Expression of Toll-Like Receptor-2, Human β -Defensin-2 and Interleukin-8 in Inflammatory Acne Vulgaris

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ABSTRACT

Background: The etiologic factors of acne are multifactorial, including ductal epidermal hyper-proliferation, excess sebum production and the presence of propionobacterium acnes (*P*. acnes). Immune response to *P*. acnes include humoral and cell- mediated immunity as well as complement activation. The burgeoning knowledge of Toll like receptors (TLR) and its effect on innate immunity are modifying our concepts regarding comedogenesis and inflammation. Much remains to be learned about the pathogenesis of acne regarding the role of *P*. acnes and TLR.

Objective: The aim of this work was to study the expression of TLR2, Human β-Defensin-2 (HβD2) and Interleukin-8 **(**IL-8) in acne vulgaris patients compared to non lesional skin in order to evaluate their role in the pathogenesis of inflammatory acne.

Patients and methods: This study was conducted on 10 patients with inflammatory acne lesions. Two biopsies (from lesional and non lesional skin) were taken from each patient. Reverse transcription-polymerase chain reaction (RT-PCR) was conducted for the skin biopsies to detect the expression of the m-RNA of TLR-2, HβD-2 and IL-8.

Results: The expression of the m-RNA of TLR-2, HβD-2 and IL-8 was statistically significantly higher in lesional skin biopsies compared with that in non lesional skin. A strong positive correlation was detected between TLR-2 and both HβD-2 and IL-8 in lesional skin biopsies.

Conclusions: TLR-2, HβD-2 and IL-8 may have a role in the pathogenesis of inflammatory acne vulgaris. In acne lesions, keratinocytes express HβD-2 and IL-8 in response to *P. acne* and this is dependent on TLR-2. So, TLR-2 could be a target of therapeutic intervention to block the inflammatory cytokine response in acne patients.

INTRODUCTION

Acne vulgaris is a common disorder that can have a significant effect on patients' physical and psychological well-being. The etiologic factors of acne are multifactorial, including ductal epidermal hyper-proliferation, excess sebum production, and the presence of *Propionobacterium acnes* (*P*. acnes). *P. acnes is* a part of the normal skin flora that can be significantly increased in the pilosebaceous units of patients with acne [(1,2)].

P. acnes contributes to the inflammatory nature of acne by inducing monocytes to secrete pro-inflammatory cytokines including TNF-α, IL-1β and IL-8 which act as a chemotactic factor for neutrophils [(3)]. In addition, *P. acnes* release lipases, proteases, and hyaluronidases which contribute to tissue injury. For these reasons, *P. acnes* have been a major target of therapy in inflammatory acne [(4)].

The mechanism by which *P. acne* activates monocyte cytokine release is unknown but is thought to involve pattern recognition receptors (PRRs) of the innate immune system. Toll-like receptors (TLRs) are one example of PRR [(3)]. TLRs recognize specific pathogen-associated molecular patterns (PAMPs) that are associated with a variety of organisms including bacteria, viruses and fungi, resulting in initiation of innate immune responses and influencing subsequent adaptive immune responses [(5)]. Keratinocytes and sebocytes, as major components of the pilosebaceous unit, may act as immune cells and may be activated by *P. acnes* via TLR and its co-receptor CD14 [(6)].

TLRs are transmembrane proteins with the extracellular portion composed of leucine-rich repeats, whereas the intracellular portion shares homology with the cytoplasmic domain of the IL-1 receptor [(7)]. The microbial ligands for many of these receptors have been identified. The ligands include molecules uniquely found in microbes such as bacterial cell wall components. More specifically, TLR4 mediates host responses to bacterial lipopolysaccharide (LPS) from Gram-negative bacteria, whereas TLR2 mediates responses to peptidoglycan from Gram-positive bacteria [(8,9)]. When TLRs are activated by ligand exposure, the intracellular domain of the TLR may trigger a myeloid differentiation protein-88 (Myd88)-dependent pathway that ultimately leads to the nuclear translocation of the transcription factor, nuclear factor kappa B (NF-kB), which then acts to modulate the expression of many immune response genes [(7)].

The human skin expresses a number of proteins that play a part in host defense [(10)].

Mammalian defensins are a family of cationic antimicrobial peptides, 28-42 amino acids long, containing three disulfide bonds. They have been divided into two subtypes, the α-defensins and the β-defensins [(11)]. The α-defensins are found in neutrophil granules or in the paneth cells of the small intestine [(12)]. To date, three human β-defensins (HβD) are shown to play a role in the defense of the skin, namely human β-defensin-1, -2 and -3 (HβD1, HβD2 and HβD3). These defensins are expressed either constitutively or up-regulated in response to microbial or pro-inflammatory stimuli [(10)]. Besides destroying various microorganisms, HβD2 contributes to the regulation of host-adaptive immunity as it is chemotactic for immature dendretic cells, induces the migration of CD45RO memory T cells [(14)], and also acts as a chemo-attractant for neutrophils [(14)]. Bacterial lipopeptide (BLP)-activation of TLR2 on human and murine monocytes results in NF-κB activation and confers anti-mycobacterial activity through nitric oxide (NO)-dependent and -independent pathways [(15)]. In addition, activation with TLR ligands has been associated with the induction of NF-κB and the antimicrobial peptide human β-defensin-2 (HβD2) [(16)]. However, a direct connection between mammalian TLR expression, TLR activation and antimicrobial peptide production has not been established [(17)].

This work aimed at studying the expression of TLR2, HβD2 and IL-8 in inflammatory acne lesions compared to non lesional skin in order to understand their role in the pathogenesis of inflammatory acne.

PATIENTS AND METHODS

Ten patients with inflammatory acne vulgaris including 6 males (60%) and 4 females (40%) were enrolled in this study. Their ages ranged from 17 to 33 years with a mean age of 22 years. They were collected from the outpatient clinic of Dermatology and Venereology Department, Tanta University Hospitals. Patients taking systemic medications (up to 4 weeks) or topical therapy (up to 2 weeks) before biopsy were excluded. Those who have been treated with systemic isotretinoin within 6 months before biopsy were also excluded.

After taking consents from all the patients, a five mm punch biopsy of the skin was taken from two sites on the back of each patient; one from the site of an inflammatory acne papule and the other from a region of clinically normal skin of the same patient to serve as control.

All skin biopsies were immediately flash frozen in liquid nitrogen and stored at –70° for subsequent RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) for mRNA of TLR2, HβD2 and IL-8.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR): Skin samples were subjected for RNA isolation using Gentra's pure script RNA isolation kits (Gentra systems, Avenue North, Minneopolis, Mx 55441, USA). Template RNA was reversely transcribed using Qiagen One Step RT-PCR Kit Technology, USA, in a thermal cycler (Cyclogen-Dri-Block Cycler Cambridge). Reverse transcription was done at 42° for 45 min, followed by an initial activation PCR step at 95° for 15 min. All primers used for TLR2 , HβD2 , IL-8 and house-keeping gene β-actin (internal control), amplification conditions in this study as well as the size of the products are listed in Table (1) [(18)].

Semi-quantitative analysis of gene expression:

Following PCR, 10-μL aliquot of each PCR product amplicon was analyzed by gel electrophoresis on 2% Agarose gel containing ethidium bromide and the bands were examined under ultraviolet light for the presence of amplified DNA. To analyze semi-quantitatively the results of RT-PCR, we scanned the gel images and measured the intensity of the PCR product through using Gel Analyzing Imager (Sharp-JX). This relies on the quantification of the amplification products on the basis of optical density of detected bands. We then determined the relative intensity (density ratio) of each of the investigated genes to that of β-actin. The relative amount of gene expression was calculated as follow:

Relative amount of gene expression =
Copy number of the target gene / Copy number of the control gene[(18)].

Statistical analysis:

The collected data were organized, tabulated and statistically analyzed using Statistical Package for the Social Sciences (SPSS for Windows version 12, SPSS Inc.). For each studied variable, the range, the mean and standard deviation were calculated. The differences of mean values between groups were statistically analyzed using Mann-Whitney test. Pearson's correlation coefficient (r) was calculated to test the correlation between TLR2 and each dependent variable. Significance was considered at $p<0.05$.

RESULTS

In the present study, using semi-quantitative RT-PCR; the expression of TLR-2 mRNA showed a statistically significant increase in inflammatory acne lesions (mean ± SD = 0.753 ± 0.39) compared to uninvolved skin (mean ± SD = 0.263 ± 0.286), $P<0.001$* (Table 2). The expression of HβD-2 mRNA showed a statistically significant increase in inflammatory acne lesions (mean ± SD = 0.864 ± 0.41) compared to uninvolved skin (mean ± SD = 0.149 ± 0.096),

P<0.001* (Table2). The expression of IL-8 mRNA was found in inflammatory acne lesions (mean ± SD = 1.043 ± 0.29) compared to uninvolved skin (mean ± SD = 0.440 ± 0.270). The difference was statistically significant, *P*<0.001* (Table 2 and Figure 1).

There were statistically significant positive correlations between the expression of TLR-2 mRNA and both HβD-2 mRNA and IL-8 mRNA in lesional skin of inflammatory acne patients (*P*=0. 001*) for each (Figure 2).

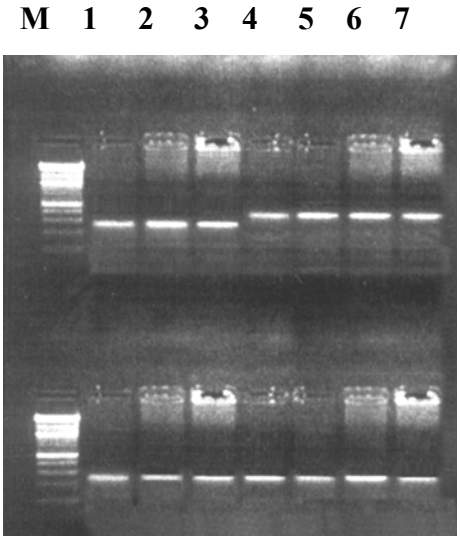

Figure (1): Agarose gel electrophoresis of PCR products stained with ethedium bromide for detection of TLR2 mRNA, HβD2 mRNA and IL-8 mRNA in lesional skin. Lanes M in both Panels A & B represent DNA marker. Lanes 4-7 in panel A represent TLR2 mRNA (347bp). Lanes 1-7 in panel B represent HβD-2 mRNA (254bp). Lanes 1-3 in panel A represent IL-8 mRNA (297 bp)

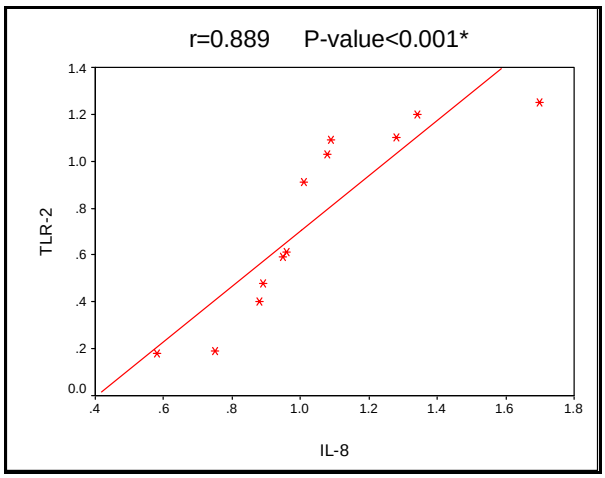

Fig2a

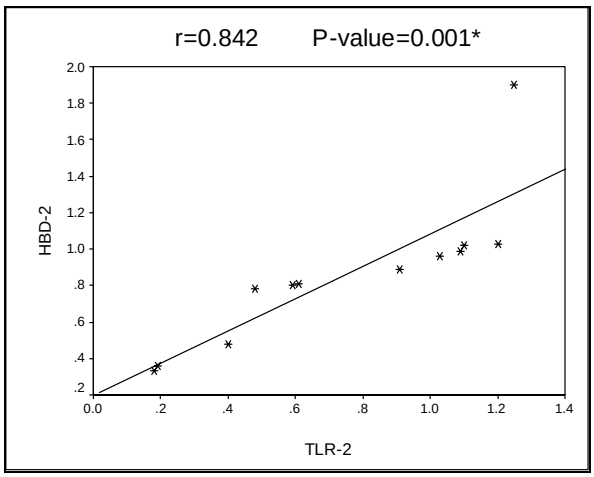

Fig2b

Figure (2): The correlation between m-RNA expression of TLR-2 and IL-8 (Fig 2a) and TLR-2 and HβD2 (Fig.2b) in inflammatory acne lesions.

Table (1): Primers used for TLR2, HβD2, IL-8 and house-keeping gene β-actin (internal control), amplification conditions and the size of the products.

Gene	Forward primer	Reverse primer	Conditions
TLR2 (347bp)	5'GCCAAAGTCTTGAT TGATTGG-3'	5'TTGAAGTTCTCCAGCTC CTG-3'	32 cycles at ; 95° for 45 sec., 54° for 45sec., 72 ° for 1 min.
HβD2 (254bp)	5'CCAGCCATCAGCCA TGAGGGT-3'	5'-GGA GCCCTTT CTGA ATCCGCA-3'	36 cycles at; 94° for 1 min, 63°for 1 min, 72 ° for 1 min .
IL-8 (297bp)	5'ATGACTTCCAAGCT GGCCGTG-3'	5'TGAATTCTCAGCCCTCT TCAAAAACTTCTC-3'	33 cycles at; 94° for 1 min, 55° for 1 min, 72° for 1 min.
β-actin(661bp)	5'-TGACGGGGTCA CCCACTGTGCCCATC TA-3'	5'-CTAGAA GCATTGC GGTGGACGA TGGAGGG-3'	35 cycles at 92° for 45 sec., 60° for 45 sec., 72° for 90 sec.

Table (2): The Expression of TLR-2mRNA, HβD-2mRNA and IL-8 mRNA in inflammatory acne lesions compared to uninvolved skin

	Lesional	Non-lesional	Mann-Whitney Test	
	Mean ± SD	Mean ± SD	z	*P*-value
TLR-2	0.753 ± 0.390	0.263 ± 0.286	2.700	<0.001*
HβD-2	0.863 ± 0.410	0.149 ± 0.096	3.705	<0.001*
IL-8	1.043 ± 0.293	0.440 ± 0.270	3.359	<0.001*

*Highly significant (*P*<0.001)

DISCUSSION

Recognition of microbial pathogens by the cells of the immune system triggers host defense mechanisms to combat infection and prevent diseases. However, activation of these pathways can also result in inflammation at the site of disease and subsequent tissue injury. In acne, the host response to P. *acnes* can result in the production of pro-inflammatory cytokines and contribute to the clinical manifestation of the disease [19]. Thus, this work aimed at studying the expression of mRNA of TLR2, HβD2 and IL-8 in inflammatory acne lesions compared to non lesional skin in a trial to understand their role in the pathogenesis of inflammatory acne.

In the current study, TLR2 mRNA expression was detected in low concentration in non lesional skin biopsies. This is in agreement with the findings of Pivarcsi et al., 2003 [20] who demonstrated TLR2 and TLR4 mRNA protein expression in cultured normal human epidermal keratinocytes (KCs). The expression of TLR at sites of host-pathogen interaction likely serves to protect the host from pathogens although unnecessary immune response to commensal bacteria may harm the host Dendretic cells (DCs) [21]. However, commensal bacteria do not continuously trigger inflammation in skin which is supposed to be due to diminution of TLR expression on Langerhans cells (LCs) found in the skin as compared with DCs [22].

In this study, a statistically significant up-regulation of TLR mRNA expression was found in inflammatory acne lesions compared to non lesional skin. This is in concordance with the results of many authors [19, 21, 23]. Kim et al., 2002[19] also demonstrated increased immuno-histochemical expression of TLRs in follicular region of acne skin lesions. Jugeau et al., 2005 [24] studied the effect of *P. acnes* on TLR activation in KCs. They found that, in vivo expression of TLR-2 and TLR-4 was increased in the epidermis of acne lesions, while in vitro, an increase in both TLR2 and TLR4 expression by human keratinocytes occurred in the first hours of incubation with bacterial fractions, as well as an increase in the expression and secretion by the KCs of matrix metalloproteinase-9 (MMP-9), which has a role in the inflammatory process. The authors concluded that the *P. acnes* induction of TLR expression may be vital in the inflammation observed in acne. These data may suggest a mechanism by which *P. acnes* induces

inflammation in acne vulgaris by activation of TLRs and subsequent release of cytokines which regulate the local immune response. Vega-Diaz et al., 2002 explained that when TLRs are activated by cell-wall components of Gram-positive bacteria, such as *P. acnes*, or by lipopolysaccharides of Gram-negative bacteria, they begin intracellular signaling of transcription factors for various pro-inflammatory mediators such as TNF-α, IL-1β and IL-8 [25].

This study demonstrated a weak expression of HβD2 mRNA in non-lesional skin. This finding is consistent with the concept that the skin is highly exposed to microbial invasion as well as the physiological skin microflora and HβD2 may play a role in protecting the pilosebaceous units from microbial invasion [26]. On the other hand, Liu et al., 2003 [27] found that HβD2 is virtually absent in normal human keratinocytes (KCs). They suggested that, the expression of HβD2 peptide by human keratinocytes (KCs) requires differentiation of the cells by growth and maturation as well as cytokine or bacterial stimulus.

In the current work, there was a statistically significant increase in the expression of HβD2 mRNA in inflammatory acne lesions compared to non lesional skin. This is consistent with the findings of many other studies [23, 26, 28-30]. It was suggested that this up-regulation of HβD2 is most probably a secondary response to the marked perilesional infiltrate and that pro-inflammatory cytokines (such as IL-1β and TNFα) and bacterial lipopolysaccharides may be responsible for this up-regulation [26]. In addition, keratinocytes might be also an important source of HβD2 in acne [23].

Human HβD2 exhibits a potent antimicrobial activity against Gram-negative bacteria and *Candida albicans*, and only a bacteriostatic activity against *Staphylococcus aureus* [18, 31]. Besides destroying various microorganisms, it contributes to regulation of host-adaptive immunity as it acts as a chemotactic for immature DCs, inducing migration of CD45RO memory T cells [13] and also acts as a chemo-attractant for neutrophils through chemokine receptor 6 (CCR6) [14,28]. On the other hand, Nagy et al., 2005 [23] in their study on different strains of *P. acnes* they found that HβD2 doesn't have any bacteriostatic or bactericidal effect against different *P. acnes* strains and they concluded that the primary contribution of HβD2 to acne pathogenesis is the regulation of adaptive immunity.

It was also suggested that up-regulation of HβD2 by *P. acnes* is strain specific. There are two reasonable explanations for this. First, keratinocytes recognize a number of *P. acnes* antigens and effective HβD2 secretion occurs only if several antigens are concurrently identified and all of the involved receptors are functional. Second, acne is a genetic disease and since HβD2 gene is polymorphic in copy number, the HβD2 mRNA level in individuals with low genomic copy number remains below detectable levels in response to *P. acnes* [23]. Also, an increase in HβD2 mRNA expression has been detected in other inflammatory conditions such as psoriasis and mastitis [32,33] and also found in abundance in lesions of superficial folliculitis, a common skin disease characterized by inflammation of hair follicles [34]. Selleri et al., 2007 [35] found that the hair follicle is equipped with TLR2, TLR4 and TLR5, and that these receptors are able to respond to microbial stimuli inducing the production of HβD2 by epithelial cells. This immune response might be important in protecting the skin from microorganism infections.

In this work, a statistically significant increase in IL-8 mRNA expression was detected in inflammatory acne lesions compared to non-lesional skin. This was in agreement with the findings of many authors [19, 21, 23], they suggested that the activation of TLR2 on monocytes release pro-inflammatory cytokines, IL-12 and IL-8. Shimada et al., 1998 [36] explained that to occur via translocation of the transcription factor nuclear factor kappa B (NF-κB) with subsequent induction of the pro-inflammatory cytokines at the mRNA and protein level.

It was hypothesized that *P. acnes* induced IL-8 secretion could have a key role in initiation of inflammatory events in acne by attracting neutrophils to the site of active lesions. The enzymes released by neutrophils lead to rupture of follicular epithelium and inflammation [23]. Moreover, the ductal hyperproliferation of keratinocytes in acne could also be associated with elevated levels of IL-8 proteins as it has a mitogenic effect on keratinocytes through their IL-8 receptors [37]. An induction of IL-8 gene expression of keratinocytes was also detected by Pivarcsi et al., 2003 [20] in response to Mycobacterium tuberculosis and this induction was also dependent on TLR2 and TLR4.

The burgeoning knowledge of Toll like receptors (TLR) and its effect on innate immunity are modifying our concepts regarding comedogenesis and inflammation[1]. It was demonstrated that some agents that cause down-regulation of TLR2 expression on monocytes such as retinoids e.g. all-trans retinoic acid, adapalene and tretinoin are used with great success in treatment of acne vulgaris [25, 38]. Not only many current acne treatments at least partially have their therapeutic effect via TLR, but novel therapies may be developed to take advantage of the inflammatory events modulated by TLR like certain lipid A analogues which have been

reported to antagonize TLR2 and TLR4 activation through interaction with shared receptor components [38].

In conclusion, TLR-2, HβD2 and IL-8 have a role in the pathogenesis of acne. In inflammatory acne lesions, keratinocytes express HβD2 and IL-8 in response to *P. acnes.* The inflammatory signaling is TLR-2 dependent. So, TLR-2 could be a target of therapeutic interventions to block inflammatory cytokine response in acne patients.

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تعبير مستقبلة شبيهة التول-٢، بيتا ديفينسين-٢ الآدمي و انترلوكين-٨ في حب الشباب الملتهب

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المقدمة :العوامل المسببة لحب الشباب عديدة و تشمل زيادة التكاثر البشروي بقناة الغدة الزهمية، زيادة انتاج الزهم بالأضافة الى وجود بروبيونوبكتيريا حب الشباب و الأستجابة المناعية لهذة البكتيريا اما أن تكون بالأجسام المضادة أو مناعة خلوية بالأضافة الى فاعلية المتمم.المعلومات المتاحة عن مستقبلة شبيهة التول و تأثيرها على المناعة الفطرية تطور المفاهيم بالنسبة الى سبب الزؤان و الألتهاب و لكن لا زال هناك الكثيرالذي يجب معرفتة عن منشأ حب الشباب و دور البكتيريا البروبيونية و مستقبلات شبيهة التول في ذلك.

الهدف من البحث: دراسة تعبير مستقبلة شبيهة التول-٢،بيتا ديفينسين-٢ الآدمي و انترلوكين-٨ في مرضى حب الشباب الملتهب و مقارنة ذلك بتعبيرهم في المناطق السليمة من الجلد لتقييم دورهم في منشأ حب الشباب الملتهب .

المرضى و طرق البحث:أجريت هذة الدراسة على عشرة من مرضى حب الشباب الملتهب و تم أخذ عينتين من جلد كل مريض (احداهما من منطقة مصابة و الأخرى من منطقة سليمة) .تم عمل تقنية تفاعل البلمرة المتسلسل ذي المستنسخ العكسي لكل العينات لأكتشاف تعبير الحامض النووي الريبوزي الرسول لكل من مستقبلة شبيهة التول-٢،بيتا ديفينسين-٢ الآدمي و انترلوكين-٨.

النتائج: كان تعبير مستقبلة شبيهة التول-٢،بيتا ديفينسين-٢ الآدمي و انترلوكين-٨ أعلى في عينا ت المناطق المصابة عنة في عينات المناطق السليمة بنسبة احصائية يعتد بها كما وجدت علاقة ايجابيه قوية بين مستقبلة شبيهة التول- ٢ و كل من بيتا ديفينسين-٢ الآدمي و انترلوكين-٨ في عينات الأماكن المصابة.

المستخلص: مستقبلة شبيهة التول-٢،بيتا ديفينسين-٢ الآدمي و انترلوكين-٨ لهم دور في منشأ حب الشباب الملتهب ففي المناطق المصابة تقوم الخلايا التقرنية بتعبير بيتا ديفينسين-٢ الآدمي و انترلوكين-٨ بالأستجابة الى بروبيونوبكتيريا حب الشباب و هذا يعتمد على مستقبلة شبيهة التول-٢ لذلك فان مستقبلة شبيهة التول-٢ يمكن أن تكون هدفا للتدخل العلاجي لحصر الأستجابة الخلوية الألتهابية في حب الشباب.