Letter to the Editor

Title: Wy14643, an agonist for PPARα, down-regulates expression of TARC and RANTES in cultured human keratinocytes.

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Short title: Wy14643 down-regulates TARC and RANTES

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ABSTRACT:

Beneficial effects of Wy14643, an agonist for peroxisome proliferator-activated receptor (PPAR)α, on permeability barrier homeostasis-related functions of keratinocytes such as up-regulation of epidermal differentiation-related molecules and lipid synthesis, have been demonstrated. The present study demonstrated that Wy14643 reduced the expression of thymus and activation-related chemokine (TARC) and regulated on activation normal T cell expressed (RANTES) in both single- and 3D-cultured human keratinocytes. The combined data of the present and previous studies support the notion that Wy14643 could be a therapeutic agent that might simultaneously and directly modulate permeability barrier dysfunction and allergic inflammation in the pathogenesis of atopic dermatitis. As for the anti-microbial barrier function, the present study demonstrated that Wy14643 up-regulated expression of the anti-microbial peptide, human β defensin 3, in cultured human keratinocytes only in mRNA levels but not in protein ones, suggesting that Wy14643 might not directly account for the up-regulation of the anti-microbial peptide which has been reported in vivo.

BACKGROUND:

Permeability barrier dysfunction, anti-microbial dysfunction, and allergic inflammation are the principal pathogenic features of atopic dermatitis (AD) (1). Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that are classified into three subclasses: PPARα, PPARβ/δ, and PPARγ (2). Reduced expression of PPARα (but not of PPARβ/δ or PPARγ) in human atopic dermatitis (AD) lesions and epidermis in hapten-induced AD-like dermatitis has been reported (3, 4). In addition, PPARα deficient mice show a more severe hapten-induced AD-like dermatitis than wild-type mice (3). These data suggest that reduction in PPARα might be involved in the pathogenesis of AD. Consistent with these data, we demonstrated that decreased PPARα not only down-regulated the expression of epidermal differentiation-related molecules but also up-regulated the expression of thymus and activation-related chemokine (TARC) and regulated on activation normal T cell expressed (RANTES) in cultured human keratinocytes (5). TARC and RANTES are chemokines produced by keratinocytes that are important in terms of cutaneous inflammation of AD (6, 7). Therefore, these results suggest that PPARα in keratinocytes might play a role in both permeability barrier dysfunction and allergic inflammation in AD. Beneficial effects of agonists of PPARα (including effects of the agonist Wy14643) on permeability barrier homeostasis-related functions of keratinocytes such as up-regulation of epidermal differentiation-related molecules and lipid synthesis have been shown. Beneficial

effects of PPARα agonists (not including the agonist Wy14643) on the anti-microbial barrier, such as up-regulation of anti-microbial peptides, have also been demonstrated (8, 9). However, effects of PPARα agonists on allergic inflammation-related functions in keratinocytes have not been addressed.

QUESTIONS ADDRESSED:

The present study addressed whether the synthetic PPARα ligand, Wy14643, could influence inflammatory functions (i.e. expression of chemokines such as TARC and RANTES) of keratinocytes to modulate the cutaneous allergic inflammation of AD. In addition, the present study addressed whether Wy14643, similar to other previously reported PPARα ligands, could augment anti-microbial barrier functions of keratinocytes. Based on our results, we consider that Wy14643 might be a therapeutic agent that modulates not only the already known skin barrier functions, such as the permeability barrier and the anti-microbial barrier, but that might also simultaneously and directly modulate cutaneous allergic inflammation in AD.

EXP. DESIGN:

We examined the effects of Wy14643 (Sigma) on the expression of TARC and RANTES in cultured human keratinocytes using both single layer and 3D-cell culture systems. Expression

levels of these molecules were evaluated by analysis of their mRNA levels in cultured keratinocytes and their protein levels in the culture supernatant using quantitative RT-PCR and ELISA, respectively. The effects of Wy14643 on the expression of the antimicrobial peptide, human β-defensin 3 (hBD3) in cultured human keratinocytes, were also examined.

Cell culture, cell stimulation, RT-PCR, ELISA, and statistical analysis were performed as described in the supplementary materials and methods.

RESULTS:

Wy14643 down-regulated the mRNA expression, and the protein level in the culture supernatant, of both TARC and RANTES not only in single layer-cultured keratinocytes (Fig. 1) but also in 3D-cultured keratinocytes (Fig. 2). In contrast, the mRNA expression of hBD3 was up-regulated by Wy14643 in both single layer-cultured and 3D-cultured keratinocytes (Fig. S1). Meanwhile, there were no difference in the protein level of hBD3 in culture supernatant in 3D-cultured keratinocytes between with and without Wy14643 (Fig. S1). The protein of hBD3 was not detected in culture supernatant in single layer-cultured keratinocytes (data not shown).

CONCLUSIONS:

The present study demonstrated that an agonist for PPARα could suppress the expression

of TARC and RANTES that are known to be involved in the pathogenesis of AD in cultured keratinocytes. These results might explain part of the mechanism of the therapeutic effects of topical treatments with PPARα agonists in AD-like dermatitis induced by hapten application in murine models (4, 10). Anti-inflammatory effects of agonists for PPARα have been demonstrated in cutaneous inflammatory models, and an antigen-presenting cell, the Langerhans cell, was reported to be a target cell for the anti-inflammatory effects of agonists for PPARα (2). The present study is the first to demonstrate that keratinocytes could be a target cell in the anti-inflammatory effects of ligands for PPARα and the results support the previously reported regulatory effects of PPARα signaling on the inflammatory functions of keratinocytes (i.e. expression of TARC and RANTES) (5).

Beneficial effects of agonists for PPARα including Wy14643 on the permeability barrier homeostasis-related functions of keratinocytes have been demonstrated (8). In addition, the present study demonstrated that Wy14643, as was reported for another ligand for PPARα, GW9578 (9), up-regulated the mRNA expression of hBD3 in cultured human keratinocytes in both single layer- and 3D cell cultures, suggesting that Wy14643 might also augment anti-microbial barrier function. Meanwhile, the protein levels of hBD3 in culture supernatant were detected only in 3D-cultured keratinocytes and there were no significant difference in those between with and without Wy14643, although it has been reported that protein levels of BD3 in

epidermis was up-regulated by topical treatment with Wy14643 in a hapten-induced AD-like dermatitis murine model (S1). There results suggest that additional factors may contribute to regulate the secretion of hBD3 in vivo.

The combined data of the present and previous studies suggest that an agonist for PPARα, Wy14643, might not only modulate permeability barrier dysfunction, but also, simultaneously, might confer anti-inflammatory effects on keratinocytes in the pathogenesis of AD, although the mechanism for such effects remains unclear. Meanwhile, the effect of an agonist for PPARα on the expressions of the anti-microbial peptide in keratinocytes remains controversial.

Acknowledgement

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Author contributions

W.Z. and Y.H. designed the experiments, analyzed the data and wrote the manuscript. T.S. contributed to the experiments using cultured keratinocytes. S.F. contributed to the design of the experiments.

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Supplementary reference

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FIGURE LEGENDS:

Figure 1: Effects of the synthetic ligand for PPARα, Wy14643, on the expression of TARC and RANTES in single layer-cultured human keratinocytes.

Twenty-four h after stimulation of single layer-cultured cells of the HaCaT human epidermal keratinocyte cell line with TNFα and IFNγ, without or with the indicted concentration of Wy14643, cells (a, c), and culture supernatants (b, d) were harvested. Expression of TARC (a, b) and RANTES (c, d) was examined using semiquantitative RT-PCR (a, c) or ELISA (b, d) as described in "Supplementary Materials and methods". n=5. P-values (vs. without Wy14643) less than 0.05 are shown.

Figure 2: Effects of the synthetic ligand for PPARα, Wy14643, on the expression of TARC and RANTES in 3D-cultured human keratinocytes.

Seventy-two h after stimulation of 3D-cultured cells of the HaCaT human epidermal keratinocyte cell line with TNFα and IFNγ, without or with the indicted concentration of Wy14643, cells (a, c), and culture medium (b, d) were harvested. Expression of TARC (a, b) and RANTES (c, d) was examined using semiquantitative RT-PCR (a, c) or ELISA (b, d) as described in "Supplementary Materials and methods". n=5. P-values (vs. without Wy14643) less than 0.05 are shown.

Figure 1

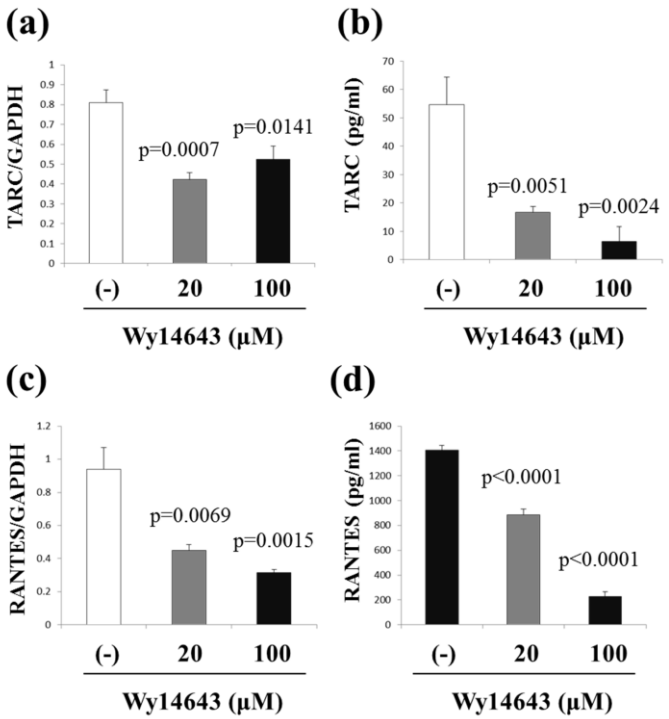
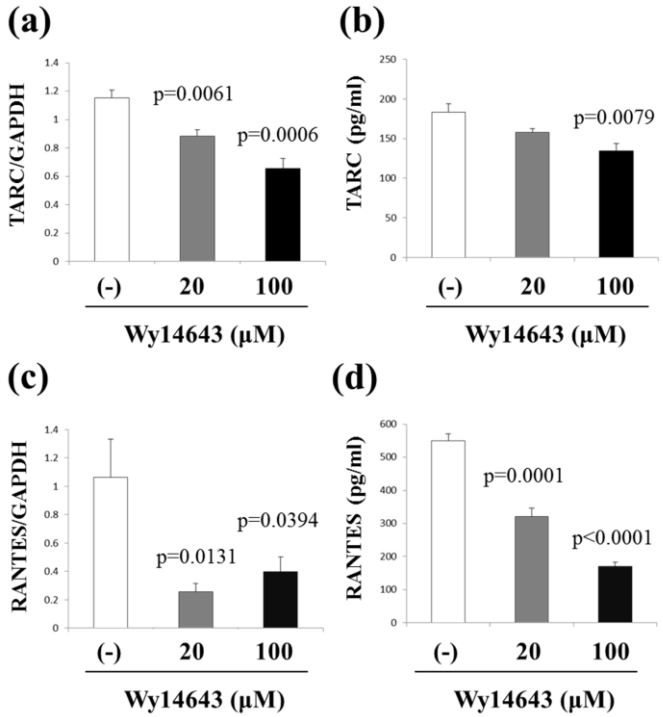
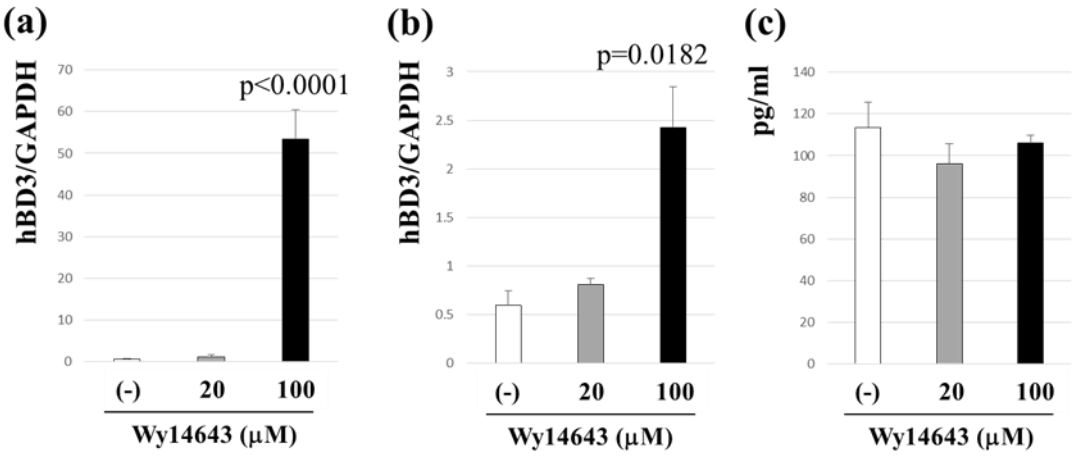

(a)
TARC/GAPDH
p=0.0007
p=0.0141
(-)
20
100
Wy14643 (μM)
(b)
TARC (pg/ml)
p=0.0051
p=0.0024
(-)
20
100
Wy14643 (μM)
(c)
RANTES/GAPDH
p=0.0069
p=0.0015
(-)
20
100
Wy14643 (μM)
(d)
RANTES (pg/ml)
p<0.0001
p<0.0001
(-)
20
100
Wy14643 (μM)

Figure 2


(a)
TARC/GAPDH
p=0.0061
p=0.0006
(-)
20
100
Wy14643 (μM)
(b)
TARC (pg/ml)
p=0.0079
(-)
20
100
Wy14643 (μM)
(c)
RANTES/GAPDH
p=0.0131
p=0.0394
(-)
20
100
Wy14643 (μM)
(d)
RANTES (pg/ml)
p=0.0001
p<0.0001
(-)
20
100
Wy14643 (μM)



Supplementary Figure S1: Effects of the synthetic ligand for PPARα, Wy14643, on the expression of human β defensin 3 in cultured human keratinocytes. Twenty-four h (a) or seventy-two h (b, c) after stimulation of single layer-cultured (a) or 3D-cultured (b, c) cells of the HaCaT human epidermal keratinocyte cell line with TNFα and IFNγ, without or with the indicted concentration of Wy14643, the cells (a, b) and the culture supernatant (c) were harvested as described in "EXP. DESIGN". Transcript levels (a, b) and protein levels (c) of human β defensin 3 were examined using semiquantitative RT-PCR (a, b) and ELISA (c) as described in "Supplementary Materials and methods". n=5. P-values (vs. without Wy14643) less than 0.05 are shown.

Supplementary Materials and Methods

Cell culture

Cells of the human epidermal keratinocyte HaCaT cell line were used for analysis of the expression of TARC and RANTES, since a previous study demonstrated that HaCaT cells but not cultured normal human keratinocytes produce TARC (1). As previously reported (1, 2, 3), constitutive expressions of mRNAs of TARC and RANTES were not detected in cultured keratinocytes (data not shown). Single-layer culture of HaCaT and induction of TARC and RANTES by recombinant human (rh)TNFα (R&D Systems) and rhIFNγ (R&D Systems) were performed as previously reported (4). Culture at an air-liquid interface (i.e. 3D-culture) was performed as previously reported (5) with some modifications. Briefly, HaCaT cells were seeded into Cell Culture Inserts (0.4 um pore size; BD Biosciences) and cultured in Keratinocyte Basal Medium 2 with Supplement Pack Keratinocyte Growth Medium 2 (PromoCell) at 37 °C in a humidified atmosphere of 5% CO2 in air. A Companion Plate (BD Biosciences) containing the same medium was simultaneously processed. The medium was exchanged every three days. The medium in the Cell Culture Inserts was aspirated when the cells in the Cell Culture Inserts reached 100% confluence, in order to move the cells into an air-liquid interface condition.

Thereafter, the medium in the Companion Plate was changed to assay medium (EPI-MODEL; J-TEC Japan Tissue Engineering Co., Ltd.). The medium in the plate was changed every three days, and the keratinocytes were cultured for 15 days.

Cell stimulation

Stimulation with rhTNFα (10 ng/ml for single layer-culture and 50 ng/ml for 3D-culture) and rhIFNγ (2 ng/ml for single layer-culture and 10 ng/ml for 3D-culture) with or without Wy14643 was performed under conditions of Keratinocyte Basal Medium 2 with Supplement Pack Keratinocyte Growth Medium 2 (PromoCell) without hydrocortisone in both single layer- and 3D-cultures. Cell stimulation was started with cells at 80% cell confluence in the single layer-culture and on day 15 of the air-liquid interface condition in the 3D-culture. The incubation periods before harvesting the cultured keratinocytes and the culture medium were 24 h for the single layer-culture and 72 h for the 3D-culture. The medium was not exchanged during the incubation.

RT-PCR

Total RNA was isolated from HaCaT cells using the High Pure RNA Isolation Kit (Roche Diagnostics GmbH) and reverse transcription was performed with the Transcriptor First

Strand cDNA Synthesis Kit (Roche Diagnostics GmbH) according to each manufacturer's instructions. Complementary DNA products were amplified on a LightCycler System (Roche Diagnostics GmbH), as described previously (5). The primers used for real-time PCR are shown in Table S1. Product specificity was evaluated by melting curve analysis, and relative gene expression was calculated from a standard curve included in each run. Relative mRNA expression levels were normalized with the housekeeping gene, GAPDH.

ELISA

Levels of TARC and RANTES in the culture supernatants were determined using ELISA quantification kits purchased from R&D Systems and all analyses were performed according to the manufacturer's instructions.

Statistical analysis

The statistical significance of differences was evaluated by Student's t-test. A P-value of less than 0.05 was regarded as significant.

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the productions of interleukin (IL)-1α, IL-8, IL-18 and granulocyte macrophage colony-stimulating factor in stratified human keratinocytes. J Dermatol Sci 2015: 80:158-60.

Supplementary Table S1. Primers used for RT-PCR

Gene product	Primer	Sequence
TARC	Upstream	5”-CCC CTT AGA AAG CTG AAG AC-3”
	Downstream	5”-CTC TCA AGG CTT TGC AGG TA-3”
RANTES	Upstream	5”-TGC CCA CAT CAA GGA GTA TTT C-3”
	Downstream	5”-TCC ATC CTA GCT CAT CTC CA -3”
hBD3	Upstream	5”-GTG AAG CCT AGC AGC TAT GA-3”
	Downstream	5”-TGT TTA TGA TTC CTC CAT GAC CTG-3”
GAPDH	Upstream	5”-TGA ACG GGA AGC TCA CTG G-3”
	Downstream	5”-TCC ACC ACC CTG TTG CTG TA-3”

TARC: human thymus and activation-related chemokine; RANTES: regulated upon activation, normal T-cell expressed and secreted; hBD3: human β defensin 3; GAPDH: glyceraldehyde-3-phosphate dehydrogenase