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Selective killing of proinflammatory synovial fibroblasts via activation of transient receptor potential ankyrin (TRPA1)

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ABSTRACT

Background: Studies in rheumatoid arthritis synovial fibroblasts (RASF) demonstrated the expression of several transient receptor potential channels (TRP) such as TRPV1, TRPV2, TRPV4, TRPA1 and TRPM8. Upon ligation, these receptors increase intracellular calcium but they have also been linked to modulation of inflammation in several cell types. TNF was shown to increase the expression of TRPA1, the receptor for mustard oil and environmental poisons in SF, but the functional consequences have not been investigated yet.

Methods: TRPA1 was detected by immunocytochemistry, western blot and cell-based ELISA. Calcium measurements were conducted in a multimode reader. Cell viability was assessed by quantification of lactate dehydrogenase (LDH) in culture supernatants and "RealTime-Glo" luminescent assays. IL-6 and IL-8 production by SF was quantified by ELISA. Proliferation was determined by cell titer blue incorporation.

Results: After 72 h, mimicking proinflammatory conditions by the innate cytokine TNF up-regulated TRPA1 protein levels in RASF which was accompanied by increased sensitivity to TRPA1 agonists AITC and polygodial. Under unstimulated conditions, polygodial elicited calcium flux only in the highest concentrations used (50 μM and 25 μM). TNF preincubation substantially lowered the activation threshold for polygodial (from 25 μM to 1 μM). In the absence of TNF pre-stimulation, only polygodial in high concentrations was able to reduce viability of synovial fibroblasts as determined by a real-time viability assay. However, following TNF preincubation, stimulation of TRPA1 led to a fast (< 30 min) viability loss by necrosis of synovial fibroblasts. TRPA1 activation was also associated with decreased proliferation of RASFs, an effect that was also substantially enhanced by TNF preincubation. On the functional level, IL-6 and IL-8 production was attenuated by the TRPA1 antagonist A967079 but also polygodial, although the latter mediated this effect by reducing cell viability.

Conclusion: Simulating inflamed conditions by preincubation of synovial fibroblasts with TNF up-regulates and sensitizes TRPA1. Subsequent activation of TRPA1 increases calcium flux and substantially reduces cell viability by inducing necrosis. Since TRPA1 agonists in the lower concentration range only show effects in TNF-stimulated RASF, this cation channel might be an attractive therapeutic target in chronic inflammation to selectively reduce the activity of proinflammatory SF in the joint.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease which is characterized by autoantibody production, joint destruction and disability [1]. Healthy synovial tissue is comprised of synovial fibroblasts (SF) and macrophages, whereas lymphocytes migrate to the joint in the course of RA [2]. Proinflammatory cytokine production leads to increased SF proliferation and transforms these cells into a "tumor-like" phenotype with the capacity to degrade cartilage and bone [2,3]. Furthermore, SF might spread RA to unaffected joints since this was already demonstrated with human RASF and cartilage in the SCID mouse co-implantation model [4]. Although SF are a major contributor to disease progression, no specific therapy targeting this cell type is currently available.

Transient receptor potential channels (TRP channels) are non-selective ion channels that increase intracellular concentrations of calcium, sodium and magnesium upon activation [5]. While some TRPs are activated by physical stimuli (e.g. heat, cold, stretch) others respond

Abbreviations: RA, rheumatoid arthritis; WIN, WIN55212-2 mesylate ((R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate); TNF, tumor necrosis factor; IL-6, interleukin-6; IL-8, interleukin-8; TRPV1, transient receptor potential vanilloid 1; TRPA1, transient receptor potential ankyrin 1; SF, synovial fibroblast/s; LDH, lactate dehydrogenase; AITC, allyl isothiocyanate

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to distinct exogenous and endogenous agonists [5,6]. Transient receptor potential ankyrin (TRPA1) and transient receptor potential vanilloid I (TRPV1) are co-expressed on the majority of sensory nerves but also in non-neuronal cells [7–9]. TRPA1 and TRPV1 have an important role in inflammation since they modulate the production of inflammatory mediators and influence arthritis severity [9–12]. Endogenous agonists for TRPA1 and TRPV1 are polyunsaturated fatty acids, aldehydes and endovanilloids which are generated in inflammatory environments [13,14]. Exogenous agonists for TRPA1 include the mustard oil and wasabi constituent allyl isothiocyanate (AITC) and the active compound in cinnamon, cinnamaldehyde, while TRPV1 is activated by the chili pepper ingredient capsaicin [5,15].

Expression of TRPV1 and TRPA1 in RASF and modulation of these receptors by endocannabinoids or capsaicin was shown to attenuate IL-6, IL-8 and MMP-3 production [9,16]. In addition, ligation of either TRPA1 or TRPV1 increases calcium flux in isolated synoviocytes [17]. The importance of TRP channels in RA is emphasized in TRPV1 knockout mice which develop milder arthritis compared to wild type animals with decreased paw swelling and disability [11]. Similarly, TRPA1 knock out mice display decreased paw volume and reduced pain sensitivity [10]. Consequently, the aim of this study is to elucidate the role of TRPA1 under proinflammatory conditions and its influence on synovial fibroblast function. We show here that TNF strongly enhances TRPA1 expression, TRPA1-mediated calcium flux and cell death in RASF. The results presented here might help to elucidate the role of TRPA1 in chronic inflammation and establish TRPA1 as an attractive molecule for selective deletion of proinflammatory synovial fibroblasts in RA, since T- and B-cells survival is not influenced by TRPA1 agonism [18,19].

2. Materials and methods

2.1. Patients

In this study, 19 patients with long-standing RA fulfilling the American College of Rheumatology revised criteria for RA [20] were included. The RA group comprised of 15 females and 4 males with a mean age of 61.1 years ± 10.7 years; C-reactive protein was 7.0 mg/dl ± 8.59 mg/dl. In the RA group, 19 out of 19 patients received non-steroidal anti-inflammatory drugs, 18 out of 19 glucocorticoids, 5 out of 19 methotrexate, 3 out of 19 sulfasalazine and 2 out of 19 biologicals. All patients underwent elective knee joint replacement surgery, and they were informed about the purpose of the study and gave written consent. The study was approved by the Ethics Committees of the University of Düsseldorf and Regensburg.

2.2. Compounds and chemicals

AITC (Allyl isothiocyanate) and cinnamaldehyde were obtained from Sigma Aldrich (St. Louis, USA). Polygodial, A967079, HC030031, AZ1047808 and INF 4E were obtained from Tocris/Bio-Techne (Wiesbaden, Germany).

2.3. Synovial fibroblast and tissue preparation

Synovial tissue samples from RA were obtained immediately after opening the knee joint capsule, the preparation of which was recently described [21]. Pieces of synovial tissue of up to 9 cm^2 were excised. One part of the tissue was cut, placed in protective freezing medium and stored at −80 °C until further use (Tissue Tek, Sakura Finetek, Zoeterwoude, The Netherlands). Another part was minced and treated with liberase (Roche Diagnostics, Mannheim, Germany) at 37 °C overnight. The resulting suspension was filtered (70 μm) and centrifuged at 300*g* for 10 min. The pellet was then treated with erythrocyte lysis buffer (20.7 g NH_4Cl, 1.97 g NH_4HCO_3, 0.09 g EDTA ad 1 l H_2O) for 5 min and again centrifuged for 10 min at 300*g*. The pellet was resuspended in RPMI-1640 (Sigma Aldrich, St. Louis, USA) with 10% FCS. Cell number was calculated using a Neubauer cell counting chamber. A total of 1,000,000 cells were transferred to a 75 cm^2 tissue culture flask. After overnight incubation, cells were supplemented with fresh medium.

2.4. Stimulation of RA synovial fibroblasts

8000 cells were seeded onto 96 well microtiter plates, grown for two days and were then stimulated with TNF (10 ng/ml) for 72 h to induce TRPA1 synthesis (in RPMI medium containing 2% FCS to minimize proliferation; for all assays). After that, cells were stimulated with respective TRPA1 agonists.

2.5. IL-6 and IL-8 ELISA

Cell culture supernatants were used for ELISAs 24 h (IL-6 and IL-8) after addition of TRPA1 ligands. Tests were conducted as described by the supplier (BD, OptEIA, Heidelberg, Germany). Inter- and intraassay coefficient of variation was below 10%.

2.6. Calcium flux assay

Tests were conducted as described by the supplier (abcam, Cambridge, UK, Fura-2 AM, ab 176766). In brief, 8000 cells were seeded onto 96 well black microtiter plates and were either left untreated or stimulated with TNF (10 ng/ml) for 72 h to induce TRPA1 synthesis. TRPA1 antagonists were added 60 min prior to agonist addition. For the detection of intracellular calcium, Fura-2 was added according to manufacturer's instructions and results were monitored using a VarioskanFlash with SkanIt Software 2.4.5 RE for Varioskan Flash (Thermo Fisher, Waltham, USA) or a Infinite 200 Pro TECAN multimode reader (TECAN, Männedorf, Switzerland).

2.7. Lactate dehydrogenase assay

Tests were conducted as described by the supplier (LDH Cytotoxicity Detection Kit, Clontech – 630117). 8000 cells were seeded onto 96 well microtiter plates and were either left untreated or stimulated with TNF (10 ng/ml) for 72 h to induce TRPA1 synthesis. After addition of TRPA1 agonist in serum-free medium (LDH is sensitive to FCS), LDH release was monitored over 4 h.

2.8. RealTime-Glo cell viability assay

Tests were conducted as described by the supplier (Promega #G9712, Mannheim, Germany). Synovial fibroblasts were either left untreated or were stimulated with TNF (10 ng/ml) in RPMI 1640 medium with 2% FCS for 72 h to induce TRPA1 synthesis. After that, TRPA1 agonists were added and cell viability was then monitored over 5 h.

2.9. PoPo-3 iodide uptake assay

Synovial fibroblasts were stimulated with TNF (10 ng/ml) in RPMI 1640 medium with 2% FCS for 72 h to induce TRPA1 synthesis. After that, TRPA1 agonist polygodial was added with PoPo-3 (Thermo Fisher, 1:500) and fluorescence was monitored over 90 min.

2.10. Immunofluorescence I (staining of synovial fibroblasts)

For immunofluorescent visualization of TRPA1, antibody ab62053, 30 μg/ml (Abcam, Cambridge, UK) was used. Cells were fixed with 2% formaldehyde and permeabilized with 0.1% Triton-X 100 in PBS. Slides were blocked with 1% BSA in PBS/0.1% Triton-X and incubated with primary antibody for 3 h at 37 °C. Cells were washed and incubated