

Analysis of Gene Expression in Atopic Dermatitis Using a Microabrasive Method

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TO THE EDITOR

We have developed a simple approach (see Supplementary Data online for the method) to analyze the differences in the gene expression profile of atopic and healthy skins in order to better characterize the skin changes associated with atopic dermatitis (AD). This method has enabled us to define gene expression profiles related to the pathogenesis of AD from microabrasive samples, which, consequently, are applicable in young children (Table 1).

During the development of the skin sampling method, we evaluated the relative expression of various constitutive genes of the epidermis or inflammatory genes, depending on the depth level measured by transepidermal water loss. Results showed that the expression levels were not correlated with the extent of abrasion and are thus independent of the sampling depth.

Moreover, even in the presence of a small quantity of mRNA, we have been able to demonstrate variations in expression between xerotic and inflammatory areas of patients with AD and the control population. In addition, the extraction of mRNA from these samples provides information about certain cell types involved in the pathophysiological process of AD. Thus, although keratinocytes predominate and express a number of inflammatory markers, we were also able to demonstrate the presence of lymphocytes (expression of CD25 and CD45Ro) and dendritic cells (expression of CD1b and langerin). Conversely, we were unable to demonstrate the presence of macrophages (CD163) and neutrophils (myeloperoxidase; data not shown), the latter

probably being present in small quantities relative to other cell types.

In this study we observed a decrease in the expression of profilaggrin in atopic skins. Several studies have demonstrated that two mutations of the filaggrin gene are highly associated with the development of AD, particularly in patients who also exhibit allergic problems and asthma (Palmer *et al.*, 2006). However, the filaggrin deficiency resulting in abnormal barrier function does not appear to be purely genetic and can also be dependent on acquired immunity. In fact, the development of an antigen-specific Th2-type immune response causes reductions in filaggrin and ceramide syntheses, which characterize the abnormalities of skin barrier function observed in AD patients (Man *et al.*, 2008). In addition to the impaired skin barrier, other mechanisms promoting potentially pathogenic microbial colonization are postulated, such as an epidermal immune deficiency that is reportedly responsible for insufficient expression of antimicrobial peptides. In fact, levels of expression of these peptides in psoriasis are clearly higher than those observed in AD, the latter being higher than those observed in healthy subjects (Gambichler *et al.*, 2008; Harder *et al.*, 2010). In agreement with these data, the proposed methodology enabled us to show a higher expression of antimicrobial peptide (HBD2, HBD3, and LL37) in AD compared with healthy samples. Conversely, the experience of Leung's group (Howell *et al.*, 2006) does not show any increase in hBD3m RNA expression in AD patients compared with healthy subjects. In fact, it showed the opposite tendency.

In addition, the increased expression of NF- κ B observed at the inflammatory sites of atopic patients highlights the key role that this factor might have in controlling the inflammatory processes in AD. The same applies to the increased expression in lesional areas of I κ B kinases, which phosphorylate NF- κ B inhibitory proteins (I κ B), thereby releasing NF- κ B, which becomes active. Activation of NF- κ B thus induces the transcription of genes of proinflammatory cytokines, chemokines, adhesion molecules, cyclooxygenase 2, and still others (Barnes and Karin, 1997). These results are consistent with the demonstration of a marked expression of IL-1 β proinflammatory cytokines and the adhesion molecule ICAM1, as well as that of cyclooxygenase 2, in the inflammatory areas of AD patients.

On the other hand, the higher expression of markers of Langerhans cells (langerin) in the inflammatory areas and of dendritic cells (CD1a and CD1b) in the inflammatory and xerotic areas of the AD population compared with healthy subjects appears to confirm the crucial role of these antigen-presenting cells in AD (Schuller *et al.*, 2001). This increase might reflect the cooperation between the dendritic cell presenting the antigen and expressing stimulatory molecules such as CD80 and CD86 and the T lymphocyte responding to it by polarizing toward the Th2 type in atopic situations (Hammad *et al.*, 2001). The expression of CD80 and CD86 is effectively higher in inflammatory areas than in xerotic areas in AD subjects.

The approach developed here once again enables us to demonstrate a more marked expression in the lesional areas of AD subjects of the CD45RO receptor, which is synthesized after activation of naive T lymphocytes and carried

Table 1. Gene expression profiles

	Marker	Lesional area	Xerotic area	Healthy	
Antimicrobial peptides	Profilaggrin	66.36 ± 89.21*	52.93 ± 45.75**	207.20 ± 169.76	
		28.74	36.41	185.61	
		(6.66–383.82)	(2.52–158.24)	(10.36–629.89)	
	HBD2	50.51 ± 35.21***	29.29 ± 28.33**,\$\$	6.23 ± 6.31	
		36.84	22.32	3.62	
		(8.60–132.24)	(1.97–114.95)	(0.12–17.99)	
	HBD3	39.93 ± 31.01***	20.83 ± 15.63***,\$\$	2.53 ± 3.51	
		33.00	16.55	1.25	
		(7.10–136.69)	(1.75–65.87)	(0.01–11.74)	
LL37	39.32 ± 31.35***	18.90 ± 17.38\$\$	4.23 ± 3.80		
	30.50	18.11	2.10		
	(6.59–144.22)	(0.18–63.61)	(0.02–9.97)		
Markers of inflammatory response	NF-κB	48.01 ± 26.57***	24.98 ± 22.58**,\$\$\$	6.87 ± 9.90	
		41.52	16.57	2.57	
		(18.90–119.99)	(2.07–85.76)	(0.09–33.94)	
	IκBKB	25.04 ± 16.18***	13.08 ± 10.25*,\$\$\$	6.02 ± 6.20	
		21.13	12.53	5.12	
		(4.49–64.30)	(1.65–38.05)	(0.10–18.41)	
	Cox-2	31.39 ± 29.3***	10.05 ± 13.7\$\$\$	5.12 ± 4.25	
		21.85	4.63	3.28	
		(0.86–103.60)	(0.08–61.88)	(0.66–13.52)	
	IL-1b	270.01 ± 303.96***	71.11 ± 129.73\$\$\$	14.22 ± 13.84	
		180.27	13.65	8.52	
		(5.69–1240.41)	(0.20–472.84)	(1.85–45.29)	
	ICAM	29.22 ± 23.89***	12.68 ± 15.39**,\$\$	2.34 ± 2.65	
		19.99	7.00	1.99	
		(5.16–99.14)	(0.23–55.32)	(0.10–9.05)	
	Dendritic cell markers	Langerin	15.33 ± 9.70***	13.00 ± 12.96	4.42 ± 2.20
			13.85	7.71	3.43
			(3.42–38.96)	(1.61–48.27)	(1.81–8.21)
CD1a		42.42 ± 27.57***	30.06 ± 25.24***	3.13 ± 4.62	
		37.57	22.32	1.54	
		(4.86–117.19)	(1.96–89.02)	(0.26–16.12)	
CD1b		15.07 ± 11.31***	10.05 ± 9.93**	2.55 ± 4.61	
		12.35	5.26	0.70	
		(2.28–42.86)	(0.26–37.83)	(0.08–14.35)	
CD80		24.67 ± 24.38***	11.02 ± 16.67\$\$\$	3.60 ± 3.01	
		18.64	4.38	2.53	
		(1.35–109.92)	(0.25–57.85)	(0.18–10.43)	
Lymphocyte markers	CD86	60.37 ± 33.88***	25.49 ± 27.2*,\$\$\$	4.84 ± 3.52	
	CD45Ro	17.20 ± 14.04***	9.80 ± 19.30\$	1.63 ± 2.38	
		13.96	2.73	0.83	
		(3.26–52.45)	(0.02–68.43)	(0.11–8.54)	
	CD25	509.43 ± 361.63***	277.32 ± 343.51***,\$	15.08 ± 14.90	

Table 1 continued on following page

Table 1. Continued

	Marker	Lesional area	Xerotic area	Healthy
		<i>436.46</i>	<i>146.73</i>	<i>9.52</i>
		(89.83–1,697.45)	(2.77–1,542.18)	(0.46–43.17)
	IL-31	29.51 ± 39.38***	7.89 ± 20.23**,\$\$	0.00 ± 0.00
		<i>14.56</i>	<i>0.42</i>	<i>0.00</i>
		(0.00–137.98)	(0.00–89.63)	(0.00–0.00)
Neuromediators and TRPV channels	CALCA	10.68 ± 12.44**	2.42 ± 4.34**,\$\$	0.00 ± 0.00
		<i>6.89</i>	<i>0.44</i>	<i>0.00</i>
		(0.00–41.94)	(0.00–15.03)	(0.00–0.00)
	TAC-1	26.63 ± 23.86**	21.17 ± 22.7**	6.61 ± 12.31
		<i>20.98</i>	<i>13.67</i>	<i>1.20</i>
		(3.23–98.10)	(1.35–97.97)	(0.04–38.16)
	POMC	6.35 ± 5.74***	8.48 ± 8.05***	164.62 ± 205.07
		<i>4.71</i>	<i>5.96</i>	<i>80.21</i>
		(0.00–18.10)	(0.00–33.05)	(15.53–592.11)
	TRPV3	27.17 ± 21.79***	12.79 ± 12.73\$\$\$	6.3 ± 8.57
		<i>23.95</i>	<i>7.86</i>	<i>3.37</i>
		(5.43–100.26)	(2.12–47.36)	(0.12–30.03)
	TRPV4	38.38 ± 30.68	27.16 ± 50.48\$\$	25.73 ± 30.31
		<i>33.66</i>	<i>14.20</i>	<i>14.53</i>
		(1.47–137.05)	(3.29–248.39)	(3.12–97.43)
	TRPV6	6.57 ± 10.55**	25.15 ± 49.72**	140.09 ± 165.41
		<i>1.61</i>	<i>7.12</i>	<i>82.54</i>
		(0.00–34.95)	(0.00–199.83)	(7.93–475.47)

* $P < 0.0170$, ** $P < 0.01$, *** $P < 0.001$ versus healthy patients (Wilcoxon test).

\$ $P < 0.0170$, \$\$ $P < 0.01$, \$\$\$ $P < 0.001$ versus lesional area of atopic patients.

Student's *t*-test has been used for the following markers: CD1a, CD1b, CD25, CD86, HBD2, NFKB, POMC, and TAC-1. In all the other cases, we have used Wilcoxon test.

A Dunn-Sidak correction was made to adjust for the multiplicity of tests.

Median values are given in italics and ranges are indicated in parentheses.

by memory T cells. In addition, it is important to note that the domiciliation of T cells in inflammatory skin is particularly affected by the secretion of IL-31, which in turn induces a profile of keratinocyte chemokine secretion (CCL17 and CCL22), promoting the migration of T cells toward the skin (Dillon *et al.*, 2004). In addition, IL-31 is also said to have a key role in the development of the pruriginous reaction, and its concentration is reported to be correlated to the severity of AD (Raap *et al.*, 2008). The demonstration of levels of expression of IL-31 in atopic samples, and particularly in inflammatory areas, in contrast to samples taken from healthy subjects, are consistent

with this. In addition, the increased expression of CD25 in inflammatory areas suggests a recruitment of T regulatory cells, dependent on chemokines secreted particularly by dendritic cells or resulting from a marked polyclonal expansion of T lymphocytes following the action of staphylococcal superantigens (Ou *et al.*, 2004).

Exacerbation of inflammatory episodes is also said to be prompted by a major local neurogenic phenomenon related in particular to substance P and to CGRP under the control of nerve growth factor (Ostlere *et al.*, 1995). Other receptors such as the TRPV3 and TRPV4 ion channels recently studied in AD might contribute to the

pathophysiology of inflammation and neurogenic perceptions, such as the pruritus characteristic of this condition (Bíró *et al.*, 2007). Thus, the overexpression of substance P, CGRP, and TRPV3 channels in AD patients (xerotic and inflammatory areas) would appear to cause sensitization of the afferent nerve fibers and increase pruriginous manifestations.

Conversely, we have shown that the expression levels of POMC (a precursor molecule that after proteolysis releases hormones such as α -melanocyte stimulating hormone) and TRPV6 were markedly reduced in AD subjects. This reduction is very pronounced in inflammatory areas and might influence the

induction of episodes in AD. In fact, several studies demonstrate the antagonistic action of α -melanocyte stimulating hormone on proinflammatory cytokines IL-1 α , IL-1 β , IL-6, and tumor necrosis factor- α (Böhm et al., 2006) and also its inhibitory role in lymphocyte activation and function (Luger et al., 1993). Similarly, the reduction in TRPV6 expression in atopic subjects might induce a reduction in the intracellular calcium content and consequently in disruption of the epidermal proliferation/differentiation balance.

In conclusion, although none of these factors are specific for AD, it is still the case that together they provide an aid to understanding the mechanisms of the pathogenesis of AD.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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Supplementary data

Clinical Cases:

This study was conducted according to the principles of the Declaration of Helsinki, and was approved by the French Ethics Committee (CPP) in Nice, France, No CCP 08.013. Child volunteers were enrolled in the study after their parents had given written informed consent. These included a population of 27 children, aged from 6 months to 7 years and suffering from AD according to the UK Working Party criteria (SCORAD mean = 39.44), who were studied alongside a group of age-matched healthy subjects (n=11) presenting no cutaneous sign and not undergoing any pharmacological treatment. Data from 23 atopic children, with SCORAD $\geq$ 15, were analysed in this monocenter open study. The use of topical corticosteroid and immunosuppressor was forbidden 15 days before the inclusion and during the study. Antifungus, antibiotics and antiseptics were forbidden during the study.

The study was carried out under the control and with the agreement of the requisite ethics committee. Selected patients refrained from using an emollient for 24h preceding skin sampling. Two skin samples were taken from lesional (edematous or inflamed) and xerotic areas in atopic patients. These areas were localized on the upper or lower limbs.

Skin Samples

Skin samples were taken by scratching the skin surface (5 times back and forth) with a sterile microabrasive tool (Vitry, Paris, France) approved by the French Ethics Committee in Nice (No CCP 08.013). Cell aggregates were transferred from the tool to a

microtube containing 500 µL of RNAProtect Cell Reagent (Qiagen, Courtaboeuf, France) and placed at –80°C.

Real Time RT-PCR Analysis

Total RNA was isolated using the iPrep PureLink kit (Invitrogen, Carlsbad, USA) according to the manufacturer's directions, including DNase I digestion. RNA was eluted in 50 µL of sterile, RNase-free water. Ten µL of RNA was reverse transcribed to single-stranded cDNA by the High Capacity cDNA kit (Applied Biosystems, Foster City, USA), using random hexamer, in a final volume of 20 µL according to the manufacturer's instructions. A minus RT reaction was performed in parallel to ensure the absence of genomic DNA contamination.

The method we developed for skin sampling enabled us to extract only small quantities of RNA, so that to optimize the quantity of cDNA we performed a preamplification PCR (Applied Biosystems, Foster City, USA). Before running the preamplification reaction, the intended assays (containing primers and probes specific to the 24 genes of interest and one endogenous control) were pooled together, as per the manufacturer's instruction. Briefly, 5 µL of cDNA was used to perform PCR with 5 µL of the pooled assay mix (0.2 x) and 10 µL of TaqMan® Preamp master mix (2 x). The preamplification reaction was performed for 10 min at 95°C, and 14 cycles of 15 s at 95 °C and 4 min at 60°C. The preamplified products were diluted 1:20 using sterile, RNase-free water.

These cDNA samples were used for real-time PCR amplification assays employing TaqMan® low density arrays (TLDA) (Applied Biosystems, Foster City, CA). The fluorogenic, FAM-labeled probes and the sequence-specific primers for the list of genes with RPLP0 as endogenous control were obtained as inventoried assays and incorporated

into the TaqMan[®] array format. cDNA template samples were amplified and analyzed. In this study, the TLDA was configured into 8 identical 24-gene sets in duplicate. A total of 100 µL reaction mixture with 50 µL preamplified cDNA template and an equal volume of TaqMan[®] universal master mix (Applied Biosystems, Foster City, CA) were loaded into each fill port of the TLDA. Fluorescence was monitored and analyzed in the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Analysis of RPLP0 was performed to standardize gene expression. Results are expressed as $2^{-(C_t \text{ RPLP0} - C_t \text{ target gene})}$, where C_t corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold.

Primers and probes used for the preamplification and the TLDA coating were designed by Applied Biosystems: CALCA Hs00266142_m1, LL37 Hs00189038_m1, CD1a Hs00233332_m1, CD1b Hs00957537_m1, langerin Hs00210453_m1, CD80 Hs00175478_m1, CD86 Hs01567025_m1, HBD2 Hs00175474_m1, HBD3 Hs00218678_m1, profilaggrin Hs00863478_g1, ICAM-1 Hs00164932_m1, NF-κB Hs00174517_m1, IκBKB Hs00395088_m1, IL-1β Hs01555410_m1, CD25 Hs00907777_m1, IL-31 Hs01098710_m1, POMC Hs00174947_m1, Cox-2 Hs00153133_m1, CD45 Ro Hs00153133_m1, Tac-1 Hs00243225_m1, TRPV3 Hs00376854_m1, TRPV4 Hs01099348_m1, TRPV6 Hs00367960_m1 and RPLP0 Hs99999902_m1.

Statistical Analysis:

The results of the clinical study are expressed as means ± standard deviation.

The within-group analysis (inflammatory area - xerotic area comparison in AD subjects) was performed using a Wilcoxon test; the median and range values were given only in case of non parametric markers. For normally distributed markers, Student's t test was used.

Data from all the studies were analyzed using a Wilcoxon test for paired data for the within-group analyses.

In order to ensure a global type I risk of 5%, an adjustment method was used because of the multiplicity of tests. The Dunn-Sidak correction: $\alpha^* = 1 - (1 - \alpha)^{1/k}$ with $\alpha = 0.05$ and $k = \text{number of tests (here 3)}$ was chosen. If the probability p is less than or equal to α^* ($=0.0170$), then the comparison is considered significant at the alpha error risk.