 37 °C under a humidified 5% CO_2 atmosphere and sub-cultivated once per week. For a further direct comparison of monocytic cells and macrophages, THP-1 cells were differentiated to a phagocytic phenotype by incubation with phorbol 12-myristate 13-acetate (PMA). Differentiation of 1×10^5 THP-1 cells/ml was induced with 5 ng/ml PMA over 48 h according to the protocol of Park *et al.* [19] and was monitored by changes in cell morphology as well as an increase in cell adhesion and phagocytosis of coated latex beads (Suppl. Fig. 1).

2.2. Primary human cells

Human monocytes were isolated from peripheral blood as described previously [20–22]. Briefly, heparinized buffy coats of human male donors were obtained from the transfusion center of the University Hospital. Written consent of the donors was obtained. The use of the blood was permitted by the local ethical committee. The buffy coat was diluted with the same volume of Hank's balanced salt solution (Biochrom AG, Berlin, Germany) and the mononuclear cells (MNC) were obtained after density gradient centrifugation (400 ×*g*, 30 min, 20 °C) using Biocoll™ (Biochrom, Berlin, Germany). The MNC were washed twice (200 ×*g*, 10 min), transferred into MACS® buffer and monocytes were prepared using CD14-beads and a LS-column (MACS®-system; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. These monocytes were used either for direct measurements after exposing them to an extracellular acidosis (see below, except: incubation time without FCS supplementation was reduced to 3 h) or were differentiated to macrophages by plating the cells for 24 h in 6-well plates in RPMI/10% FCS leading to unpolarized M0 macrophages [23]. Polarization of the macrophages to M1 or M2 cell was performed as described previously [24]. In brief, cells were either incubated for 24 h with LPS (50 ng/ml) and IFNγ (20 ng/ml) resulting in M1 macrophages or with IL-4 and IL-10 (20 ng/ml each) leading to M2 macrophages [24]. After polarizing the cells, macrophages were kept for another 24 in RPMI without FCS supplementation after which the cells were incubated in an acidic or a control environment (see below).

2.3. Experimental setup (acidosis challenge)

The cells were transferred to medium without additional FCS supplementation for 24 h prior to the measurements and were incubated afterwards for 3 h (mRNA and signaling measurements) or up to 24 h (protein expression) in Ringer solutions, respectively. Control cells were exposed to bicarbonate HEPES-buffered Ringer solution adjusted to pH 7.4 ($NaHCO_3$ 24.0 mM, Na_2HPO_4 0.8 mM, NaH_2PO_4 0.2 mM, NaCl 86.5 mM, KCl 5.4 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 0.8 mM, HEPES 20 mM; pH adjustment with 1 N NaOH). Extracellular acidosis (pH 6.6) was applied using isoosmotic bicarbonate MES-buffered Ringer solution ($NaHCO_3$ 4.5 mM, Na_2HPO_4 0.8 mM, NaH_2PO_4 0.2 mM, NaCl 106 mM, KCl 5.4 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 0.8 mM, MES (morpholinoethanesulfonic acid) 20 mM; pH adjustment to 6.6 with 1 N NaOH). For incubation periods longer than 6 h (COX-2 and IL-1ß protein expression measurements) DMEM medium with 1,5 g/l $NaHCO_3$ was used and adjusted to pH 7.4 or pH 6.6 with 1 M HCl. Extracellular pH (pH_e) was checked with a blood gas analyzer (ABL5, Radiometer, Copenhagen, Denmark) and only minor pH changes of Ringer solutions and DMEM medium were observed after the incubation periods. For experiments on the role of MAP kinase p38 10 μM of the p38 inhibitor SB203580 or DMSO (control) was added during the incubation period.

2.4. Quantitative PCR

Total RNA was isolated using the InviTrap Spin Tissue RNA Mini kit (Invitek, Berlin, Germany). 1 μg RNA was subjected to reverse transcription with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and analyzed by qPCR using the Platinum SYBR Green qPCR Supermix (Invitrogen, Carlsbad, CA, USA); each step according to the manufacturer's instructions. The obtained data was normalized against *Rn18S*. The obtained data was normalized against Rn18S and related to the respective control (pH 7.4) [25]. The obtained ΔΔCq values for each experiment were averaged and subjected to statistical analysis. The following primers were used:

Target-murine-	Forward primer	Reverse primer
Ptgs2/COX2	AGGACTCTGCTCACGAAGGA	TCATACATTCCCCACGGTTT
Il1b	CCTGCTGGTGTGTGACGTTCCC	CAGGGTGGGTGTGCCGTCTT
Il6	CCGGAGAGGAGACTTCACAG	TTCTGCAAGTGCATCATCGT
Nos2/iNos	ACTGGAGGTGGGTGGCCTCG	CTCCACGGGCCCGGTACTCA
Ccl2/MCP1	AGGTCCCTGTCATGCTTCTG	TCTGGACCCATTCCTTCTTG
SPP1/osteopontin	ATTTGCTTTTGCCTGTTTGG	TGGCTATAGGATCTGGGTGC
Tnf	CACACTCAGATCATCTTCTCAAAA	GTAGACAAGGTACAACCCATCG
Target-human-	Forward primer	Reverse primer
PTGS2/COX2	CTTACAATGCTGACTATGGCTAC	AAACTGATGCGTGAAGTGCTG
IL1B	ACGCTCCGGGACTCACAGCA	TGAGGCCCAAGGCCACAGGT
IL6	CCTCGACGGCATCTCAGCCC	TGTGGTTGGGTCAGGGGTGGT
NOS2/INOS	ACAAGCCTACCCCTCCAGAT	TCCCGTCAGTTGGTAGGTTC
CCL2/MCP1	GTCTCTGCCGCCCTTCTGTGC	AACAGCAGGTGACTGGGGCA
Spp1/osteopontin	AGCAGAATCTCCTAGCCCCA	CTGGATGTCAGGTCTGCGAA
TNF	AGTTGTGTCTGTAATCGCCCTAC	CTAAGCAAACTTTATTTCTCGCC
Rn18s	CTG AGA AAC GGC TAC CAC ATC	CCC AAG ATC CAA CTA CGA GC

2.5. IL-1ß ELISA and nitrate/nitrite production

Secretion of IL-1ß was analyzed by mouse IL-1 beta ELISA Ready-SET-Go (eBioscience, San Diego, CA, USA), while nitrite and nitrate production was studied using nitrate/nitrite fluorometric assay kit (Cayman Chemical, Ann Arbor, MI, USA). Prior to both measurements cell supernatant was centrifugated briefly to remove any cells. Respective media and Ringer's solutions without cells were used as references. Measurements were made according to the manufacturer's instruction and the obtained data was normalized to overall protein content in μg before calculating relative acidosis-induced effects. The secreted IL-1ß concentration measured in the assay was within 0–5 pg/ml.