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TOLL 7 AND TOLL 9 IN *PSORIASIS VULGARIS* BEFORE AND AFTER PHOTOTHERAPY

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Abstract
Introduction: Psoriasis is a common chronic inflammatory, recurrent, immune mediated disease of the skin and joints. Toll-like receptors are pattern recognition receptors for conserved molecular patterns of pathogenic microorganisms.Under certain circumstances, self nucleic acids can trigger TLR 7 and TLR 9, which can lead to autoimmune diseases such as psoriasis.
Materials and Methods: The study included 15 psoriatic patients (plaque type) and 15 controls, patients received 36 sessions of phototherapy (NB-UVB). Skin biopsies were taken from all the patients (before & after NB-UVB) and controls and were assessed for TLR 7 and TLR 9 by PCR.
Results: Showed significant difference between patients and controls as regards TLR 7 and TLR 9. In addition a significant decrease in thier levels in patients after phototherapy with NB-UVB.
Conclusion: TLR 7 and TLR 9 may play a role in the pathogenesis of poriasis. Decrease in their levels after NB-UVB may be one of the therapeutic mechanisms of NB-UVB in psoriasis.

Key words: Psoriasis; Toll-like receptors 7 and 9; NB-UVB

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Introduction

Toll-like receptors are a recently identified group of pattern recognition receptors (PRRs) that recognize distinct conserved microbial components and permit cells to recognize self from non-self in immune activation [1]. By far, ten different receptors have been identified and have unique tissue distribution, ligand binding properties, cellular signaling pathways, and cytokine production profiles. A subgroup of TLRs, namely TLR3, 7, 8, and 9, are located within the cell in endosomal compartments and recognize pathogen derived nucleic acid components [2].
Psoriasis is a chronic inflammatory skin disease mediated by T cells, which trigger keratinocytes to hyperproliferate and perpetuate the disease. Psoriasis has been associated with Th-1 and Th-17 cytokine profiles [3], and angiogenesis. Inappropriate recognition of self-nucleic acids in addition to type I IFNs (IFN-α/β) production by plasmacytoid DCs (PDCs) can lead to psoriasis. PDCs are an important cell population in this condition, because they are 16% of the total dermal infiltrate in psoriatic skin lesions whereas they are nonexistent in normal skin [4]. PDCs and B cells express high levels of TLR 7 and 9 [5,6]. Thus it is possible that abberent expression of both TLR7 and 9 could contribute in the pathogenesis of psoriasis vulgaris. Narrow-band UVB radiation (NB-UVB) therapy offers a well-established treatment modality for psoriasis. However, despite the common use of this form of treatment, the mechanism of action of NB-UVB in psoriasis is not well understood [7]. UVB radiation is a potent immunosuppressive agent that inhibits cell-mediated immune responses. The mechanisms by which UVB radiation influences cell-mediated immune responses have been the subject of extensive investigation. However, the role of innate immunity on photoimmunological processes has received little attention.
The aim of the present study was to find out the effect of NB-UVB on the expression of TLR9 and TLR7 gene expression ` in psoriatic skin, in an attempt to add another possible mechanism of action by which NB-UVB improves psoriatic lesions.

Material and Methods

Patients

The current study was conducted on 15 patients (8 females and 7 males) suffering from psoriasis vulgaris (chronic plaque type). Their age ranged from 14-63 years. They were selected from the outpatient clinic of the Dermatology Department, Faculty of medicine, Cairo University from July 2011 till July 2012, with lesional extent ranging from 20-80%. The duration of the treatment was 3 months (36 sessions) for each patient. An informed consent was signed by each patient and ethical committee approval was fulfilled before the start of the study.
Diagnosis was done on clinical basis and confirmed by skin biopsy.

Inclusion criteria:

· Patients over 12 years.
· Either male or female.
· Psoriasis vulgaris

Exclusion criteria:

· Children less than 12 years old.
· Pregnant and lactating females.
· Patients with systemic diseases e.g. hepatic, renal, and cardiac diseases.
· Erythrodermic and pustular psoriasis (only patients with psoriasis vulgaris).

Before initiation of therapy, each patient was subjected to the following:

I. History taking:

A) Personal history: (name, age, sex, weight, residence (far residence decreases patient's compliance), occupation (sun exposed or not), pregnancy, lactation and any special habits of medical importance).
B) Present history: (onset, course and duration of psoriasis).
C) Precipitating factors:
e.g infection, psychic stress or trauma ,drugs, such as; steroids, β blockers, lithium, antimalarials or nonsteroidal anti-inflammatory drugs. Photosensitizers, such as; tolbutamide, sulfonamides, tetracycline, griseofulvin, phenothiazides or others.
D) Previous treatment and any past history of medical importance
F) Associated disease:
Such as: vitiligo, diabetes mellitus, arthritis or others.
G) Family history:
Psoriasis, vitiligo, arthritis, diabetes mellitus, or others.

II. Examination and investigations:

· Skin examination: to determine the distribution, clinical variant, skin type and extent of psoriasis.
· PASI score was calculated for each patient. The PASI score includes assessment of erythema, infilteration, desquamation and extent of lesions [8].
· Ophthalmologic examination by slit lamp: to exclude lens opacity.

Patients who were treated before by topical treatment (had stopped it 2 weeks) before starting the phototherapy, and those who were treated before by systemic treatment had stopped the last treatment one month before starting the phototherapy.

Control group:

A control group of 15 healthy individuals (age and sex matched with the patient group) with no history of skin or autoimmune diseases, were included in our study.

Methods

Skin Biopsies:

· Two 5mm punch skin biopsies were taken from all the patients, one before treatment and the other after 36 sessions of phototherapy and only one biopsy was taken from each control.
· On taking the biopsy, one half portion of the biopsy specimen was fixed in formalin solution and the other half of the specimen was kept frozen in an empty epindorphe for PCR studies.
The patients received NB-UVB (36 sessions) three times per week (for 3 months).

UVB light:

UVB light was delivered by a UV cabin (waldmann) equipped with an integrated UV photometer equipped with 13 TL-01/100 w fluorescent lamps producing a narrow band UVB peak emission at 311nm.

Dosing schedule:

· The initial UVB dose was 70% of the minimal erythema dose for all skin types.
· The dose of UVB was increased by 20% of the last dose every other session.

Duration of the study:

The study was conducted for 3 months so that the maximum number of sessions was 36 sessions (patients comes 3 times/ week). However, patients showing complete clearance (complete disappearance of all lesions with residual hypo- or hyperpigmentation) before 3 months were biopsied and assessed for TLR 7 and 9 by RT-PCR.

Detection of TLR7 and TLR9 gene expression by Polymerase Chain Reaction (PCR):

Detection of TLR7 and TLR9 by semiquantitative reverse transcriptase- polymerase chain reaction (RT-PCR).
For the detection of TLR7 and TLR9, RNA was extracted, reversely transcribed into cDNA, and amplified by PCR.

Detection of TLR7&TLR9 Gene Expression Level by Real-Time PCR

Total RNA Extraction.

Total RNA was isolated from skin tissue homogenates using RNeasy Purification Reagent (Qiagen, Valencia, CA) according to manufacturers instruction . The RNA sample was dissolved in RNase-free water and. RNA quantity was characterized using a UV spectrophotometer (Beckman, USA), the isolated RNA has an A 260/280 ratio of 1.9–2.1. The integrity of the RNA was studied by gel electrophoresis on a 1% agarose gel, containing ethidium bromide.

cDNA Synthesis

First-strand cDNA was synthesized from 1 μg of total RNA by reverse transcription with a superscript first-strand synthesis system kit (Life Technologies, Breda, the Netherlands) according to according to manufacturers instruction.

Real-time quantitative polymerase chain reaction (PCR)
The sequences of the PCR primers for TLR7&TLR9 and the housekeeping gene glyceraldehydes-3- phosphate dehydrogenase (GAPDH) are listed in Table I. PCR was carried out in a reaction mixture containing iQTM SYBR Green Supermix (Bio Rad Laboratories, CA, USA) and cDNA template. The PCR was performed in an Step one plus Real-Time PCR system (AppliedBiosystems) using the following cycle parameters of 95°C for 10 min (1 cycle), 94°C for 15s, and 60°C for 1min (40 cycles). Data were analyzed with system software and quantified using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the GAPDH genes (Tabl. II).

Number of patients	Toll-9		Toll-7	
	Before	After	Before	After
1	1.3	0.9	1.74	1.06
2	1.06	0.92	0.81	0.4
3	1.04	0.68	0.99	0.27
4	2.7	1.04	1.9	0.6
5	1.9	0.73	1.6	0.43
6	2.01	1.2	1.4	0.66
7	1.12	0.96	0.88	0.28
8	1.8	0.42	1.2	0.35
9	2.03	1.5	1.4	0.47
10	1.2	0.46	0.73	0.51
11	2.6	1.02	1.9	1.04
12	2.8	0.8	2.06	1.5
13	1.2	0.4	1.6	0.8
14	2.7	0.6	2.01	1.6
15	2.4	0.49	2.06	0.59

Table I. Levels of both Toll-9 and Toll-7 in patients before and after NB-UVB sessions.

Primer	Sequence
TLR7	Forward primer : 5′- AAACTCCTTGGGGC-TAGATG -3′Reverse primer:5′- AGGGT-GAGGTTCGTGGTGTT -3according to gene bank accession numberNM_016562.3
TLR9	Forward primer : 5'- CGCCCTGCACC-CGCTGTCTCT -3'-Reverse primer:5'-C G G G G T G C T G C C A T G G A G A A G -3'according to gene bank accession num-berNM_017442.3
GAPDH	Forward 5′ GGATTTGGTCGTATTGGG 3′Reverse 5′ GGAAGATGGTGATGGGATT 3' according to gene bank accession number DQ403057.1

Table II. Primer sequences used for RT-PCR.

Statistical Methods
Data were statistically described in terms of range, means ± standard deviation (± SD), median, frequencies (number of cases) and percentages when appropriate. Comparison of TLR 9 and TLR 7 pre and post between cases and controls was done using Mann Whitney U test for independent samples. Comparison between pre and post treatment values was done using Wilcoxon signed rank test for paired (matched) samples. A probability value (p value) less than 0.05 was considered statistically significant.Correlation between various variables was done using Pearson correlation equation for linear relation. All statistical calculations were done using computer programs Microsoft Excel version 7 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the social science; SPSS; Inc., Chicago, IL, USA) statistical program.

Results
Clinical data
The current study included 15 patients with psoriasis vulgaris and 15 healthy individuals serving as a control group. The patient group included 8 females (53.33%) and 7 males (46.67%). Their ages ranged from 14 to 63 years with a mean of 35.13 ± 16.10 years. The duration of the disease ranged from 1 to 35 years with a mean of 8.3 ± 8.5 years. The mean value of PASI score was15.60 + 7.31 before therapy which was reduced significantly after therapy to be 7.5 + 3.5.

TLRs values before therapy (Fig. 1)
The mean value of Toll-like receptor-9 in patients before treatment with NB-UVB was (1.85 + 0.66 µg/dl) which was higher than that of controls (0.23 + 0.005 µg/dl), with a significant P value (P value 0.001).
On the other hand, the mean value of Toll-like receptor-7 in patients before treatment with NB-UVB was (1.48+0.47 µg/dl) and when compared to that of controls (0.13+ 0.002 µg/dl), it also showed higher levels with a significant P value (P value 0.001).

After NB-UVB sessions
On comparing the mean value of TLR-9 after treatment with NB-UVB (0.80 ± 0.31) and that of controls (0.23 ± 0.005), still higher levels were detected in the patients with a significant P value (0.002).

In addition on comparing the mean value of TLR-7 after treatment with NB-UVB (0.70 ± 0.41) and that of controls (0.13 ± 0.002), still higher levels in the patients were detected with a significant P value (0.001). There was a significant decrease of the mean levels of both TLR-9 and 7 after therapy when compared to their levels before therapy (P value 0.001) (Tabl. I).

No significant correlation was detected between levels of both TLR 7 & TLR9 (before and after therapy) and any of the clinical data of the patients (age, sex, extent, severity and duration).

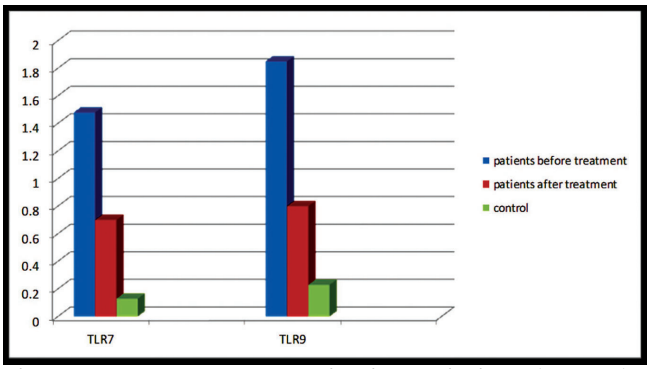

Figure 1. PPARγ gene expression in psoriasis and control.

Discussion

The current study showed significant higher levels of both TLRs 9 and 7 genes in psoriasis vulgaris patients compared to healthy individuals. Besides a significant reduction of their levels was reported after NB-UVB therapy of the same patients. Psoriasis is considered to be a genetically determined disease of dysregulated inflammation, which is driven and maintained by multiple components of the immune system. The pathologic collaboration between innate immunity (mediated by antigen-presenting cells and natural killer T lymphocytes) and acquired immunity (mediated by T lymphocytes) results in the production of cytokines, chemokines, and growth factors that contribute to the inflammatory infiltrate seen in psoriatic plaques [9].

Toll-like receptors are a recently identified group of pattern recognition receptors (PRRs) that recognize distinct conserved microbial components and permit cells to recognize self from non-self in immune activation [1]. There is a growing interest in the role of innate and adaptive immunity in inflammatory diseases such as psoriasis [10]. It is conceivable that certain microorganisms could induce or exacerbate psoriasis through activation of keratinocyte TLRs (innate immunity), leading to the secretion of cytokines which activate the acquired immunity by effects on T cells, antigen presenting cells and endothelial cells [11].

Another study [12], demonstrated that human keratinocytes constitutively express mRNA for TLR1, 2, 3, 4, 5, 6, 9, and 10, but not for TLR7 or 8, confirming previously published studies on the expression of TLR1, 2, 3, 4, 5, and 9 in keratinocytes [13-15]. However, both TLR7 and 8 expression was proved to be achieved by plasmacytoid DCs and monocyte-derived DC in the same study [12].

In addition, others demonstrated that plasmacytoid pre-DCs and B cell express high levels of TLR 7 and 9 [5,6]. PDCs are an important cell population in psoriatic skin lesions, because they constitute 16% of the total dermal infiltrate. Whereas they are nonexistent in the normal skin [4].

The aim of the present study was to find out the effect of NB-UVB on the expression of TLR9 and TLR7 in psoriatic skin, so that we may add another possible mechanism of action by which NB-UVB improves psoriatic lesions.

Our results showed that the mean values of both TLR9 and TLR7 were significantly higher in patients before therapy compared with controls.

Some investigators studied the expression of TLR9 alone in psoriatic patients and found also similar results as Miller et al. [16] who demonstrated an increased expression of TLR9 throughout the epidermis in psoriatic patients. Based on the immunoperoxidase labeling for TLR9, they concluded that the level of expression of TLR9 may depend upon the differentiation stage of the keratinocytes as they mature from the basal layer in the epidermis. They reported that the growth and differentiation factor, TGF-α, which is important during wound healing and is found at increased levels in psoriasis, regulates the expression and function of TLR9 on human keratinocytes.

Moreover, others [3] demonstrated that, keratinocytes from psoriatic plaques express high levels of TLRs 1, 2, 4, 5, and 9, compared with normal skin.

The results of the present study differ from others [17,18] who reported the low expression of TLR9 in psoriatic lesions compared to normal skin.

As far as we know, no previous studies investigated the expression of TLR7 in psoriatic lesions. However some studies [3,4] observed aggravation and spreading of a psoriatic plaque when treated topically with the toll-like receptor (TLR) 7 agonist imiquimod. The exacerbation of psoriasis was accompanied by a massive induction of lesional type I interferon activity.

Furthermore, they found large numbers of PDCs infiltrated psoriatic skin as well, which play as a target for the TLR7 agonist (imiquimod) and hence producing interferon I that augments more the psoriatic lesionss. Those findings support as well the results of the present study verifying the presence of high levels of TLR7 in lesional skin compared to the control.

The use of PCR technique in the current study, did not enable us to detect the exact source of producing such receptors.

It only showed us the upregulation of TLRs 7 and 9 genes in psoriatic skin.However previous studies confirmed the production of both receptors by pDCs and B cells [5,6]. In addition keratinocytes express as well TLR-9 as verified by others [3].

Regarding the possible role played by the abnormal expression of TLRs-7,9 several studies stated that the inappropriate recognition of self-nucleic acids (self-DNA and ssRNA) by both receptors, pushed them to bind to the antimicrobial peptide LL-37 to form aggregated and condensed structures (DNA/LL-37 and RNA/LL-37) which are delivered to and retained within early endocytic compartments in pDCs leading to pDC activation [19,20], and trigger type I IFN production. Type I IFNs produced by pDCs support myeloid dendritic cell maturation and eventual autoreactive T cell activation leading to psoriatic skin lesions [21].

Owing to our findings and previous data results (mentioned above) we suggest that TLRs 7 and 9 have an important impact on the pathogenesis of psoriasis and possibly explaining the role of autoimmunity that is probably involved to induce the psoriatic lesions.

NB-UVB phototherapy is an effective treatment for psoriasis. Owing to its limited penetration, the direct effect of UVB is mostly restricted to cells residing in the epidermis and papillary dermis, and is associated with depletion of epidermal LCs and T cells [22]. To the best of our knowledge no pervious studies investigated the effect of nb-UVB on the expression of TLR7 and TLR9.

In the current study we compared the mean value of both TLR9 and TLR7 in patients before and after phototherapy. Both mean values of TLR9 and TLR7 after therapy were still higher than controls yet they showed significant reduction following thirty six sessions of nb-UVB phototherapy.

One study [23] reported that UVB radiation induces the up-regulation and secretion of endogenous Toll-like receptor ligands such as Heat Shock Proteins (HSPs) from the UV-exposed keratinocytes. These secreted stress signals are transmitted via Toll-like receptors 2 and 4 in an autocrine-paracrine manner, activating the Toll-IL-1 receptor signaling cascade and the subsequent elaboration of IL-10 and TNF-α. Given that regulatory T cells appear to express abundant Toll-like receptors on their surfaces, some of the UV-induced ligands may directly activate these T-cell subtypes to induce IL-10, thereby further augmenting the immunosuppressive cytokine milieu.

Hence we can deduce that the downregulation of both TLRs 7 and 9, following UVB therapy, could be a secondary effect to the this immunosuppressive state induced by the UV-induced ligands possibly by downregulating pDCs, the main producers of both receptors. Such an event leads to suppression of autoreactive T cells and hence the reduction of cytokines needed for psoriatic lesion formation. Keratinocyte proliferation thus, is markedly reduced and hence again more downregulation of all TLRs (especially TLR9) produced by the keratinocytes. Although this current study showed no significant correlation between the levels of both receptors and the clinical severity of the lesions,yet the limited number of the patients may be possibly the cause. Thus further studies with larger scales are highly recommended to elaborate more the exact played role of TLRs 7 and 9 in the aetiopathogenesis of psoriasis.

In conclusion, TLRs 7 and 9 play an important role within the sequence of events taking place in psoriasis vulgaris. Downregulation of those receptors induced by nb-UVB ,may possibly add to the therapeutic mechanisms of nb-UVB in psoriatic patients. TLR 7 and 9 antagonists could be new future therapeutic tools for psoriasis and need further studies to elaborate their possible benefit for treating such a chronic disease.

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