Gene Array Expression Profiling in Acne Lesions Reveals Marked Upregulation of Genes Involved in Inflammation and Matrix Remodeling

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The pathogenesis of acne has been linked to multiple factors such as increased sebum production, inflammation, follicular hyperkeratinization, and the action of *Propionibacterium acnes* within the follicle. In an attempt to understand the specific genes involved in inflammatory acne, we performed gene expression profiling in acne patients. Skin biopsies were obtained from an inflammatory papule and from normal skin in six patients with acne. Biopsies were also taken from normal skin of six subjects without acne. Gene array expression profiling was conducted using Affymetrix HG-U133A 2.0 arrays comparing lesional to nonlesional skin in acne patients and comparing nonlesional skin from acne patients to skin from normal subjects. Within the acne patients, 211 genes are upregulated in lesional skin compared to nonlesional skin. A significant proportion of these genes are involved in pathways that regulate inflammation and extracellular matrix remodeling, and they include *matrix metalloproteinases 1* and *3*, *IL-8*, human *β-defensin 4*, and *granzyme B*. These data indicate a prominent role of matrix metalloproteinases, inflammatory cytokines, and antimicrobial peptides in acne lesions. These studies are the first describing the comprehensive changes in gene expression in inflammatory acne lesions and are valuable in identifying potential therapeutic targets in inflammatory acne.

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INTRODUCTION

Acne is the most common skin condition affecting millions of people worldwide. The pathogenesis of acne vulgaris is complex and incompletely understood. Inflammation is a key component of the pathogenesis of acne (Webster, 1995; Harper and Thiboutot, 2003; Farrar and Ingham, 2004). An immunological reaction to the Gram-positive microbe *Propionibacterium acnes* may play a major role in the initiation of the inflammatory reaction (De Young *et al.*, 1984; Jappe *et al.*, 2002). Recently published studies also implicate toll-like receptor 2 in inflammatory acne. *P. acnes* triggers proinflammatory cytokine release from inflammatory cells via activation of toll-like receptor 2, which in turn initiates an intracellular signaling cascade resulting in the transcription of genes such as *IL-12* and *IL-8* (Kim *et al.*, 2002). Furthermore, viable *P. acnes* and not heat-killed organisms can stimulate the release of cytokines such as IL-1β, GM-CSF, and IL-8 (Nagy *et al.*, 2005; Schaller *et al.*, 2005).

Although the initiating events causing acne still remain a mystery, there exists a debate as to whether hyperkeratinization of the follicular duct precedes the influx of inflammatory cells or *vice versa*. Recent studies support the latter hypothesis by demonstrating that an increase in *IL-1* activity occurs before the hyperproliferation around uninvolved follicles and this triggers the ''keratinocyte activation cycle''. (Freedberg *et al.*, 2001; Jeremy *et al.*, 2003).

The skin expresses various antimicrobial peptides in response to pathogens as part of cutaneous innate immunity (Schroder, 2004; Braff *et al.*, 2005; Selsted and Ouellette, 2005). These include members of the human β-defensin family and granulysin-derived peptides (Harder *et al.*, 2004; Deng *et al.*, 2005; McInturff *et al.*, 2005). Human β-defensin 1 and 2 (DEFB1 and DEFB2) are expressed in the pilosebaceous unit and their expression is upregulated in acne lesions (Chronnell *et al.*, 2001). Recent studies also note that select strains of *P. acnes* can activate *DEFB2* through toll-like receptors, further confirming the importance of these peptides in inflammatory acne (Nagy *et al.*, 2005).

Acne research during the last 25 years has significantly increased our understanding about the etiological factors giving rise to acne. With the advent of gene array expression profiling however, new opportunities have arisen to

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Abbreviations: DEFB1, DEFB2, DEFB3, DEFB4, human β-defensin 1, 2, 3, 4; MMP-1, MMP-3, matrix metalloproteinase 1, 3

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re-examine the disease and potentially identify novel targets in its treatment. The primary goal of this study is to identify the specific genes expressed in inflammatory acne lesions compared to normal skin from acne patients and to test the hypothesis that differences in gene expression exist between normal skin from acne patients and skin from subjects without acne that may account for the predisposition to the disease.

RESULTS

Expression of inflammatory mediators, antimicrobial peptides, and matrix metalloproteinases is increased in acne lesions compared to uninvolved skin

A distinct pattern of gene expression was seen from inflammatory acne lesions compared to uninvolved skin from the same patients. Analysis of the gene expression profiles revealed that 211 genes were upregulated at the site of the acne lesion, whereas 18 genes were downregulated (false discovery rate = 0.01) (see Table S1 for a complete list). A majority of the genes whose expression is increased in acne lesions are involved with the inflammatory processes, and these included a variety of chemokines, antimicrobial peptides, apoptosis-inducing proteins, and interstitial collagenases (Table 1 and Figure 1).

Genes with the greatest fold increases in expression in acne lesions included the matrix metalloproteinases *MMP-1* and *MMP-3*, which had 92- and 64-fold higher expression patterns, respectively. Other genes that were significantly upregulated included the proinflammatory cytokines *IL-8* (52-fold) and *CXCL-2* (16-fold). Significant increases in the expression of *chemokine receptor 1*, *IL-7 receptor*, *IL-13 receptor*, and *IL-1* family members *5* and *9* were also noted. These data highlight the prominent role of these cytokines in inflammatory acne, which is in agreement with other studies that demonstrate the role of *IL-8* in inducing the recruitment of chemotactic mediators at the site of acne lesions (Vowels *et al*., 1995).

The expression of several antimicrobial peptides was also significantly increased in acne lesions compared to normal skin from the same patients. These include DEFB4 and granulysin, which were upregulated by more than 33- and two-fold, respectively. Genes involved in apoptosis and immune pathways were also significantly upregulated in acne lesions. These included granzyme B, which is responsible for target cell lysis in cell-mediated immune responses, and GPR65, which is involved with the differentiation of T cells as well as their apoptosis. Granzyme B was upregulated more than 10-fold in acne lesions when compared to normal skin, whereas GPR65 was increased by approximately 2.7-fold.

Although very few genes were downregulated in acne lesions compared to normal skin, some important genes regulating key pathways were identified (Table 2). The frizzled related proteins, which function as part of the ''Wnt'' signaling pathway, were downregulated by approximately two-fold. Inhibition of ''Wnt'' signaling in the skin has been shown to influence stem cell fate in favor of development of sebocytes rather than hair (Merrill *et al*., 2001). Also downregulated in acne lesions were three genes of the secretoglobin family. *Secretoglobin family 1D member 2* and *secretoglobin family 2A member 1* are transcriptionally regulated by steroids and bind to androgens and other steroids. The function of *secretoglobin family 2A member 2* is unclear.

Gene expression profiles from biopsy samples of normal skin from six subjects without acne were compared to profiles obtained from normal skin (uninvolved sites) of six patients with acne. No significant changes in gene expression patterns were noted in this analysis (data not shown). A subset analysis of target genes involved in inflammation (*MMP-1*, *MMP-3*, *IL-8*, *DEFB4*, and *granzyme B*) was also performed in normal skin from normal subjects and uninvolved skin from acne patients. No statistically significant differences were identified between the two groups.

Cluster analysis

Hierarchical clustering of the entire set of genes (229) that were significantly upregulated or downregulated from our microarray data demonstrated that samples from inflammatory acne lesions clustered into a separate group from the normal skin from the same group of patients (Figure S1). Using the NetAffix analysis center, we identified 41 genes from a total of 211 genes upregulated in inflammatory acne to be involved in inflammation. Figure 1 represents a cluster diagram of the inflammatory genes and shows the unique clustering pattern of the samples into two groups, one corresponding to involved skin from acne lesion and another corresponding to normal skin from the same group of six patients. In the clustering diagram, each row represents a gene and each column represents a sample. Clustering of patient samples (columns) is indicated at the top of the diagram. Genes with higher correlation coefficients among the standardized gene expression values across samples are clustered together by rows (for complete clustering tree, refer to Figure S1). Therefore, genes in the same cluster should have more similar expression patterns, whereas genes in different clusters should have less similar expression patterns.

Quantitative PCR confirms gene array expression data of select genes

We selected five genes of interest based on their fold changes and involvement in inflammation from five acne subjects to validate the microarray findings using qPCR. These genes include, *MMP-1*, *MMP-3*, *IL-8*, *β-defensin 4*, and *granzyme B*. qPCR results were normalized to the internal control gene, *TATA binding protein* (*TBP*). A robust increase in mRNA expression was seen for all five genes tested, with the magnitude of the fold change greater than that observed with the microarray (Table 3).

Immunohistochemistry demonstrates tissue localization of select proteins in inflammatory acne lesions and normal skin

To further confirm the differences in tissue expression and distribution patterns of MMP-1 (interstitial collagenase), IL-8, and DEFB4, we performed immunohistochemistry on inflammatory acne lesions, uninvolved skin from acne subjects, and

Table 1. Genes significantly upregulated in acne lesions compared to normal skin

Probe set ID	Accession	Fold change	Gene title	Gene symbol
(A) Genes upregulated by more than 10-fold in acne lesions				
204475_at	NM_002421	92.166	*matrix metalloproteinase 1*	*MMP1*
205828_at	NM_002422	64.020	*matrix metalloproteinase 3*	*MMP3*
202859_x_at	NM_000584	52.521	*interleukin 8*	*IL8*
207356_at	NM_004942	33.300	*defensin, beta 4*	*DEFB4*
203691_at	NM_002638	19.354	*protease inhibitor 3, skin-derived (SKALP)*	*PI3*
41469_at	L10343	17.495	*protease inhibitor 3, skin-derived (SKALP)*	*PI3*
204470_at	NM_001511	16.152	*chemokine (C-X-C motif) ligand 2*	*CXCL2*
210873_x_at	U03891	15.921	*apolipoprotein B mRNA editing enzyme*	*APOBEC3A*
216841_s_at	X15132	11.529	*superoxide dismutase 2, mitochondrial*	*SOD2*
210164_at	J03189	10.630	*granzyme B*	*GZMB*
(B) Top 10 inflammatory genes upregulated in acne lesions				
204475_at	NM_002421	92.166	*matrix metalloproteinase 1*	*MMP1*
205828_at	NM_002422	64.020	*matrix metalloproteinase 3*	*MMP3*
202859_x_at	NM_000584	52.521	*interleukin 8*	*IL8*
207356_at	NM_004942	33.300	*defensin, beta 4*	*DEFB4*
202833_s_at	NM_000295	8.649	*serine proteinase inhibitor, clade A*	*SERPINA1*
204563_at	NM_000655	6.796	*selectin L (lymphocyte adhesion molecule 1)*	*SELL*
205098_at	AI421071	4.057	*chemokine (C-C motif) receptor 1*	*CCR1*
220322_at	NM_019618	3.343	*interleukin 1 family, member 9*	*IL1F9*
201645_at	NM_002160	3.022	*tenascin C*	*TNC*
203645_s_at	NM_004244	2.979	*CD163 antigen*	*CD163*
(C) Top apoptosis-associated genes upregulated in acne lesions				
210164_at	J03189	10.630	*granzyme B*	*GZMB*
214467_at	NM_003608	2.772	*G protein-coupled receptor 65*	*GPR65*
204860_s_at	AI817801	2.272	*baculoviral IAP repeat-containing 1*	*BIRC1*
201743_at	NM_000591	2.211	*CD14 antigen*	*CD14*
201739_at	NM_005627	1.663	*serum/glucocorticoid regulated kinase*	*SGK*
206545_at	NM_006139	1.658	*CD28 antigen (Tp44)*	*CD28*
219366_at	NM_020371	1.627	*apoptosis, caspase activation inhibitor*	*AVEN*
208315_x_at	NM_003300	1.393	*TNF receptor-associated factor 3*	*TRAF3*
(D) Top genes involved in lipid and steroid metabolism upregulated in acne lesions				
206561_s_at	NM_020299	8.389	*aldo-keto reductase family 1, member B10*	*AKR1B10*
203649_s_at	NM_000300	5.518	*phospholipase A2, group IIA (platelets, synovial fluid)*	*PLA2G2A*
215125_s_at	AV691323	2.804	*UDP glycosyltransferase 1 family, polypeptide A10*	*UGT1A10*
202430_s_at	NM_021105	2.959	*phospholipid scramblase 1*	*PLSCR1*
208607_s_at	NM_030754	2.659	*serum amyloid A2*	*SAA2*
202345_s_at	NM_001444	2.268	*fatty acid binding protein 5 (psoriasis-associated)*	*FABP5*
204446_s_at	NM_000698	2.128	*arachidonate 5-lipoxygenase*	*ALOX5*
203879_at	U86453	1.461	*phosphoinositide-3-kinase, catalytic, delta polypeptide*	*PIK3CD*

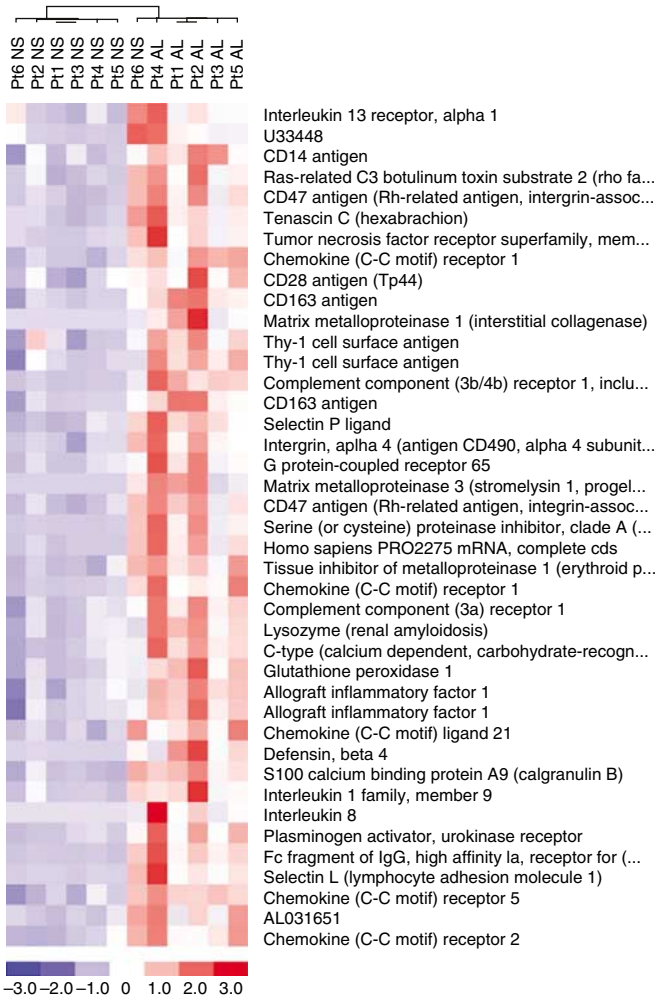

Figure 1. Hierarchical clustering of 41 inflammatory genes upregulated in inflammatory acne identifies genes whose expression patterns segregate on the basis of sample (normal skin vs acne lesion) and magnitude of change in expression. Hierarchical clustering of genes involved in inflammation from six patients with skin biopsies taken at the site of the acne lesion (AL) and at a corresponding site of normal skin (NS) from the same patient. Each row represents a gene labeled with gene name or accession number and each column represents the patient sample. The color in each cell reflects the level of expression of the corresponding gene in the corresponding sample, relative to its mean level of expression in the entire set of biopsy samples. Expression levels greater than the mean are shaded in red and those below the mean are shaded in blue.

on normal skin from subjects without acne. Increased expression of all three proteins was noted in inflammatory acne lesions when compared to normal skin from the acne subjects. The expression of DEFB4 was greater in the epidermis of inflammatory acne lesions when compared to the epidermis in the uninvolved skin (Figure 2a and b). Immunoreactivity with antibody to IL-8 was noted at the follicular and perifollicular sites of the inflammation in the acne lesion (Figure 2c). IL-8 immunoreactivity was relatively absent in the normal skin (Figure 2d).

Whereas we found high levels of MMP-1 expression in the epidermis and sebaceous glands in biopsy sections from the inflammatory acne lesions (Figure 3b), we found variation in MMP-1 expression in skin biopsies from the uninvolved skin from the same acne patient (Figure 3c–e). In one of the six biopsies of normal skin from acne patients, we observed perifollicular inflammation, suggestive of a clinically unapparent acne lesion. MMP-1 immunoreactivity was increased in the epidermis adjacent to perifollicular inflammation (Figure 3c), whereas very little MMP-1 immunoreactivity was observed in those areas that had no microscopically visible inflammation (Figure 3e). The higher levels of MMP-1 expression at some sites of normal skin need to be further examined to determine if changes in MMP-1 expression are among the earliest changes before the development of comedones and inflammatory acne or if an increase in MMP-1 persists during involution of an inflammatory acne lesion.

Additional immunohistochemistry studies on skin biopsies of psoriasis and folliculitis revealed that MMP-1 is also expressed in the epidermis overlying inflammation and in follicular epithelium in folliculitis lesions (Figure 4). These data suggest that the rise in MMP-1 may be a part of a generalized inflammatory reaction that is not specific to acne.

DISCUSSION

Acne is a chronic inflammatory disease, characterized by increased sebum production, abnormal follicular differentiation, and bacterial colonization. Although significant advances have been made in the last decade in identifying the pathophysiological mechanisms involved in acne, there are no published gene array studies analyzing the differential pattern of gene expression in inflammatory acne lesions and normal skin. Ours is the first study to provide such a comprehensive comparison. In this regard, 211 genes were significantly upregulated in inflammatory acne lesions, many of which, as expected, are involved in inflammation. The major genes whose expression was increased have been implicated in acne. These include matrix metalloproteinases, *β-defensin 4*, *IL-8*, and *granulysin*. In contrast, a much smaller set of genes (18) were downregulated, three of which are in the secretoglobin family. Despite evidence of small foci of inflammation in clinically normal skin from acne patients, no differences were noted between normal skin from acne patients and that from patients without acne in the array analysis. This is most likely due to the very small contribution of a focus of inflammation to overall gene expression within a 5-mm biopsy of whole skin.

The recognition of microbial pathogens by immune cells triggers host defense mechanisms to combat infection. These defenses include antimicrobial peptides, inflammatory cytokines, and proapoptotic enzymes. However, activation of these same mechanisms also results in tissue injury and scarring, a feature commonly observed in inflammatory acne. The data generated in this study support many of the recent findings regarding inflammatory mediators in acne, for example, the extent to which *P. acnes* induces the expression of the antimicrobial peptide, β-defensin 2 (now known as DEFB4), and IL-8 (Nagy *et al.*, 2005). As *P. acnes* is routinely present in the skin of most individuals, and no correlation

Table 2. Genes significantly downregulated in acne lesions compared to normal skin

Probe set ID	Accession	Fold change	Gene title	Gene symbol
Complete list of genes that were downregulated in acne lesions				
206799_at	NM_006551	−2.889	*secretoglobin, family 1D, member 2*	*SCGB1D2*
205979_at	NM_002407	−2.883	*secretoglobin, family 2A, member 1*	*SCGB2A1*
203824_at	NM_004616	−2.677	*transmembrane 4 superfamily member 3*	*TM4SF3*
212913_at	BE674960	−2.586	*mutS homolog 5 (E. coli)*	*MSH5*
206378_at	NM_002411	−2.227	*secretoglobin, family 2A, member 2*	*SCGB2A2*
221406_s_at	NM_025259	−1.991	*mutS homolog 5 (E. coli)*	*MSH5*
203697_at	U91903	−1.939	*frizzled-related protein*	*FRZB*
219127_at	NM_024320	−1.879	*hypothetical protein MGC11242*	*MGC11242*
209842_at	AI367319	−1.766	*SRY (sex determining region Y)-box 10*	*SOX10*
201596_x_at	NM_000224	−1.656	*keratin 18*	*KRT18*
206606_at	NM_000236	−1.571	*lipase, hepatic*	*LIPC*
205620_at	NM_000504	−1.547	*coagulation factor X*	*F10*
219717_at	NM_017741	−1.522	*hypothetical protein FLJ20280*	*FLJ20280*
205388_at	NM_003279	−1.509	*troponin C2, fast*	*TNNC2*
207265_s_at	NM_016657	−1.442	*KDEL endoplasmic reticulum protein retention receptor 3*	*KDELR3*
206747_at	NM_014696	−1.328	*KIAA0514*	*KIAA0514*
210006_at	BC002571	−1.325	*DKFZP564O243 protein*	*DKFZP564O243*
31637_s_at	X72631	−1.310	*nuclear receptor subfamily 1, group D, member 1*	*NR1D1*

Table 3. Quantitative PCR confirms an increase in the expression of select inflammatory genes

	Fold change in mRNA expression (quantitative real-time PCR) (mean±SD)	Fold change in gene expression (Gene Microarray data) (mean)
MMP-1	+170±18	+92
MMP-3	+165±32	+64
IL-8	+181±23	+52
DEFB4	+167±20	+33
Granzyme B	+16±2	+11

The table represents the fold changes in mRNA expression for *MMP-1*, *MMP-3*, *IL-8*, *DEFB4*, and *granzyme B* in inflammatory acne lesions and biopsies of normal skin from the same group of patients. Statistical analysis was performed by paired *t*-test: *MMP-1*, $P<0.007$; *MMP-3*, $P<0.05$; *DEFB4*, $P<0.02$; **IL-8*, $P<0.03$; *granzyme B*, $P<0.008$; $n=5$, $*n=4$, one sample being an outlier was upregulated by more than 3,500-fold in acne lesion. Comparative values of changes in gene expression are also represented in the table.
MMP-1, matrix metalloproteinase 1; *MMP-3, matrix metalloproteinase 1*; *DEFB4, human β-defensin 4.*

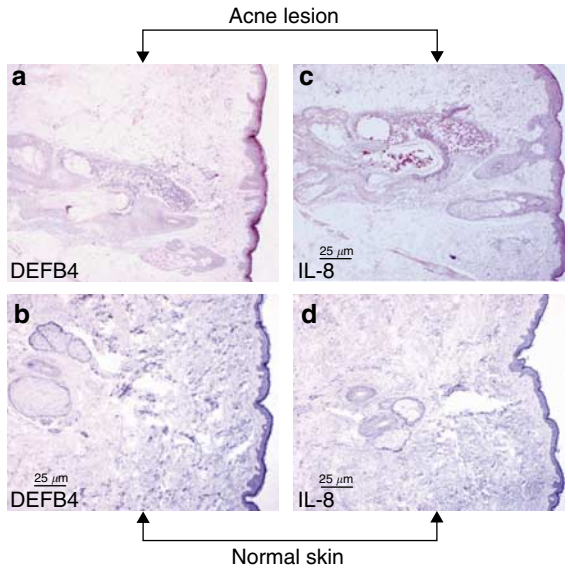

Figure 2. Immunoreactivity to DEFB4 and IL-8 in skin biopsies confirms array findings. Immunohistochemistry was performed on multiple sections of acne skin and compared to those of clinically normal skin from the same patient. (**a**, **c**) Expression of DEFB4 and IL-8 in biopsies from inflammatory acne lesions. (**b**, **d**) Comparative staining for DEFB4 and IL-8 in biopsies from clinically normal skin from the same patients.

between the number of bacteria and the severity and type of acne has been found, differences in strain variations may account for the differences in expression of β-defensin 4 or IL-8 (Nagy *et al.*, 2005). β-Defensin 4 demonstrates antimicrobial activity against both Gram-positive and Gram-negative bacteria. Our data demonstrate that it is expressed in the epidermis of inflammatory acne lesions, but not in normal skin. These results are in agreement with other studies demonstrating an increase in expression of β-defensin 1 and 2

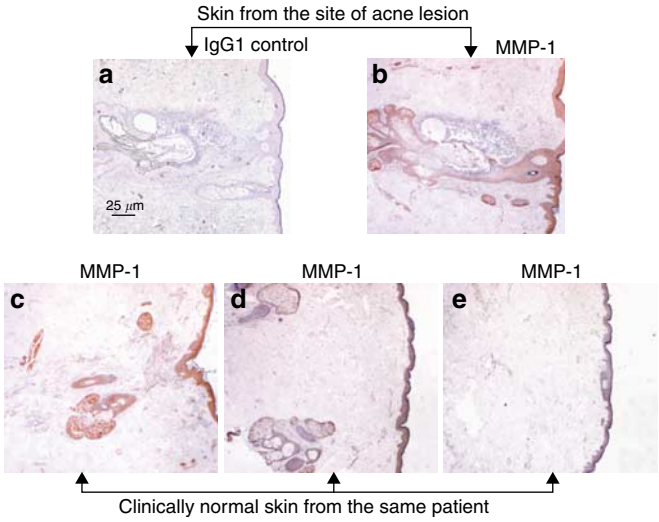

Figure 3. Immunoreactivity to MMP-1 is increased in acne lesions and in areas of microscopic inflammation in clinically normal skin. MMP-1 expressed in the epidermis and sebaceous glands in inflammatory acne shown in (**b**) compared to IgG1 control shown in (**a**). Variable levels of MMP-1 staining can be seen in serial sections of clinically normal skin from the same acne patient (**c**–**e**). A higher expression level of MMP-1 staining is seen in the epidermis in sections close to microscopically visible perifollicular inflammation (**c**) and progressively lower levels of MMP-1 staining is seen in sites distal from it (**d**, **e**).

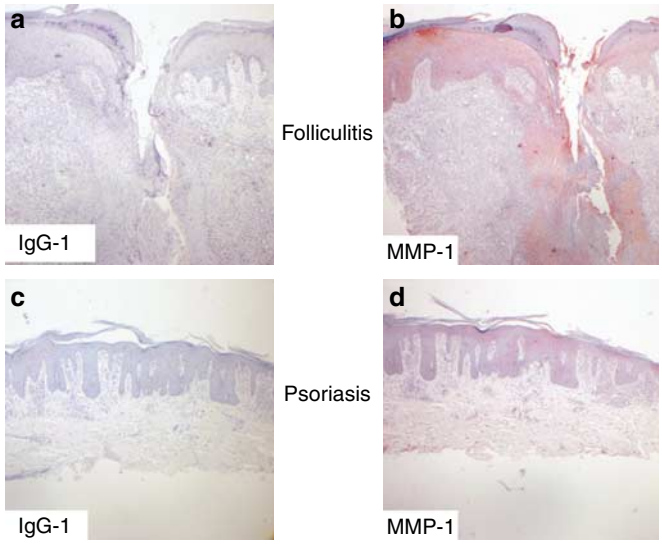

Figure 4. Increased levels of MMP-1 expression in other inflammatory skin conditions. Increased levels of MMP-1 expression are seen in the epidermis and perifollicular area of folliculitis (**b**) and in the epidermis of psoriasis (**d**), compared to IgG1 controls (**a**, **c**).

(DEFB4) in the epidermis of the inflammatory acne lesions (Chronnell *et al.*, 2001).

Granulysin is an antimicrobial peptide whose expression is significantly increased in inflammatory acne lesions in our study. It functions both as a cytotoxic agent against pathogenic bacteria and as a chemoattractant that activates monocytes to produce cytokines (Deng *et al.*, 2005). Antibiotics to combat *P. acnes* and other bacteria have been commonly used in the treatment of acne and thus the upregulation of these antimicrobial peptides produced by the body can be helpful in killing the bacteria. Recent studies have shown, however, that although some chemokines are reduced as a consequence of the microbicidal effect of granulysin peptides, the levels of IL-8 remain unchanged (McInturff *et al.*, 2005).

IL-8 is a major inflammatory mediator and a strong chemotactic factor for neutrophis, basophils, and T cells (Zachariae, 1993). Our study demonstrates that it is markedly upregulated in inflammatory acne lesions. The activation of this chemokine is regulated by a combination of three major mechanisms, namely (a) the transcriptional activation by the NF-κB and JUN protein kinase pathways, (b) stabilization of the mRNA by the p38 mitogen-activated protein kinase pathway, and (c) derepression of the gene promoter (Mukaida *et al.*, 1994; Harant *et al.*, 1996; Hoffmann *et al.*, 2002). As in several inflammatory diseases, IL-8 has been implicated in mounting an inflammatory response in acne lesions. Several studies have focused on the role of *P. acnes* bacteria in inducing IL-8 and discovered that this process may be mediated by the activation of transcription factor NF-κB (Vowels *et al.*, 1995; Cheng *et al.*, 2002). Thus, a detailed understanding of the pathways governing IL-8 production may help us to identify newer targets and better therapeutics for acne treatment.

The more recent studies by Kang *et al.* (2005) have focused on identifying the various intracellular signaling cascades associated with transcription factors involved in inflammation and matrix degradation in acne lesions. The activation of NF-κB pathway as evidenced by nuclear localization of p65 and p50, and the subsequent regulation of inflammatory cytokines such as tumor necrosis factor-α, IL-1β, and IL-8 have provided clues that help our understanding of the molecular pathways governing inflammation in acne (Kang *et al.*, 2005). Our results are in agreement with studies by Kang *et al.* in demonstrating increased expression of matrix metalloproteinases, such as MMP-1 and MMP-3, and cytokines, especially IL-8, in the inflammatory acne lesions. In order to confirm if the upregulation of MMP-1 was specific to acne, we performed immunohistochemistry on sections of isolated folliculitis and psoriasis and found high levels of expression of this metalloproteinase in the epidermis, suggesting a role of MMP-1 in several inflammatory conditions.

Our study also demonstrates significant fold increases in the expression of *granzyme B* in inflammatory acne lesions. *Granzyme B* is an essential component of the apoptotic pathway that is necessary for target cell lysis in cell-mediated immune response (Heibein *et al.*, 2000). It is commonly found in granules produced by cytolytic T lymphocytes and natural killer cells. Cytolytic T lymphocytes and natural killer cells use perforin- and granzyme B-containing granules to destroy cells infected with intracellular pathogens (Trapani and Smyth, 2002). Although very little is known about the role of *granzyme B* in the pathogenesis of acne, the strong upregulation of *granzyme B* in acne lesions as observed in our studies will help foster interest into a more in-depth study of this protease in acne.

The upregulation of antimicrobial peptides is of interest, further supporting their role in the endogenous inflammatory response to bacterial pathogens and perhaps also providing a rationale for their potential therapeutic use in acne. Critical questions remain, however, as to the nature of the initiating events in the development of acne lesions. It is likely that the profiles of gene expression in any inflammatory process in the skin are quite similar and that many of the changes observed in inflammatory lesions are likely to be secondary to as yet unidentified primary pathogenic events. The challenge lies ahead in identifying these primary events in acne as well as in other inflammatory diseases.

MATERIALS AND METHODS

Materials

The gene arrays ''HG-U133A 2.0'' were purchased from Affymetrix (Santa Clara, CA). The *MMP-1*, *MMP-3*, *IL-8*, human *β-defensin 4* (*DEFB4*), and *granzyme B* primers for real-time PCR were obtained from Applied Biosystems (Foster City, CA). The primary antibodies for immunohistochemistry of MMP-1 and IL-8 were purchased from R&D systems (Minneapolis, MN) and DEFB4 from Abcam Inc. (Cambridge, MA).

Patient selection and tissue biopsies

Twelve patients, including men and women, were enrolled in this study. All patients signed a written informed consent. The study was approved by the Institutional Review Board of the Pennsylvania State University College of Medicine and was conducted according to the Declaration of Helsinki Principles. The mean $\pm$SD ages of the six acne patients and the six normal subjects were 29 ± 9 and 39 ± 6.8 years, respectively. The inclusion criteria for the acne lesion group included (a) men and women aged 18–45 years with inflammatory acne on their back, (b) subjects without other skin disease in the biopsy area, (c) subjects who were willing to have skin biopsies performed from their back, and (d) subjects who have not been treated with isotretinoin for acne within the previous 6 months. The inclusion criteria for subjects without acne included (a) men and women aged 18–45 years who were willing to have a skin biopsy performed from their back and (b) subjects without other skin disease in the biopsy areas. The exclusion criteria included subjects who were taking oral medications that might influence gene expression in the skin or applying topical medications in the target areas on the back. A punch biopsy (5 mm) of the skin was performed at two sites on the back of acne patients, one at the site of an inflammatory acne papule and other from a region of clinically normal skin. The acne lesions that were selected for biopsy were new-onset inflammatory papules ranging in size from 3 to 4 mm that were present on the mid-upper back. The diagnosis of acne was substantiated by the presence of facial acne in addition to the presence of comedones on the back of the subjects enrolled. Biopsies of normal skin were taken at sites on the upper mid-back that were as remote as possible from any visible acne lesions. Normal subjects without acne were subjected to only one biopsy taken from the mid-upper back.

Extraction of RNA, labeling, and hybridization to probe arrays

Total RNA was isolated from skin samples and DNase treated using the RNeasy Fibrous Tissue Kit (Qiagen Inc., Valencia, CA). RNA was ethanol precipitated and quantified using a spectrophotometer. Approximately 2 μg of total RNA from each sample was used to generate double-stranded cDNA using a T7-oligo (dT) primer. Biotinylated cRNA, produced through *in vitro* transcription, was fragmented and hybridized to an Affymetrix human U133A 2.0 microarray. The arrays were processed on a GeneChip Fluidics Station 450 and scanned on an Affymetrix GeneChip Scanner (Santa Clara, CA).

Quantitative real-time PCR

Quantitative real-time PCR was performed to confirm changes in the level of select genes from the array data. cDNA was generated from 1 μg of total RNA, primed with oligo-dT, using the Superscript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen, Carlsbad, CA). Applied Biosystems' Assays-on-Demand Taqman Universal PCR Master Mix and primer probe sets were used to run real-time PCR on ABI's 7900HT Fast Real-Time PCR System with 384-well plate block module (Applied Biosystems, Foster City, CA). Samples corresponding to 80 ng of total RNA input were run in triplicate for the reference gene *TBP* as well as six genes of interest: *MMP-1*, *MMP-3*, *DEFB4*, *IL-8*, *GZMB*, and *GATA6*. No template and no amplification controls were also run.

Immunohistochemistry

In order to verify changes in gene expression at the protein level, a cohort of four additional subjects with acne were recruited to undergo biopsies of an inflammatory acne papule and of uninvolved skin from the back in a site remote from visible acne lesions. In addition, skin samples from additional subjects without acne were obtained to further assess the expression of the proteins of interest. Immunohistochemistry was performed on paraffin-embedded sections following antigen retrieval. Sections were blocked with 1% BSA and incubated with monoclonal antibody to MMP-1 (1:200) and *β*-defensin 4 (1:100), and polyclonal antibody to IL-8 (1:50) overnight at 4°C. Slides were incubated with biotinylated secondary antibodies against the respective primary antibodies at a dilution of 1:500 for 60 minutes at room temperature followed by 30 minutes incubation with the ABC reagent (Vector Labs, Burlingame, CA). The AEC kit (Vector Labs) was used as the chromogen, which stains as a red color. The sections were counterstained with hematoxylin and slides analyzed by microscopy. Negative controls consisted of sections incubated with IgG1 in place of the primary antibody. Archived paraffin-embedded specimens of inflammatory folliculitis and psoriasis were also examined for the presence of MMP-1 immunoreactivity.

Cluster analysis

Out of the 211 genes that were upregulated in acne lesions, 41 were identified as part of the inflammatory probe set generated from the NetAffix Analysis Center from the Affymetrix website (http://www.affymetrix.com/analysis/index.affx). Hierarchical clustering of patient samples and genes involved in inflammation was performed using the Computer software dChip version 1.3 (Li and Wong, 2003). Patient samples included skin biopsies taken at the site of the acne lesion and at a site of clinically normal skin from the same patient. Normalized chip intensity data were imported into dChip. The gene information file for Affymetrix human genome HG-U133A 2.0 array was obtained from dChip's website at www.dChip.org. Hierarchical

clustering is used to compute a dendrogram that assembles all genes or samples into a single tree.

Statistical analyses

Before testing significant changes in gene expression, the expression signals were normalized by using the R-Affy package from Bioconductor version 1.1 (Irizarry *et al.*, 2003a, b) to remove background noise and non-biological variations among arrays. The background noise was removed from the PM probe intensities using the ''RMA'' method (Irizarry *et al.*, 2003a, b), which assumes a global model for the distribution of probe intensities and models the PM probe intensities as the sum of a normal noise component and an exponential signal component. Normalization was performed using the quantile normalization method (Bolstad *et al.*, 2003). Quantile normalization assumes that the expression of the majority of genes on the arrays does not change in different treatments and the distribution of probe intensities for each array in the data set is the same. To remove outlier probes and summarize probe intensities within one probe set into a single expression value, the ''Tukey Biweight'' method was applied to the background adjusted, normalized PM intensities. Expression values were obtained based on PM intensities and not PM–MM intensities, because PM and MM intensities were found to be highly correlated, which suggested that MM intensities were composed of background noise as well as probe-specific signals. Significant gene expression alterations were identified using the computer software Significance Analysis of Microarrays (Tusher *et al.*, 2001). Significance Analysis of Microarrays assigns a score to each gene on the basis of gene expression change relative to the standard deviation of repeated measurements and identifies genes with statistically significant changes in expression using a permutation procedure. Significance Analysis of Microarrays controls the false positives resulting from multiple comparisons through controlling the false discovery rate (Benjamini and Hochberg, 1995). False discovery rate is defined as the proportion of false positive genes among all genes that are considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Comprehensive list of genes upregulated/downregulated in inflammatory acne, compared to clinically normal skin from the same patients.

Figure S1. Hierarchical clustering of 229 genes involved in inflammation from six patients with skin biopsies taken at the site of the acne lesion (AL) and at a corresponding site of normal skin (NS) from the same patient.

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