Cytokine 61 (2013) 161–167

Contents lists available at SciVerse ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Citrullination of TNF-α by peptidylarginine deiminases reduces its capacity to stimulate the production of inflammatory chemokines

Eva A.V. Moelants, Anneleen Mortier, Karolien Grauwen, Isabelle Ronsse, Jo Van Damme, Paul Proost *

Laboratory of Molecular Immunology, Rega Institute, KU Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium

ARTICLE INFO

Article history:
Received 18 April 2012
Received in revised form 10 August 2012
Accepted 18 September 2012
Available online 15 October 2012

Keywords:
TNF
Citrullination
Cytokine
IL-1β
Posttranslational modification

ABSTRACT

Citrullination, a posttranslational modification (PTM) recently discovered on inflammatory chemokines such as interleukin-8 (IL-8/CXCL8) and interferon-γ-inducible protein-10 (IP-10/CXCL10), seriously influences their biological activity. Citrullination or the deimination of arginine to citrulline is dependent on peptidylarginine deiminases (PADs) and has been linked to autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA). Chemokines are to date the first identified PAD substrates with receptor-mediated biological activity. We investigated whether cytokines that play a crucial role in RA, like interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α), may be citrullinated by PAD and whether such a PTM influences the biological activity of these cytokines. IL-1β and TNF-α were first incubated with PAD *in vitro* and the occurrence of citrullination was examined by Edman degradation and a recently developed detection method for citrullinated proteins. Both techniques confirmed that human TNF-α, but not IL-1β, was citrullinated by PAD. Citrullination of TNF-α reduced its potency to stimulate chemokine production *in vitro* on human primary fibroblasts. Concentrations of the inflammatory chemokines CXCL8, CXCL10 and monocyte chemotactic protein-1 (MCP-1/CCL2) were significantly lower in supernatants of fibroblasts induced with citrullinated TNF-α compared to unmodified TNF-α. However, upon citrullination TNF-α retained its capacity to induce apoptosis/necrosis of mononuclear cells, its binding potency to Infliximab and its ability to recruit neutrophils to the peritoneal cavity of mice.

1. Introduction

Cytokine and chemokine activity is regulated at multiple levels including posttranslational modification (PTM) [1,2]. Reduced or enhanced receptor affinity/specificity and chemokine activity have been reported, depending on the chemokine and on the type of PTM [3,4].

In addition to NH_2- and COOH-terminal proteolytic processing, and glycosylation, citrullination, i.e. deimination of arginine (Arg) to citrulline (Cit), is a recently discovered PTM on the natural chemokines interleukin-8 (IL-8/CXCL8) and interferon-γ-inducible protein-10 (IP-10/CXCL10) [5,6]. For both chemokines Arg at position 5 was converted into Cit. The enzymes responsible for the conversion of peptidylarginine to peptidylcitrulline are peptidylarginine deiminases (PADs) [7]. Citrullination may seriously influence the biological activity of proteins since citrullination may change ionic interactions in macromolecules resulting in altered protein folding [8,9]. Moreover, citrullinated proteins and autoantibodies to citrullinated peptides have been implicated in autoimmune diseases such as rheumatoid arthritis (RA) [10,11] and multiple sclerosis (MS) [12]. Chemokines are to date the first identified PAD substrates with receptor-mediated biological activity.

Interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) are two major inflammatory cytokines sharing several biological actions [1]. Both cytokines have a pivotal role in the pathophysiology of RA. They act on a range of cell types in the joint space to amplify and perpetuate the inflammatory process [13]. Levels of IL-1β and TNF-α are elevated in synovial fluid of RA patients compared to healthy controls [14,15]. PAD2 mRNA or protein have been detected in lymphocytic and monocytic cells [16]. PAD4 is widely

Abbreviations: Arg, arginine; CHX, cycloheximide; Cit, citrulline; [Cit]TNF-α, citrullinated TNF-α; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; E/S, enzyme-substrate; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; HPLC, high performance liquid chromatography; IL-1β, interleukin-1β; IL-8/CXCL8, interleukin-8; i.p., intraperitoneal; IP-10/CXCL10, interferon-γ-inducible protein-10; LPS, lipopolysaccharide; MCP-1/CCL2, monocyte chemotactic protein-1; MS, multiple sclerosis; PAD, peptidylarginine deiminases; PBS, phosphate buffered saline; PTM, posttranslational modification; RA, rheumatoid arthritis; TFA, trifluoroacetic acid; TMB, 3,3′,5,5′-tetramethylbenzidine; TNF-α, tumor necrosis factor-alpha; TNFR, TNF receptor.

* Corresponding author. Address: Laboratory of Molecular Immunology, Rega Institute for Medical Research, KU Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium. Tel.: +32 16 337341; fax: +32 16 337340.

E-mail addresses: eva.moelants@rega.kuleuven.be (E.A.V. Moelants), anneleen.mortier@rega.kuleuven.be (A. Mortier), karolien.grauwen@remynd.com (K. Grauwen), isabelle.ronsse@rega.kuleuven.be (I. Ronsse), jo.vandamme@rega.kuleuven.be (J. Van Damme), paul.proost@rega.kuleuven.be (P. Proost).

1043-4666/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cyto.2012.09.011

expressed in T cells, B cells, macrophages, neutrophils and fibroblast-like cells. Both isotypes are found in synovium of RA patients [16,17]. Furthermore a haplotype of *PADI4* associated with susceptibility to RA and with increased mRNA stability was identified [18]. This haplotype is associated with increased levels of autoantibodies to citrullinated peptides in sera from individuals with RA. The co-localization of the cytokines IL-1β and TNF-α, and PAD-4 combined with the higher stability of PAD4 mRNA in RA make these cytokines potential targets for citrullination. Therefore we investigated if citrullination of these cytokines by PAD occurs *in vitro* and whether this PTM influences the biological activity of these inflammatory cytokines.

2. Materials and methods

2.1. Reagents and materials

Recombinant human TNF-α and IL-1β were purchased from R&D Systems (Abingdon, UK) and PeproTech (Rocky Hill, NJ, USA), respectively. PAD purified from rabbit skeletal muscle was obtained from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from HyClone (Rockford, IL, USA), Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium from Lonza (Basel, Switzerland). Cycloheximide (CHX) was obtained from Sigma–Aldrich. The humanized monoclonal anti-TNF-α antibody Infliximab (Remicade®, Janssen Biotech, Horsham, PA, USA) was kindly provided by Dr. I. Arijs of the IBD Leuven research group (KU Leuven).

2.2. Citrullination, modification and detection of IL-1β and TNF-α

IL-1β and TNF-α were incubated with PAD for 1.5 h at 37 °C at enzyme–substrate (E/S) molar ratio of 1/10 and a substrate concentration of 0.6 μM. Incubations with PAD were carried out in 40 mM Tris with 2 mM $CaCl_2$ (pH7.4). Enzymatic citrullination was stopped with 0.1% (v/v) trifluoroacetic acid (TFA). PAD-treated IL-1β and TNF-α were chemically modified by adding 50 mM antipyrine, 16% (v/v) TFA and 12.5 mM 2,3-butanedione and incubated for 2 h at 37 °C in the dark, as described in [19,20]. The strong acidic reaction mixture was dialyzed using Slide-A-Lyzer®MINI Dialyse Units (Pierce, Rockford, IL, USA) overnight against phosphate buffered saline (PBS) containing 0.05% (v/v) Tween20 (pH7.4) at room temperature protected from light. Citrullinated IL-1β and TNF-α were quantified by a specific sandwich enzyme-linked immunosorbent assay (ELISA) developed in our laboratory as recently described for CXCL8 [21]. A 96-well plate was coated with mouse monoclonal anti-human IL-1β/TNF-α antibody (R&D Systems), followed by blocking with PBS containing 0.1% (w/v) casein and 0.05% (v/v) Tween20. Chemically modified human citrullinated IL-1β/TNF-α were detected by specific rabbit antibodies against chemically modified citrulline residues (generated in our laboratory) and by a secondary peroxidase-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Peroxidase activity was quantified by measuring the conversion of 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma–Aldrich) at 450 nm.

In addition, after incubation with PAD the NH_2-terminal sequences of IL-1β and TNF-α were determined by Edman degradation on a 491 Procise cLC protein sequencer (Applied Biosystems, Foster City, CA, USA) to verify if citrullination occurred on the more NH_2-terminally located arginines.

2.3. Citrullination and purification of TNF-α

1.5 μM TNF-α was incubated with PAD for 15 min at 37 °C at an E/S molar ratio of 1/10. Incubations with PAD were carried out in 40 mM Tris with 2 mM $CaCl_2$ (pH7.4). Enzymatic citrullination was stopped with 0.1% (v/v) TFA and the reaction mixture was loaded on an Aquapore butyl (C4) column (2.1 × 220 mm; Applied Biosystems) and eluted with an acetonitrile gradient in 0.1% (v/v) TFA at a flow rate of 400 μl/min. UV absorption was monitored at 214 nm. Part of the column effluent (2%) was analyzed by online ion trap mass spectrometry (Esquire LC, Bruker Daltonics, Bremen, Germany). The NH_2-terminal sequence of the protein fractions with correct M_r was determined by Edman degradation to confirm citrullination. TNF-α concentrations in the high performance liquid chromatography (HPLC) fractions were determined by ELISA and SDS–PAGE.

2.4. Cell cultures and induction experiments

Human diploid skin/muscle-derived fibroblasts (E_1SM) were grown in DMEM supplemented with 10% (v/v) FBS [22]. Monolayers were grown to confluency in 24-well plates and TNF-α isoforms (0.5, 1.5 or 4.5 ng/ml) were added in fresh DMEM containing 2% (v/v) FBS. Conditioned media were harvested after 24 h and 72 h and stored at −20 °C until further analysis.

2.5. Immunoassays

Levels of human CXCL8, CXCL10 and monocyte chemotactic protein-1 (MCP-1/CCL2) were quantified by specific sandwich ELISAs developed in our laboratory as previously described [23–25].

For human CXCL8 a 96-well plate was coated with goat polyclonal anti-human CXCL8 antibody generated in our laboratory [22], followed by blocking with PBS containing 0.1% (w/v) casein and 0.05% (v/v) Tween20. Human CXCL8 in supernatants was detected by mouse monoclonal anti-human CXCL8 antibody (R&D Systems), followed by a secondary antibody, peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories).

The sandwich ELISA for human CXCL10 consisted of mouse monoclonal anti-human CXCL10 as coating antibody, biotinylated rabbit polyclonal anti-human CXCL10 as detecting antibody and peroxidase-conjugated streptavidin as secondary antibody (all R&D Systems).

The sandwich ELISA for human CCL2 consisted of mouse monoclonal anti-human CCL2 as coating antibody, mouse monoclonal biotinylated anti-human CCL2 as detecting antibody and peroxidase-conjugated streptavidin as secondary antibody (all R&D Systems). Peroxidase activity was quantified by measuring the conversion of TMB (Sigma–Aldrich) at 450 nm. Statistical comparison between production levels of induced chemokine concentrations was performed using the Wilcoxon matched-pairs test.

2.6. Cell death assay

THP-1 cells were seeded and grown for 24 h in RPMI 1640 medium supplemented with 10% (v/v) FBS. Cells were treated with different TNF-α isoforms (20 ng/ml) in the absence or presence of CHX (1 μg/ml) and harvested after 24 h for further analysis. Cells were stained with Annexin V and propidium iodide with the Annexin V-FITC Apoptosis detection Kit (eBioscience, Vienna, Austria). Intact cells ($FITC^-/PI^-$), early apoptotic cells ($FITC^+/PI^-$), late apoptotic/necrotic cells ($FITC^+/PI^+$) and damaged cells ($FITC^-/PI^+$) were quantified by fluorescence-activated cell sorting (FACS) flow cytometry. The percentages of different cell populations were statistically compared for THP-1 cells induced with TNF-α or citrullinated TNF-α ([Cit]TNF-α) and control cells (non-induced cells) using the Mann–Whitney U test.