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Attenuation of the acute inflammatory response by dual specificity phosphatase 1 by inhibition of p38 MAP kinase

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ABSTRACT

Dual specificity phosphatase 1 (DUSP1) dephosphorylates and, hence, regulates the activity of MAP kinases. The present study investigated the effect of DUSP1 on inflammatory gene expression and on the development of carrageenan-induced inflammation. It was found that DUSP1 expression was increased by LPS, and the down-regulation of DUSP1 by siRNA enhanced the phosphorylation of p38 MAPK, while JNK phosphorylation was not affected in murine macrophages. LPS-induced interleukin (IL)-6, tumor-necrosis factor (TNF) and cyclooxygenase-2 (COX2) expression were enhanced in bone marrow-derived macrophages (BMMs) from DUSP1(−/−) mice as compared to those from wild-type mice. In addition, down-regulation of DUSP1 by siRNA enhanced IL-6, TNF and COX2 expression in J774 macrophages, while p38 MAPK inhibitors SB202190 and BIRB 796 inhibited the expression of those inflammatory factors. *In vivo*, the intensity of the carrageenan-induced paw edema reaction was increased in DUSP1(−/−) mice as compared to the wild-type animals. In conclusion, DUSP1 is an important negative regulator of the acute inflammatory response by limiting p38 MAPK, and compounds which enhance DUSP1 expression or activity may hold a promise as anti-inflammatory drugs.

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1. Introduction

There are several mitogen-activated protein kinases (MAPKs) including stress-activated protein kinases p38 MAPK and Jun N-terminal kinase (JNK) and extracellular-signal regulated protein kinase (ERK). MAPKs are activated in response to various extracellular stimuli, such as growth factors and cellular stress through G-protein coupled receptor activation. The substrates of MAP kinases include transcription factors, such as Jun, Myc, Fos, activating transcription factor 2, and several other regulatory proteins, such as mitogen-activated protein kinase-activated protein kinases (MAPKAPKs) (Johnson and Lapadat, 2002).

There are four p38 MAPK isoforms, namely p38α, p38β, p38γ and p38δ. p38α and p38β MAPKs are widely expressed, while p38γ expression is restricted to skeletal muscle and that of p38δ to testes, pancreas, small intestine, and $CD4^+$ T cells. p38 MAPK is known to regulate cellular growth and differentiation and it has been shown to participate in the regulation of immune response (Rincón and Davis, 2009).

Inflammatory stimulation leads to the activation of p38 MAPK pathway, and the expression of inflammatory genes, such as those coding for tumor necrosis factor (TNF) and interleukins (IL), is regulated by p38 MAPK (Ono and Han, 2000; Mahtani et al., 2001; Turpeinen et al., 2010). Genetic deficiencies in mitogen-activated protein kinase kinase (MAP2K) 3 and MAP2K6, upstream activators of p38 MAPK, inhibits the production of IL-12 by macrophages and T helper (Th)1 differentiation and interferon-γ (IFNγ) production (Lu et al., 1999). Mice lacking MAPKAPK2, a direct substrate for p38 MAPK, exhibit an impaired response to lipopolysaccharide (LPS) and cells isolated from their spleens produce markedly lower amounts of TNF, IL-6, IL-10 and IFNγ (Kotlyarov et al., 1999). p38 MAPK inhibitors have been shown to inhibit the disease progression in a rodent arthritis models and to suppress the inflammatory response during endotoxemia in humans (Branger et al., 2002; Burnette et al., 2009).

Dual specificity phosphatases (DUSPs) are a large group of protein phosphatases that are able to dephosphorylate phosphotyrosine and phosphoserine/phosphothreonine residues in their

Abbreviations: BMMs, bone marrow-derived macrophages; COX2, cyclooxygenase-2; DUSP, dual specificity phosphatase; ERK, extracellular-signal-regulated-kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte/macrophage-colony stimulating factor; IFNγ, interferon-γ; IL, interleukin; JNK, Jun N-terminal kinase; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MAPK, mitogen-activated protein kinase; MAPKAPK, mitogen-activated protein kinase-activated protein kinase; siRNA, small interfering RNA; TNF, tumor necrosis factor.

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target proteins (Patterson et al., 2009). There is one subgroup of DUSPs that target MAP kinases; these are also known as MAP kinase phosphatases (MKPs). This subgroup of DUSPs includes more than ten members that differ from each other in their expressional pattern, cellular location and substrate specificity (Liu et al., 2007; Boutros et al., 2008). DUSP1 belongs to the MKP subgroup of DUSPs. It is a nuclear phosphatase widely expressed in various cell types and tissues, and its expression is induced by a range of stimuli, including cellular stress, cytokines and LPS. DUSP1 displays specificity towards p38 and JNK over p42/44 ERK (Franklin and Kraft, 1997; Liu et al., 2007; Boutros et al., 2008). Macrophages from DUSP1(−/−) mice display increased and prolonged activation of p38 in response to LPS, and LPS-induced inflammatory responses are amplified in DUSP1(−/−) mice as compared to wild-type animals (Zhao et al., 2005; Hammer et al., 2006; Salojin et al., 2006).

In the present study, we investigated the effect of DUSP1 on the expression of inflammatory genes TNF, IL-6 and COX2 in activated macrophages and on the development *in vivo* of an acute carrageenan-induced edema response. DUSP1 acted as a negative regulator of the expression of inflammatory genes and acute inflammatory response by limiting p38 MAPK.

2. Materials and methods

2.1. Materials

Reagents were obtained as follows: LPS from *Escherichia coli* strain 0111:B4 (Sigma–Aldrich Inc., St. Louis, MO, USA), SB202190 (Tocris Biosciences, Bristol, UK), BIRB 796 (Axon Medchem, Groningen, The Netherlands), JNK inhibitor VIII (Calbiochem, Merck Chemicals, Darmstadt, Germany) and macrophage colony-stimulating factor (M-CSF) (RD Systems Inc., Minneapolis, MA, USA) were purchased as indicated. All other reagents were from Sigma Aldrich Inc. unless otherwise stated below.

2.2. Cell culture

J774 murine macrophages (ATCC, Rockville Pike, MD, USA) were cultured at 37 °C in 5% CO_2 atmosphere in Dulbeccoïs modified Eagle's medium supplemented with glutamax-1 containing 5% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (all from Invitrogen, Paisley, UK). For cytokine measurements and Western blot experiments, cells were seeded on 24-well plates at density of 2×10^5 cells/well. Cell monolayers were grown for 72 h before the experiments were started. BIRB 796 and SB202190 were dissolved in DMSO. BIRB 796 and SB202190 at concentrations indicated or DMSO (0.1%, v/v) were added to the cells in fresh culture medium containing 5% FBS and the above antibiotics 30 min prior to the stimulation with LPS (10 ng/ml). Cells were further incubated for the time indicated. The effect of LPS and the tested chemicals on cell viability was evaluated by visual assessment in a microscope and by the XTT test using Cell Proliferation Kit II (Roche Diagnostics, Mannheim, Germany) (Korhonen et al., 2001; Hämäläinen et al., 2009). Neither LPS nor the other chemical used in the experiments were found to evoke cytotoxicity.

2.3. Isolation of the bone-marrow-derived and peritoneal macrophages and cell culture

The study was approved by the Animal Care and Use Committee of the University of Tampere and the respective public provincial authority committee for animal experiments. Inbred C57BL/6 DUSP1(−/−) mice were originally generated by the R. Bravo laboratory at Bristol–Myers Squibb Pharmaceutical Research Institute (Dorfman et al., 1996). Bone marrow-derived macrophages (BMMs) were isolated from femur and tibia of the hind legs of mice aged 10–12 weeks. Briefly, mice were anesthesized by intraperitoneal injection of 0.05 mg/100 g body weight of medetomidine (Domitor® 1 mg/ml, Orion Corp., Espoo, Finland) and 7.5 mg/100 g body weight of ketamine (Ketalar® 10 mg/ml, Pfizer Oy Animal Health, Helsinki, Finland), and mice were terminated by cervical dislocation. BMMs were generated from bone marrow hematopoietic stem cells with 5–7 days of incubation in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml M-CSF. Thereafter, BMMs (1×10^6 cells/well) were seeded on a 24-well plates, cultured overnight in complete culture medium followed by serum starvation overnight. At the beginning of the experiment, LPS was added to the cells along with the culture medium containing 10% FCS and antibiotics, and BMMs were incubated for the time indicated. Primary mouse peritoneal macrophages were obtained by peritoneal lavage with sterile PBS supplemented with 0.2 mM EDTA. Cells were washed, resuspended in RPMI 1640 medium supplemented with 2% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and seeded on 24-well plates (5×10^5 cells/well). The cells were incubated overnight and washed with PBS to remove non-adherent cells before the experiments.

2.4. Carrageenan induced paw edema

The study was approved by the Animal Care and Use Committee of the University of Tampere and the respective provincial committee for animal experiments. Male C57BL/6 mice (20–25 g) were used with six animals per group. Prior to the administration of carrageenan, the mice were anesthesized by intraperitoneal injection of 0.05 mg/100 g of medetomidine (Domitor® 1 mg/ml. Orion Oyj, Espoo, Finland) and 7.5 mg/100 g of ketamine (Ketalar® 10 mg/ml, Pfizer Oy Animal Health, Helsinki, Finland). The mice received a 30 μl intradermal injection in one hind paw of carrageenan (1.5%) dissolved in normal saline. The contralateral paw received 30 μl of saline and it was used as a control. Paw volume was measured before and 3 h after the carrageenan injection with a plethysmometer (Ugo Basile, Comerio, Italy).

2.5. Preparation of cell lysates and Western blot analysis

At the indicated time points, the culture medium was removed. Cells were rapidly washed with ice-cold PBS and solubilized in cold lysis buffer containing 10 mM Tris–HCl, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 μM *n*-octyl-β-D-glucopyranoside. After incubation for 20 min on ice, lysates were centrifuged, supernatants were collected and mixed in a ratio of 1:4 with SDS loading buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue, and 5% β-mercaptoethanol) and stored at −20 °C until analyzed.

COX2, actin, DUSP1, JNK, phospho-c-Jun, c-Jun, polyclonal anti-rabbit and polyclonal anti-goat antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-p38, p38, phospho-JNK and JNK antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA).

Prior to Western blot analysis, the samples were boiled for 10 min. Equal aliquots of protein (10–20 μg) were loaded on a 10% SDS-polyacrylamide gel and separated by electrophoresis. Proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham, Buckinghamshire, UK) by semidry electroblotting. After transfer, the membrane was blocked in TBS/T (20 mM Tris–base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat milk for 1 h at room temperature. For detection of phospho-proteins, membranes were blocked in TBS/T

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