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Lymphotoxin α stimulates proliferation and pro-inflammatory cytokine secretion of rheumatoid arthritis synovial fibroblasts

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ABSTRACT

Objective: TNFα plays a crucial role in rheumatoid arthritis (RA) by stimulating fibroblast-like synoviocytes (FLS). Lymphotoxin α (LTα) is a pro-inflammatory cytokine with significant homology to TNFα. We compared the effects of both cytokines on cultured RA FLS.
Methods: Receptor expression on RA FLS was analyzed by FACS. Cells were stimulated with LTα or TNFα and proliferation was measured by [3H]thymidine incorporation and secretion of inflammatory cytokines and metalloproteinase 3 by ELISA. Activation of MAP kinases and Akt was analyzed by Western blotting. Nuclear translocation of NFκB was visualized by immunofluorescence.
Results: 60–80% and 30–50% of the RA FLS tested expressed TNF receptors I and II, respectively, and 70–75% expressed HVEM. LTα induced RA FLS proliferation at the same level of TNFα, which was blocked by etanercept. Both LTα and TNFα induced activation of MAP kinases ERK1/2 and p38 as well as Akt. 95–98% of FLS showed nuclear translocation of NFκB after stimulation with either cytokines. LTα and TNFα were potent to induce secretion of IL-6, IL-8 and metalloproteinase 3 in FLS.
Conclusion: LTα is as effective as TNFα in stimulating RA FLS. Blocking both cytokines might allow a better control of inflammation and synovial proliferation in RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting joints leading to irreversible destruction of articular cartilage and bone. The pseudo-tumoral expansion of synovial tissue that lines the capsule of the joint is a key characteristic of RA [1]. The synovial tissue is mainly composed of fibroblast-like synoviocytes (FLS), which play a crucial role in the physiopathology of RA through the secretion of metalloproteinases (MMPs) and pro-inflammatory cytokines that finally destroy the joint structure [2]. The pro-inflammatory cytokine TNFα is present in rheumatoid synovium and stimulates directly proliferation and activation of RA FLS, a pivotal event in the pathogenesis of RA [3].

TNFα regulates cell survival, death and proliferation [4]. TNFα binds and signals through TNFRI and TNFRII, which are expressed in higher levels on RA FLS when compared to healthy or osteoarthritis (OA) patients [5]. Evidences that TNFα drives most of the physiopathology in RA came from studies of neutralization or overexpression of TNFα in animal models of RA. Transgenic mice expressing high concentrations of TNFα spontaneously developed arthritis [6], while collagen-induced arthritis model demonstrated that blockade of TNFα was efficient in ameliorating the disease [7,8]. The observation that TNFα plays a central role in the pathogenesis of RA led to the introduction of TNFα antagonists to treat RA in patients.

LTα belongs to the TNF superfamily and share common features with TNFα, although there are significant molecular and biologic differences [9]. LTα is a soluble homotrimer (LTα3), which binds specifically to TNFRI and TNFRII. LTα additionally binds to HVEM (Herpesvirus Entry Mediator) [10,11]. LTα is mainly produced by T and B lymphocytes that can also generate TNFα and mediates a large variety of inflammatory responses [12]. LTα is involved in regulating development of secondary lymphoid organs, e.g. LTα$^{-/-}$ mice lack lymph nodes, in contrast to TNFα$^{-/-}$

Abbreviations: ERK, extracellular signal-regulated kinases; FLS, fibroblast-like synoviocytes; HVEM, Herpesvirus Entry Mediator; IgG, immunoglobulin G; IL, interleukin; LTα, Lymphotoxin α; MAP, mitogen-activated protein; MMP, matrix metalloproteinase; NFκB, nuclear factor κB; RA, rheumatoid arthritis; SEM, standard error of the mean; TNFα, tumor necrosis factor; TNFR, TNF receptor.

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mice [13]. The fact the same cell types express both LTα and TNFα and that knock out mice for either cytokines can manifest different phenotypes suggests that both cytokines have overlapping and different functions. Although the participation of TNFα is well characterized in RA, until now, little is known about the involvement of LTα.

Elevated levels of LTα were detected in RA synovium and serum of RA patients compared to OA patients [14,15]. Moreover, in an animal model of collagen-induced arthritis, blocking of LTα with a monoclonal antibody significantly ameliorated the disease [16], suggesting that LTα contributes to the pathology of RA.

We therefore aimed to better characterize the role of LTα in RA context by testing its direct effect in RA FLS.

2. Materials and methods

2.1. Reagents

Recombinant human LTα and TNFα, monoclonals mouse anti-human TNFRI and TNFRII, human MMP3 ELISA kit and annexin V-fluorescein isothiocyanate were purchased from R&D Systems (Lille, France). TO-PRO-3 was obtained from Invitrogen (Eragny sur Oise, France). Monoclonal mouse anti-human HVEM and isotypes controls mice IgG1 and IgG2a from eBiosciences (Montrouge, France). Control MOPC2.1 IgG and peroxidase-conjugated secondary antibodies were obtained from Sigma (St. Quentin Fallavier, France). Anti-phospho-ERK1/2 and anti-phospho-p38 (Thr(P)-180/Tyr(P)-182) were obtained from BD Biosciences (Le Pont de Claix, France). The monoclonal mouse anti-phospho-Akt (Ser-473) antibody was obtained from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-NFκB p65 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cyan3-conjugated goat anti-rabbit was obtained from Jackson ImmunoResearch (West Grove, Pennsylvania). The specific chemical inhibitors LY294002 (PI3 kinase), PD98059 (ERK1/2) and SB203580 (p38) were purchased from Euromedex (Souffelweyersheim, France). All inhibitors were dissolved in DMSO. Human IL-6, IL-8 and RANTES ELISA kits were obtained from PeproTech (Rocky Hill, NJ). Fusion protein between a fragment of TNF receptor II attached to the constant (Fc) region of human immunoglobulin G (FcIgG) (etanercept) was kindly provided by Wyeth Laboratories and diluted in distilled sterile water.

2.2. Preparation of fibroblast-like synoviocytes from rheumatoid arthritis patients

Fibroblasts were isolated from synovium obtained from patients who met the American College of Rheumatology criteria for RA (revised in 1987) and who had undergone to synovectomy or total joint replacement surgery [17]. Fresh synovial tissues were minced and digested in a solution of dispase (2.4 mg/ml) (Gibco, Cergy Pontoise, France), collagenase (250 U/ml) (Sigma) and DNAse (10,000 U/ml) (Calbiochem, Fontenay sous Bois, France). Synovial fibroblasts were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS). Cells were used from passages 4 to 10, when they constitute a homogeneous population of fibroblasts, free of detectable T cells and macrophages. Upon reaching confluence, cells were expanded by brief trypsinization. For experimentation, the content of FCS in the culture medium was progressively decreased from 10% to 1% with final starvation for 24 h in medium containing 1% FCS, as previously described [18].

2.3. Flow cytometric analysis

RA FLS were detached with PBS EDTA 1 mM and suspended in FACS buffer (PBS/0.1% bovine serum albumin/0.01% NaN_3). One hundred thousand cells were incubated for 30 min on ice in with mouse PE-conjugated anti-TNFRI antibody, FITC-conjugated anti-TNFRII antibody and APC-conjugated anti-HVEM antibody. As a negative control, cells were incubated with correspondent isotype control antibodies. Cells were then washed, resuspended in FACS buffer and analysis was performed using FACSCalibur (BD Biosciences).

2.4. Cell proliferation assay

Proliferation was evaluated by measuring DNA synthesis through the incorporation of tritiated [3H]thymidine. FLS were stimulated with increasing concentrations of LTα or TNFα (0.005–0.5 nM) or medium (RPMI/1% FCS) for 72 h. All conditions were tested in triplicate. [3H]Thymidine (1 μCi/well) was added 24 h before the end of the assay. FLS were lysed using a round of freeze–thaw cycle and then transferred onto a membrane filter using Harvester 96 (TOMTEC, Hamdem, CT). [3H]Thymidine incorporated into DNA was quantified using a scintillation counter 1450 MicroBeta Trilux (Wallac, Freiburg, Germany). For experiments with protein kinases inhibitors, FLS were pre-incubated for 1 h with the specific inhibitors (or the solvent DMSO alone) and then cultured with or without LTα or TNFα (0.5 nM). For the inhibition using soluble human TNFRII fusion protein (etanercept), inhibitor and cytokines were added simultaneously. Proliferation is expressed as *stimulation index* (arithmetic mean of cpm from triplicate of stimulated culture/arithmetic mean of cpm from triplicate of non-stimulated culture).

2.5. Western blot analysis

FLS were stimulated with 0.5 nM of LTα or TNFα and the reaction was stopped on ice. Cells were washed twice with cold PBS and harvested with a cell scraper in lysis buffer (100 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM NaF, 20 mM NaP_2O_7, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS and 0.5% desoxycholate). Equal protein amounts were loaded into a 10% polyacrylamide gel, and immunoblot analysis was done as described previously [19]. Blots were developed using Lumi-Light Western Blotting Substrate (Sigma) according to the manufacturer's instructions. Blots were scanned and densitometric signals were quantified using NIH image 1.63 software. Data were expressed as arbitrary units (AU) after normalization to levels of β-actin.

2.6. Immunofluorescence staining

To determine the nuclear translocation of NFκB, RA FLS were seeded at 2×10^4 cells/well in 8-well Lab-Tek chamber slides (Falcon). Cells were stimulated with LTα or TNFα at 0.5 nM for 30–120 min, washed with cold PBS, and then fixed in PBS with 4% paraformaldehyde for 15 min. Cells were permeabilized with PBS and 0.5% Triton X-100 for 15 min. Unspecific binding was blocked with 5% goat serum diluted in PBS. Cells were incubated with the rabbit polyclonal anti-NFκB p65 antibody at 5 μg/ml or isotype control for 60 min at room temperature. Cyan3-conjugated goat anti-rabbit antibody was used at 20 μg/ml for 60 min at room temperature. Slides were mounted with mounting medium (Shandon PermaFluor™, Thermo Scientific), and the subcellular localization of NFκB was visualized by fluorescence microscopy (Leica Microsystems, Heidelberg, Germany).

2.7. Cytokine detection in cell culture supernatants

FLS were seeded in 96-well, flat-bottom culture plates at a density of 1×10^4 cells/well. Cells were cultured in RPMI with decreasing concentrations of FCS (10% and 5%) and then synchronized for