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SHORT COMMUNICATION

HMGB1 decreases CCR-2 expression and migration of M2 macrophages under hypoxia

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Abstract

Objective The hypoxic milieu at tumor microenvironment is able to drive the behavior of infiltrating tumor cells. Considering that hypoxia-mediated HMGB1 release is known to promote tumor growth, as well to enhance the pro-tumoral profile of M2 macrophages by a RAGE-dependent mechanism, it is tempting to evaluate the potential contribution of HMGB1 under hypoxia to restrain M2 macrophages mobility.

Methods CCR-2 expression was evaluated in M2 polarized macrophages by western blotting and immunocytochemistry. The secreted levels of CCL-2 and the migration capability were evaluated using an ELISA and a chemotaxis assay, respectively.

Results HMGB1, under hypoxic conditions, markedly reduce both the production of CCL-2 and the expression of its receptor CCR-2; and reduced the migration capacity of M2 macrophages.

Conclusions These results provided new insights into the mechanisms that regulate M2 macrophages mobility at the tumor microenvironment.

Keywords Tumor-associated macrophages · M2 macrophages · Hypoxia · HMGB1 · CCR-2 · RAGE

Introduction

Most human solid tumors contain hypoxic areas [1]. Tumor-associated macrophages (TAMs), are poorly cytotoxic against neoplastic cells and may actually favor tumor cell survival and proliferation [2]. Of note, immunosuppressive M2 macrophages are abundantly found in hypoxic regions of solid tumors compared to M1 macrophages [3]. Additionally, monocyte chemoattractants, such as the monocyte chemoattractant protein-1 (MCP-1/CCL-2), has been shown to be responsible of the increased TAMs number in solid tumors [4].

HMGB1 is a nuclear chromatin-associated protein, which also has others important extranuclear functions, particularly those mediated by its release from damaged or dying cells [5]. Once released, HMGB1 works as a damage-associated molecular pattern molecule binding to receptors such as RAGE and TLRs [6]. Interestingly, hypoxia has been reported to induce HMGB1 release, and thus supporting several tumour-promoting events [7].

Because of HMGB1 enhances pro-tumoral profile of M2 macrophages by a RAGE-dependent mechanism [8], we aimed at evaluating the potential contribution of HMGB1 under hypoxia to restrain M2 macrophages mobility.

Materials and methods

Macrophage polarization

Macrophage polarization was performed as previously described [8]. Once polarized, macrophages were cultured in hypoxia/normoxia for different times in the presence or absence of HMGB1 at 1 μg/mL.

Hypoxia induction

To induce hypoxia, cells were placed in a GasPak EZ Gas generating Pouch System [9]. As control, cells were

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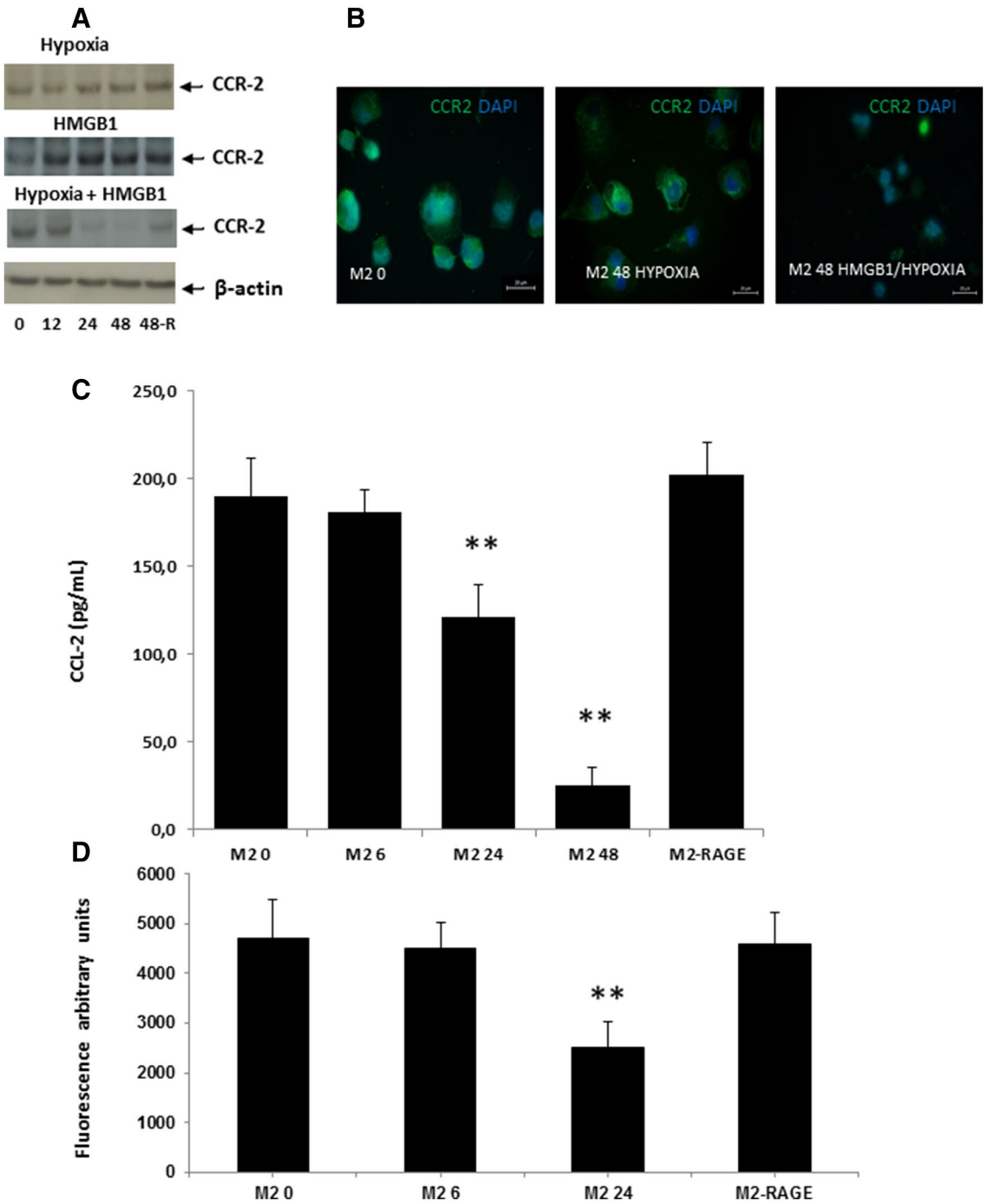

Fig. 1 HMGB1 decreased the expression of CCR-2 in M2 macrophages cultured under hypoxia conditions. M2-polarized macrophages were obtained by treating THP-1 cells with 320 nM PMA for 6 h, and then cultured with PMA plus 20 ng/mL IL-4 and 20 ng/mL IL-13 for extra 18 h. Once polarized, macrophage populations were cultured in hypoxia/normoxia for different times (0, 6, 24, 48 h) in the presence or absence of HMGB1 at 1 μg/mL. CCR-2 expression was assessed by western blot (**a**) and immunocytochemistry analysis (**b**) using an anti-CCR2 antibody and an Alexa Fluor 488 anti-mouse secondary antibody. **c** The levels of CCL2 secreted by M2 macrophages and their migration capacity assessed by a transwell cell migration assay is depicted in **d**. $*p < 0.05$, $**p < 0.01$

incubated for the corresponding times in normoxia condition (21% O_2).

Western blots

The expression of CCR2 was analyzed by western blots. Cell lysates were obtained with RIPA lysis buffer and equal amounts of proteins were subjected to electrophoresis on 10% SDS-polyacrylamide gels and subsequently transferred onto a PVDF membrane. After blocking, membrane was incubated with primary antibodies at 4 °C overnight. After washing, membranes were incubated with the secondary antibodies at room temperature for 1.5 h, washed and developed with ECL system. Blots were normalized with β-actin.

CCL-2 ELISA

CCL-2 levels in cell supernatants were quantified by a commercial ELISA (Invitrogen).

Chemotaxis assay

Chemotaxis was quantified using the fluorimetric Chemicon QCM™ Chemotaxis 5 µm 24-well migration assay (Millipore), and performed following manufacturer´s instructions. After a period of 24 h for migration, cells in the lower chamber were lysed and cell migration was determined using CyQUANT® GR dye. The fluorescence signal was read with a plate reader using a 480/520 nm filter set.

Immunofluorescence analysis

Immunofluorescence was performed on cells growing onto polylysine-treated sterile cover-slips and then submitted to differentiation and treatments. Afterwards, cover-slips were washed and fixed with 4% paraformaldehyde, blocked with 0.5% albumin/PBS, and further incubated with anti-CCR2 antibody (Thermo-Fisher) at 1:50 dilution overnight. After washing samples were then incubated for 1 h with Alexa 488 anti-mouse conjugate (Life Technologies) at a 1:400 dilution in blocking buffer. As mounting medium, Prolong Gold with DAPI (Invitrogen) was used.

Lentiviral-mediated RNA interference

THP-1 cells were infected with lentiviral particles (Santa Cruz Biotechnology) containing 3 RAGE-specific constructs that encode short hairpin RNA shRNA. After puromycin selection, stable cultures were selected, cloned by limiting dilution and checked out for cell viability, as previously described [8].

Statistical analysis

Data are represented as mean ± SEM. Statistical significance was determined by the Student's *t* test. All experiments were conducted three times.

Results

As depicted in Fig. 1a, HMGB1 decreased the expression of CCR-2 in M2 macrophages cultured under hypoxia at protein levels. HMGB1 or hypoxia alone did not produce any effect on CCR-2 expression levels. Significant CCR-2 expression remained in RAGE-targeted knock-down cells at 48 h. Immunofluorescence analysis revealed changes in expression for CCR-2, where low staining appeared in HMGB1-stimulated M2 macrophages under hypoxia (Fig. 1b).

The treatment with HMGB1 under hypoxia decreased the levels of CCL2, being maximal at 48 h (over 85% inhibition), as show in Fig. 1c. No changes were observed in RAGE-targeted knock-down cells. Finally, M2 macrophage migration was markedly decreased (over 45%) at the end of the incubation time (24 h), as shown in Fig. 1d.

Discussion

TAMs secrete mediators, such as CCL2/MCP-1, which is involved in the recruitment and retention of these cells at tumor microenvironment [10].

The present report suggests that hypoxia and the concomitant presence of HMGB1, two factors highly expectable at tumor core, promote a type of cellular hijacking trough a RAGE-dependent mechanism by decreasing both the expression of CCR2 and the migration capacity of M2 macrophages and thus supporting their retention at the tumor hypoxic core.

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