Mechanisms of IFN-γ–induced apoptosis of human skin keratinocytes in patients with atopic dermatitis

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Background: Enhanced apoptosis of keratinocytes is the main cause of eczema and spongiosis in patients with the common inflammatory skin disease atopic dermatitis (AD).
Objective: The aim of the study was to investigate molecular mechanisms of AD-related apoptosis of keratinocytes.
Methods: Primary keratinocytes isolated from patients with AD and healthy donors were used to study apoptosis by using annexin V/7-aminoactinomycin D staining. Illumina mRNA Expression BeadChips, quantitative RT-PCR, and immunofluorescence were used to study gene expression. *In silico* analysis of candidate genes was performed on genome-wide single nucleotide polymorphism data.
Results: We demonstrate that keratinocytes of patients with AD exhibit increased IFN-γ–induced apoptosis compared with keratinocytes from healthy subjects. Further mRNA expression analyses revealed differential expression of apoptosis-related genes in AD keratinocytes and skin and the upregulation of immune system–related genes in skin biopsy specimens of chronic AD lesions. Three apoptosis-related genes (*NOD2*, *DUSP1*, and *ADM*) and 8 genes overexpressed in AD skin lesions (*CCDC109B*, *CCL5*, *CCL8*, *IFI35*, *LYN*, *RAB31*, *IFITM1*, and *IFITM2*) were induced by IFN-γ in primary keratinocytes. The protein expression of IFITM1, CCL5, and CCL8 was verified in AD skin. In line with the functional studies and AD-related mRNA expression changes, *in silico* analysis of genome-wide single nucleotide polymorphism data revealed evidence of an association between AD and genetic markers close to or within the *IFITM* cluster or *RAB31*, *DUSP1*, and *ADM* genes.
Conclusion: Our results demonstrate increased IFN-γ responses in skin of patients with AD and suggest involvement of multiple new apoptosis- and inflammation-related factors in the development of AD. (J Allergy Clin Immunol 2012;129:1297-306.)

Key words: *Cytokine, mRNA expression array, atopic eczema, inflammation, allergy*

Atopic dermatitis (AD) is a common chronically relapsing skin disease that is characterized by the disturbance of epidermal barrier function, recurrent skin inflammation, and accompanying apoptosis of keratinocytes.[1-3] Linkage and association studies have identified several candidate genes possibly linked to either epidermal barrier function or to immune processes.[2,4] For instance, variants of *IL4/IL13* receptor,[5,6] *IL13*,[7] and the gene encoding the α-chain of the high-affinity receptor for IgE *(FCER1A)* have been shown to be associated with AD.[8] Concordantly, a predominant T_H2 bias with increased IgE levels is a widely recognized hallmark of AD. Nonetheless, in the chronic phase of skin inflammation, IFN-γ, as the characteristic cytokine for T_H1 cells, is dominant in the skin of patients with AD.[2,9,10] The presence of IFN-γ, excess of other cytokines and often accompanying skin infection lead to enhanced and disease-related apoptosis of keratinocytes in the eczematous lesions of patients with AD.[1-3] In contrast, keratinocytes in patients with psoriasis, the most closely analogous skin disease, undergo hyperproliferation and altered differentiation.[2,11] Apoptosis is known to occur through death receptors that are activated by their ligands. Keratinocytes have been shown to express TNF-α receptor 1 (TNF-R1), TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 (TRAIL-R1 and TRAIL-R2), fibroblast growth factor–inducible 14 (FN14), and TNF receptor superfamily

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Abbreviations used
7-AAD: 7-Aminoactinomycin D
AD: Atopic dermatitis
FAS: TNF receptor superfamily member 6
FN14: Fibroblast growth factor–inducible 14
KORA: Cooperative Health Research in the Region of Augsburg
qRT-PCR: Quantitative RT-PCR
SNP: Single nucleotide polymorphism
TNF-R: TNF receptor
TRAIL: TNF-related apoptosis-inducing ligand
TWEAK: TNF-like weak inducer of apoptosis

member 6 (FAS).[12] Previously, it was shown that IFN-γ–induced apoptosis occurs through FAS both in keratinocytes and IFN-γ–producing T cells.[13-15] Although IFN-γ appears to be a key factor, other cytokines, such as TNF-α, TNF-like weak inducer of apoptosis (TWEAK), and IL-32, can contribute to keratinocyte apoptosis in patients with AD.[16,17]

In the present study molecular mechanisms of AD-related apoptosis of keratinocytes were investigated. Interestingly, we found enhanced IFN-γ–stimulated apoptosis of keratinocytes from patients with AD, whereas no difference between the studied groups was found when other death ligands were used. To search for genes responsible for the increased sensitivity of keratinocytes from patients with AD to IFN-γ–stimulated apoptosis, we performed mRNA array analyses of keratinocytes and skin of patients with AD. Our results show that several differentially regulated immune system– and apoptosis-related genes are stimulated by IFN-γ in keratinocytes, which might be associated with AD.

METHODS

Keratinocyte cultures and apoptosis detection

Generation and maintenance of primary keratinocytes from healthy subjects, patients with AD, and patients with psoriasis and apoptosis assays are described previously.[16,17] In brief, all included subjects were older than 18 years and did not receive systemic treatment and topical corticosteroids during 1 week before the study. Keratinocytes were collected from unlesional skin from the atopic subjects. Viability represents the percentage of annexin V– and 7-aminoactinomycin D (7-AAD)–negative cells (ie, cells that were early apoptotic [annexin V-positive] and late apoptotic and necrotic [annexin V and 7-AAD-positive] were excluded).

Skin biopsy specimens for mRNA expression analysis

This study was approved by the Ethical Review Committees on Human Research of the University of Tartu and the University of Szeged. All participants signed a written informed consent form. In total, 10 patients with chronic AD and 10 healthy subjects older than 18 years were included. Eight patients had 6- to 14-day-long severe exacerbation of the disease, and 2 patients had more than 4-week-old dermatitis. No patients had been treated with systemic antihistamines and topical corticosteroid for at least 1 week before inclusion into the study. All the patients had blood eosinophilia. Two skin biopsy specimens (diameter, 4 mm), one from lesional skin and the second from uninvolved skin, were obtained from each patient. One biopsy specimen was taken from each healthy control subject. All biopsy specimens were snap-frozen in liquid nitrogen and stored at −80°C.

mRNA array analysis

mRNA profiling was performed on Illumina HumanHT-12 Expression BeadChips (Illumina, San Diego, Calif). Keratinocyte and skin mRNA array data are available at ArrayExpress as E-TABM-728 and E-MTAB-729, respectively.

For more information on proliferation assay, flow cytometry, immunofluorescence, RNA isolation, and quantitative RT-PCR, see the Methods section in this article's Online Repository at www.jacionline.org.

Statistics

For apoptosis, viability, and proliferation assays, statistical analysis between paired conditions (noninduced and induced keratinocytes from the same subject) was performed by using the Wilcoxon signed-rank test. The comparison between the groups was performed with nonparametric Mann-Whitney *U* tests (Fig 1, *B* and *C*, and see Fig E1, *B*, in this article's Online Repository at www.jacionline.org). Statistical analysis of quantitative RT-PCR (qRT-PCR) results was performed by using the nonparametric Mann-Whitney *U* test. The results were considered significant at a *P* value of less than .05 and highly significant at *P* values of less than .01 and .001.

mRNA array data were analyzed with Genomestudio software by using the custom rank invariant method (Illumina) for normalization. Genes with differential expression *P* values of less than .05 were considered differentially expressed. Pathway analysis was performed with g:Profiler (http://biit.cs.ut.ee/gprofiler/) by using default parameters. Detailed description of analysis and visualization procedures can be found in the Methods section in this article's Online Repository at www.jacionline.org.

For *in silico* candidate gene analysis, differentially regulated genes grouped by means of pathway analyses were investigated by using existing single nucleotide polymorphism (SNP) data from 533 patients with AD recruited in Munich and Kiel and were part of a recently published genome-wide association study.[18] As control subjects, we chose 1996 subjects from the population-based Cooperative Health Research in the Region of Augsburg (KORA) S4/F4 survey.[19] We extracted SNPs from identified genes plus a surrounding region of ±50 kb by using the UCSC genome browser (assembly GRCh37/hg19, February 2009).[20] SNPs were filtered according to a call rate of greater than 0.97, a Hardy-Weinberg equilibrium deviation *P* value of less than .001, and a minor allele frequency in control subjects of greater than 0.05. The case-control analysis was carried out with PLINK[21] by using a χ^2 test for the 2×2 table for each SNP. Odds ratios were derived from a 2×2 contingency table. Because we investigated candidate genes with *a priori* evidence from mRNA expression analysis, we defined a significance threshold *P* value of less than .01 according to the χ^2 test. Haplotype analysis was performed with the R-package haplo.stats[22] within R 2.13.0 software (http://www.R-project.org/).

RESULTS

Increased IFN-γ–induced apoptosis of keratinocytes from patients with AD

To study AD-related keratinocyte apoptosis, we first evaluated the viability of healthy primary keratinocytes, which revealed a strong influence of IFN-γ on keratinocyte apoptosis that was further enhanced when keratinocytes were exposed to IFN-γ in combination with TRAIL or TWEAK. TRAIL and TWEAK alone did not influence the viability of primary keratinocytes (Fig 1, *A*), whereas the HaCat cells were sensitive to these cytokines (see Fig E1, *A*). We next investigated whether there is a difference in susceptibility to apoptosis between keratinocytes from healthy subjects, patients with AD, and patients with psoriasis. Keratinocytes from 5 different donors in each group were studied. Interestingly, significantly more healthy keratinocytes (67.6 ± 8.1) were viable in comparison with keratinocytes from patients with AD (51.4% ± 5.4%). Of keratinocytes from patients with psoriasis, 62.4% ± 7.2% were viable. Less cells were viable when treated with TWEAK or TRAIL in combination with IFN-γ, although no differences among the 3 keratinocyte groups were observed (Fig 1, *B*). According to annexin V–positive/7-AAD–negative staining, 27.1% ± 13.8% keratinocytes from patients with AD,

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