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TSLP expression in the skin is mediated via RARγ-RXR pathways

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Abstract:

TSLP is an important trigger and initiator for various atopic diseases mainly atopic dermatitis (AD). Activators of nuclear hormone receptors like bioactive vitamin A and D derivatives are known to induce TSLP up-regulation in the skin. In this study various combinations of synthetic specific agonists and antagonists of the retinoic acid receptors (RARs), retinoid X receptors (RXRs) and vitamin D receptor (VDR) were topically administered to mice. The aim of the study was to elucidate via which nuclear hormone receptor pathways TSLP is regulated and how this regulation is connected to the development and phenotype of atopic dermatitis. TSLP expression was monitored using QRT-PCR and serum TSLP levels using ELISA. Synthetic agonists of the VDR and RARγ as well as the natural agonist all-*trans* retinoic acid (ATRA) increased TSLP expression in the skin, while an RXR agonist was not active. Treatments with antagonists of RXRs and RARs in addition to RARα-agonists reduced skin TSLP expression. Strong activation was found after a combination of a VDR and an RXR agonist (ca. 5 times induction) and even stronger by an RARγ and an RXR agonist treatment (ca. 48 times induction). We conclude that besides VDR-mediated signaling mainly RARγ-RXR mediated pathways in the skin are important patho-physiological triggers for increased skin TSLP expression. We conclude that topical synthesized retinoids stimulated by internal or external triggers or topically applied induce TSLP production and are thereby important triggers for atopic dermatitis prevalence.

Introduction

Atopic dermatitis (AD) is a highly pruritic, chronic and common inflammatory disease of the skin being often associated with strong hereditary background (1, 2). AD develops in early infancy and childhood and can persist till adulthood (3) and mainly Th2 pathways play a critical role during pathogenesis (1). Thymic stromal lymphopoietin (TSLP) is an IL-7 like cytokine and was shown to be a master switch of allergic inflammation at the epithelial cell - dendritic cell interface leading to allergic sensitization (4). TSLP expression is highly expressed in keratinocytes and myeloid dendritic cells in acute and chronic AD skin (5-7). It plays a critical role during initiation of allergic diseases in mice and humans (6, 8, 9) and elevated TSLP levels were associated with a Th2 polarisation in numerous inflammatory diseases (10).

Topical and systemic application of lipid ligands of the nuclear hormone receptors vitamin D receptor (VDR), retinoid X receptor (RXR) and retinoic acid receptor (RAR) trigger TSLP expression (11-14). Previously, it was reported that even selective ablation of the retinoid X receptor α (RXRα), which is predominant in skin, triggers AD and results in the development of a chronic dermatitis in mice with similarities to human AD (15). A Th2-like inflammatory reaction after increased TSLP expression was observed, suggesting that TSLP is involved in the AD-like skin syndrome.

The aim of our study was to find out how selective agonists and antagonists for the nuclear hormone receptors VDR, RARs and RXRs influence skin TSLP expression as well as serum TSLP levels and what is the consequence of this regulation on atopic dermatitis phenotype and development.

Materials and methods

Sensitization of mice

8-12 weeks old female C57BL6 and BALBc mice were obtained from and housed within the animal facility of the University of Debrecen, Hungary. Animals were maintained in single cages on standard animal chow and water *ad libitum*. All experimental procedures were approved by the Committee of Animal Research of the University of Debrecen, Hungary (Approval number: 25/2006 DEMÁB).

C57BL6 mice were anesthetized and subsequently shaved on dorsal skin sites using an electric razor. Retinoid receptor-specific agonists and antagonists were applied topically each other day in 25 µl acetone (vehicle/control; Merck, Darmstadt, D) per treatment for two weeks. According to previous studies by other groups (16, 17) agonists and antagonists were applied in the following concentrations: ATRA 40 nmol; LG268 (RXR-agonist) 100 nmol; BMS753 (RARα agonist) 40 nmol; BMS189961 (RARγ agonist) 40 nmol; BMS493 (RAR pan-antagonist) 100 nmol; UVI3003 (RXR pan-antagonist) 100 nmol; MC903 (VDR agonist) 1 nmol. On day 14, four hours after the last treatment, mice were sacrificed, sera and full thickness skin biopsies were collected, skin specimens were shock frozen in liquid nitrogen and all samples were kept at -80 °C until analyses. Skin samples were obtained from equal body sites by means of the same procedure for each mouse in order to control for variability among specimen. Samples were visibly controlled to ensure no excessive adipose tissue remained, though some contamination with remaining adipose tissue cannot be excluded.

Sensitization of BALBc mice was performed by repetitive systemic application of OVA. Briefly, mice were sensitized at days 47, 60 and 67 with 10 µg OVA intraperitoneally (i.p.) adsorbed to 1.5 mg aluminium hydroxide ($Al(OH)_3$) or with phosphate-buffered saline (PBS, control). For combined treatment mice were sensitized i.p. on days 1, 14 and 21 with 10 µg OVA adsorbed to 1.5 mg $Al(OH)_3$. This was followed by topical application of 100 µg OVA adsorbed to 1.5 mg $Al(OH)_3$ in 100 µl PBS onto shaved back skin, divided into four applications of 25 µl every other day of one week.

Epicutaneous treatment was repeated for a total exposure of three weeks separated by two-week intervals. Three days after the last treatment mice were euthanized; serum samples were collected and kept at -80 °C until analyses.

Study using human atopic dermatitis volunteers

After informed consent and the approval of the local Ethics Committee of the University of Debrecen, Hungary, Medical and Health Science Center, peripheral blood was collected from 20 AD-patients (8 male, 12 female; mean age 20 years, range 15-32 years). A group of 20 healthy age-matched volunteers (6 males, 14 females, mean age 21 years, range 19-24 years) served as controls in this study (18). All AD-patients fulfilled the diagnostic criteria established by Hanifin and Rajka (19).

RNA preparation and reverse transcription

Total RNA was isolated from frozen skin using Tri® reagent (Molecular Research Center Inc., Cincinnati, OH) following the manufacturer’s instructions. 750 ng of total RNA were reverse transcribed into cDNA in a 30 µl reaction using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Budapest, H) according to the manufacturer’s protocol.

Analysis of mRNA expression

mRNA expression in skin was determined by means of quantitative real time-PCR (qRT-PCR) and TaqMan® Low Density Arrays (TLDA) on an ABI Prism 7900. qRT-PCR measurements were performed in triplicate using pre-designed TaqMan® Gene Expression Assays and reagents; TaqMan® Low Density Array cards were used for duplicate determinations using TaqMan® Gene Expression Master Mix (all Applied Biosystems Applera Hungary, Budapest, H). Relative quantification of mRNA expression was achieved using the comparative C_T method and values were normalized to cyclophilin A mRNA. Gene expression values below detection limit were assumed to be zero for the purpose of statistical analysis.

ELISA

Quantikine human TSLP and mouse TSLP kits (RnD-Sytems, Budapest, H) were used for the quantitative determination of human and mouse TSLP in serum. All samples and standards were assayed in triplicate. 100 µl of Assay Diluent RD1X was added to each well and 50 µl standard, control or sample was added per well. The plate was incubated for 2 h at RT and covered securely with a foil plate sealer. Each well was aspirated and washed 4 times and 200 µl of conjugate was added to each well and incubated for 2 h at RT. The aspirating and washing step was repeated 4 times and 200 µl of substrate solution was added to each well and incubated for 30 min being protected from light. The reaction was stopped by adding 50 µl of stop solution to each well. The results were read within 30 min at 450 nm, with a λ correction 540 or 570 nm. The average of the duplicate readings has been calculated for each standard and sample.

Statistics

Data are indicated as mean and standard error mean. Statistical analysis of QRT-PCR and ELISA data was performed using student t-test and differences were considered significant at $p<0.05$.

Results

Increased TSLP expression upon synthetic RARγ-agonist (BMS189961), combinative RARγ-agonist-RXR-agonist and ATRA treatment in mouse skin (Fig. 1): Topical application of the RXR-agonist (LGD268) resulted in no significant change of TSLP expression in mouse skin, while the application of an RXR-antagonist (UVI3003) decreased TSLP expression ($p<0.01$) (Fig. 1A). An RARα-agonist (BMS753) reduced, while an RARγ-agonist increased dermal TSLP expression. Topical application of ATRA also significantly increased TSLP expression, while an RAR-pan antagonist (BMS493) significantly reduced it (Fig. 1B). Combinative application of an RARγ-agonist and an RXR-agonist significantly increased TSLP expression up to ca. 50 times (Fig. 1C).

Increased TSLP expression upon combinative VDR-agonist-RXR-agonist treatment in mouse skin (Fig. 2): Topical application of a VDR-agonist (calcitriol, MC903) showed a tendency of increased TSLP expression ($p=0.08$), while the combination with an RXR-agonist significantly increased TSLP expression ca. 5 times. Respectively, an RXR-agonist alone did not display significant influence on TSLP expression.

Non-elevated serum TSLP concentration in human atopic dermatitis and in a mouse atopic dermatitis model (Fig. 3): TSLP levels were comparable in the serum of AD-patients in comparison to healthy volunteers. In mice after intraperitoneal OVA treatment we also observed comparable serum TSLP levels compared to controls. After intraperitoneal and epicutaneous OVA sensitization the serum TSLP concentration also remained comparable to PBS treated mice.

After topical treatments with an RXR-agonist or an RARα-agonist comparable TSLP levels were found in serum, while after treatment with an RXR-antagonist a tendency of reduced TSLP levels were observed ($p=0.08$). Topical treatments with RARγ-agonist, VDR-agonist and ATRA result in increased serum TSLP levels.

Discussion

TSLP is implicated in the pathogenesis of AD as well as the atopic march starting from skin inflammation towards other allergic diseases (20-23). Mainly epithelial cells, especially keratinocytes, express TSLP (5) and stimulation with allergens increases dermal TSLP expression (9). Various studies report that nuclear hormone mediated signaling via RARs, RXRs and VDR-mediated pathways is involved in atopic sensitization, atopic phenotype and potential atopic dermatitis treatment. In this study we found that besides VDR-RXR signaling pathways mainly RARγ-RXR-pathways are of major importance for increased skin TSLP expression and serum TSLP levels (24).

Atopic dermatitis is a chronic inflammatory skin disease with a strong hereditary background (25). Local inflammation and missense mutated epidermal structural proteins are responsible for epidermal barrier dysfunction resulting in increased percutaneous penetration of exogenous substances. Its consequences are increased allergic sensitization towards normally harmless allergens and further chronification (26, 27). Serum TSLP levels were low in serum of healthy volunteers and non altered in serum of AD-patients (28, 29), while just in adults with a loss-of-function mutations within the filaggrin gene resulted in increased TSLP serum levels (27). In serum of atopic children during early sensitization increased TSLP levels were found (30) displaying a more skin dependent and acute phase relevant influence of TSLP. In our study we confirmed that serum TSLP levels were non-altered in human atopic dermatitis patients as well as in mice with systemic or systemic plus topical sensitization (Figure 3).

Nuclear hormone receptors, which mainly form heterodimers with RXRs, play important roles for in skin physiology (31, 32). Especially the retinoid receptors (RAR) RARα and RARγ are crucial for skin homeostasis and skin inflammation (16) and application of selective synthetic RARγ ligands induced epidermal hyperproliferation (33). In addition, ablated RXRαβ (ep-/-) keratinocytes in mice generate a chronic dermatitis which displays high similarity to human AD (34). In VDR -/- animals (35) and treatments with the endogenous VDR-agonist 1,25-$(OH)_2$-vitamin D3 (1,25VD3) induced AD-like syndromes in mice (36). Topical application of a synthetic RAR-ligand and especially co-treatments of an RAR- and a VDR-agonist strongly increased skin TSLP expression and serum TSLP levels (11). Our study and others (29) could not or just partly confirm this effect of 1,25VD3 treatment on TSLP expression in the skin. This displayed that VDR- and RAR-agonists are involved in increased skin TSLP expression. In our studies we found; a) that a synthetic VDR-agonist displays just a tendency for increasing skin TSLP expression and that co-application of an RXR-agonist, which is not modifying skin TSLP expression and serum TSLP levels, results in increased skin TSLP expression and increased serum TSLP levels. b) In addition that an RARγ-agonist induced strong TSLP expression in the skin as well as increased TSLP levels in serum and that co-administration with an RXR-agonist resulted in strong dermal expression of TSLP and increased serum TSLP levels. c) We also found that an RARα-agonist and pan-RAR- and RXR-antagonists can reduce skin TSLP expression. As a conclusion, antagonizing specific RAR and RXR pathways may result in potential treatment strategies for AD prevention and therapy.

We conclude that skin TSLP expression is minor influenced by vitamin D-mediated signaling but mainly via RARγ-mediated signaling in the skin. Increased RARγ-RXR mediated signaling may be initiated in the skin via internal or external triggers (33, 37). We confirm by this that retinoids / vitamin A are important triggers for atopic sensitization (38-40). Based on that, we postulate that reducing dermal TSLP expression via interfering RARγ-RXR-mediated signaling and further serum TSLP levels might be an important strategy for prevention and therapy of AD or other TSLP-linked diseases.

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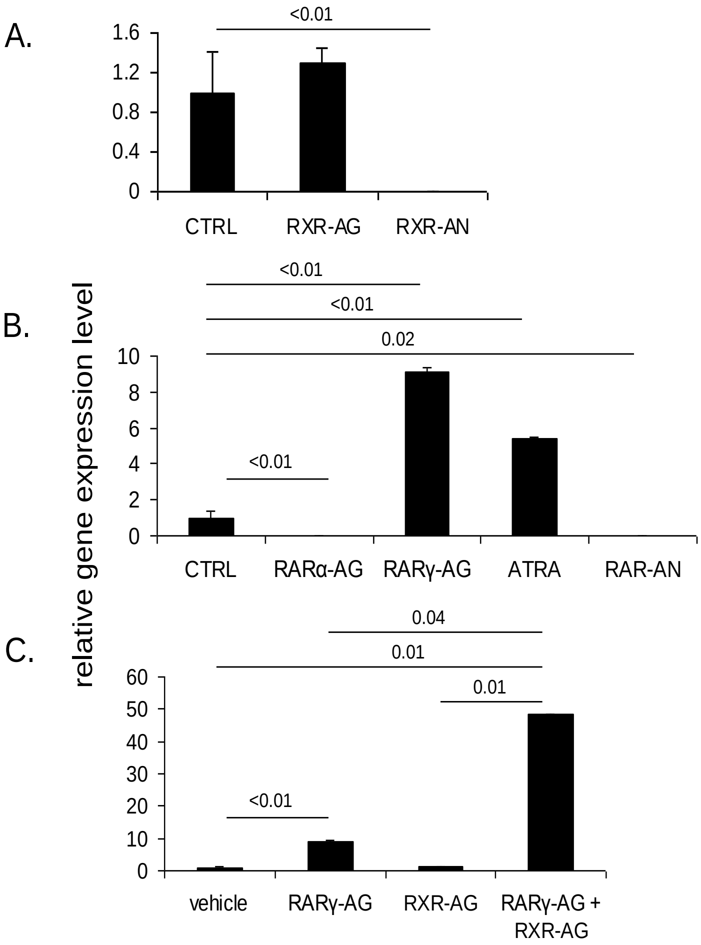

Figure 1: (A) Gene expression of TSLP in mouse skin upon topical treatment with RXR-agonist (LG268, RXR-AG), RXR-antagonist (UVI3003, RXR-AN) vs. control treatment (CTRL); (B) Gene expression of TSLP in mouse skin upon topical treatment with RARα-agonist (BMS753, RARα-AG), RARγ-agonist (BMS189961, RARγ-AG), all-*trans* retinoic acid (ATRA), RAR-pan-antagonist (BMS493, RAR-AN) vs. control treatment; (C) Gene expression of TSLP in mouse skin upon topical treatment with RARγ-agonist, RXR-antagonist and combinative treatment of RARγ-agonist and RXR-agonist vs. control treatment. All experiments were performed with n=6 female animals, * - $p < 0.05$.

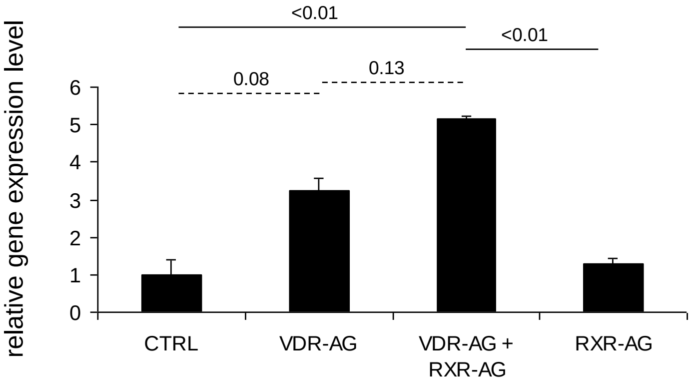

Figure 2: Gene expression of TSLP in mouse skin upon topical treatment with VDR-agonist (MC903, VDR-AG), VDR-agonist and RXR-agonist, RXR-agonist vs. control treatment, experiments were performed with n=6 female animals- Significant differences were indicated by a solid line over the bars including p-values in addition to non-significant differences marked by a dashed line including the p-value.

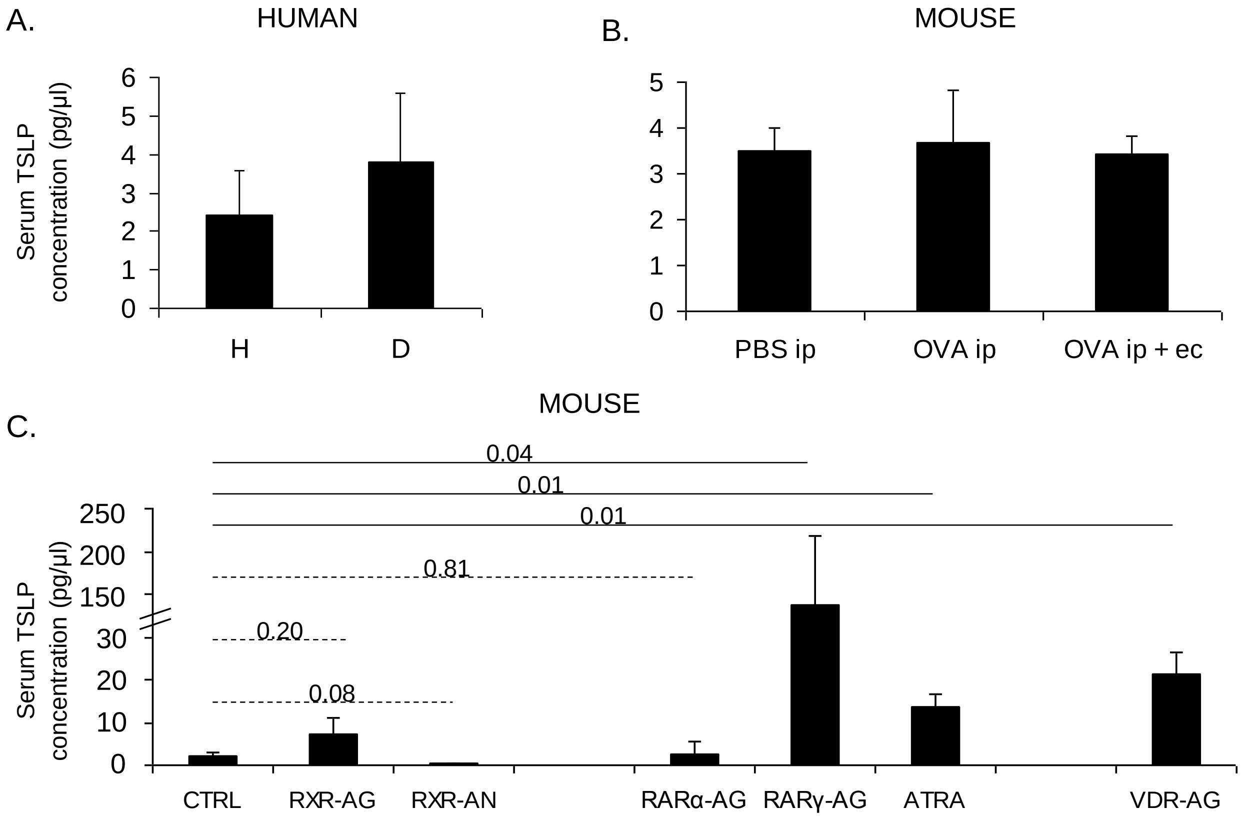

Figure 3: (A) TSLP concentration (pg/µl) in serum of AD patients (D) vs. healthy volunteers (H) by ELISA method, n=20; (B) TSLP concentration (pg/µl) in mouse serum by ELISA method after intraperitoneal OVA administration (OVA i.p.), intraperitoneal and epicutaneous OVA administration (i.p. + e.c) vs. intraperitoneal PBS administration (PBS i.p.), n=4; (C) TSLP concentration (pg/µl) in mouse serum by ELISA method after topical treatment with RXR-agonist, RXR-antagonist, RARα-agonist, RARγ-agonist, ATRA, VDR-agonist treatment vs. control treatment, n=4. Significant differences were indicated by a solid line over the bars including p-values in addition to non-significant differences marked by a dashed line including the p-value.