Apoptotic markers in spongiotic dermatitis
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Background
Keratinocyte (KC) apoptosis is believed to play an important role in the pathogenesis of spongiotic dermatitis, in particular for the formation of spongiosis.

Objective
To investigate changes in the expression level of the apoptosis regulatory proteins cleaved caspase-3, Fas, Bcl-2, nuclear factor NF-κB and p53 in skin samples of patients with spongiotic dermatitis.

Patients and methods
The present study included a total of 50 patients with spongiotic dermatitis and 10 healthy controls. Patients were classified into five groups (10 patients each): group A, atopic dermatitis; group B, allergic contact dermatitis; group C, irritant contact dermatitis; group D, nummular eczema; and group E, dyshidrotic eczema. Using immunohistochemistry, we investigated the expression of apoptotic regulatory proteins cleaved caspase-3, Fas, Bcl-2, NF-κB and p53 in skin biopsies taken from all patients and controls.

Results
The mean values of cleaved caspase-3 and Fas expression were statistically increased in patients with acute spongiotic dermatitis compared with the controls (P<0.001).

In the spinous cell layer, cleaved caspase-3 and Fas expression was observed in all specimens taken from lesional skin with different intensities. The strongest positive staining was noticed in areas of spongiosis. Bcl-2 and NF-κB expression was absent or weak in suprabasal cells in the lesional skin with no statistically significant difference between the patient group and the control group (P=0.74 and 0.38, respectively).

p53 expression was absent or weak in suprabasal cells in the lesional skin, with no statistically significant difference between the patient group and the control group (P=0.97). There was no difference in the mean expression of cleaved caspase-3, Fas, Bcl-2, NF-κB and p53 between different subsets of eczematous dermatitis (P=0.60, 0.14, 0.68, 0.76 and 0.30, respectively).

Conclusion
Apoptosis of KC through an extrinsic pathway is the initiating event in the development of the epidermal pathology seen in spongiotic dermatitis. Most notably, KC apoptosis occurs in suprabasal cells, where spongiosis takes place.

Keywords:
apoptosis, Bcl-2, cleaved caspase-3, Fas, nuclear factor κB, p53, spongiotic disorders

Introduction
Programmed cell death plays a role in the homeostasis of the epidermis and in the terminal differentiation of keratinocytes (KC), resulting in a cornified layer that is finally shed from the skin surface [1]. Many molecules or genes involved in the regulation of apoptosis have been identified either by preventing or by promoting apoptosis. The ratio of antiapoptotic versus proapoptotic proteins determines the inherent susceptibility of a given cell to respond to apoptotic signals [2].

Fas, Bcl-2, nuclear factor κB (NF-κB) and p53 proteins are known to play a central role in the regulation of apoptosis. Fas is a cell surface receptor that is highly expressed on a variety of cells. FasL is a membrane protein, usually restricted to activated T cells and natural killer cells. Binding of FasL to Fas on Fas-sensitive target cells causes apoptosis of the target cells by triggering a caspase cascade [3]. Caspase-3 is considered to be the most important of the executioner caspases and is activated (cleaved) by any of the initiator caspases (caspase-8, caspase-9 or caspase-10). This results in disruption of the cytoskeleton, intracellular transport, cell division and signal transduction [4].

p53 is a tumor-suppressor gene that controls cellular proliferation and can eliminate the cells by directing them down an irreversible apoptotic pathway [5]. In contrast, NF-κB and Bcl-2 are proto-oncogenes that protect cells from apoptosis [6].
Spongiosis refers to intercellular edema between the KC of the stratum malpighii. It is characterized by widening of the intercellular space and a sponge-like appearance of the epidermis. Spongiotic dermatitis is a broad category of inflammatory skin disease in which spongiosis is the microscopic hallmark [7]. Apoptosis of KC has been implicated as a key mechanism of spongiosis, which represents a main histopathologic feature in many dermatomes [8].

The aim of this study was to evaluate the expression of apoptosis markers, cleaved caspase-3, Fas, Bcl-2, NF-κB and p53, in the skin of patients with spongiotic disorders such as atopic dermatitis (AD), allergic contact dermatitis (ACD), irritant contact dermatitis (ICD), nummular eczema (NE) and dyshidrotic eczema (DE) with respect to their role in spongiosis formation.

**Patients and methods**

**Study population**

This case control study was carried out on 50 patients and 10 healthy controls. The patients were selected from patients attending the Dermatology outpatient clinic of Vanderbilt University Hospital, Tennessee State, USA, and Benha University Hospital, Egypt. Patients were divided into five groups, and each group included ten patients:

Group A: AD patients were diagnosed on the basis of Hanifin and Rajka criteria [9].
Group B: ACD was diagnosed clinically and confirmed by patch testing.
Group C: ICD was diagnosed clinically and confirmed by a negative patch test.
Group D and E: NE and DE were diagnosed clinically.

**Inclusion criteria**

Patients with active lesions of any severity were included in the study. Duration of the lesions was less than 6 weeks, and patients were 18 years or older.

**Exclusion criteria**

(1) Patients who were pregnant, using systemic medications within the last 4 weeks or using topical medications within the last week, and those who have received immunotherapy within the last year.
(2) Conditions that impact the biopsy procedure, for example history of bleeding disorders or known lidocaine allergy.
(3) Patients with infected lesions and patients with AD or CD of the face only.
(4) Patients with other illnesses that may affect apoptotic markers in the skin: for example autoimmune disorders or liver and renal diseases.

Informed consent was obtained from all the patients, and the study was approved by both the Ethical Committees of Vanderbilt University, and that of Benha University Hospital.

All the patients and control individuals were subjected to history taking and general and local examination.

**Immunohistologic examination**

Punch skin biopsies (4 mm) were taken from sun-protected skin lesions and fixed in 10% neutral-buffered formalin, routinely processed and paraffin embedded. All biopsies were subjected to histopathologic evaluation to confirm the diagnosis of spongiotic dermatitis.

Immunohistochemical (ABC) staining for cleaved caspase-3, Fas, Bcl-2, NF-κB and p53 antibodies was carried out in the Dermatology Research Laboratory, Dermatology Department, Faculty of Medicine, Vanderbilt University.

**Primary antibodies' specificity and sensitivity**

**Caspase-3**

Cleaved caspase-3 (Asp175) (5A1) (Cell Signaling Technology, Beverly, Massachusetts, USA) is a rabbit monoclonal antibody that detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175. This antibody does not recognize full-length caspase-3 or other cleaved caspases.

**Fas**

Fas (C18C12; Cell Signaling Technology) is a rabbit monoclonal antibody that detects endogenous levels of total human Fas protein.

**Bcl-2**

Bcl-2 (R&D Systems, Minneapolis, Minnesota, USA) is a goat polyclonal antibody that detects endogenous levels of Bcl-2. No cross-reactivity was detected with other family members at physiological levels.

**NF-κB**

NF-κB p65 (E498; Cell Signaling Technology) is a rabbit polyclonal antibody that detects endogenous levels of total NF-κB p65/RelA protein. It does not cross-react with other NF-κB/Rel family members.

**p53**

p53 (DO-2; Santa Cruz Biotechnology, Santa Cruz, California, USA) is a mouse monoclonal antibody raised against amino acids 10–16 of p53 of human origin. The p53 antibody detects endogenous levels of total p53 protein. The antibody does not cross-react with other p53-related proteins.

Biotinylated secondary antibody (Vectastain ABC Kit; Vector Laboratories Inc., Burlingame, California, USA): Antirabbit biotinylated secondary antibody for cleaved caspase-3, Fas and NF-κB. Antimouse biotinylated secondary antibody for p53. Antigoat biotinylated secondary antibody for Bcl-2. Antibody diluent: BSA 10% in PBS. All antibodies were used in a dilution of 1 : 50.
Immunostaining steps
Primary antibody diluted in PBS was applied to each section, incubated overnight at 4°C in a humidity chamber, and then washed with wash buffer three times for 5 min each. Biotinylated secondary antibody (100–400 μl) diluted in PBS as per the manufacturer’s recommendation, was applied to each section, and then incubated 30 min at room temperature. The secondary antibody was removed, and then sections were washed three times with wash buffer for 5 min each. 100–400 μl ABC reagent was added to each section and incubated for 30 min at room temperature. The ABC reagent was removed, and then sections were washed three times in wash buffer for 5 min each. The chromogenic reaction was developed with diaminobenzidine, and all sections were counterstained with Myer’s haematoxylin. All tissue sections were stained under similar conditions to ensure equal staining quality. Positive and negative controls were stained appropriately in the same settings. Negative control sections were treated in the same way as above, using the strain-specific blocking serum while omitting the primary antibody labeling.

Evaluation of immunostaining
The site of the staining was determined (cytoplasmic, nuclear or membranous), and the distribution of positive cells was noted. To semiquantitatively evaluate the expression of antibodies, the numbers of positively stained cell layers was evaluated in accordance with the scoring system devised by Liang et al. [10]. Each score represents the mean value of different fields from three sections of each specimen. This system results in a score ranging from 0 to 3 for both the degree of positivity (percentage of positively stained epidermal cells: 0, <1%; 1, 1–10%; 2, 10–50%; 3, >50%) and the degree of intensity of staining [from faint-brown (score 1) to deep-brown (score 3)]. The sum of the two scores was taken as the level of expression. The results were graded as follows:

- Negative expression: the sum of the two scores is 0.
- Weak expression: the sum of the two scores is 1 or less than 4.
- Moderate expression: the sum of the two scores is 4 or less than 6.
- Strong expression: the sum of the two scores is 6.

Statistical analysis
The statistical analysis system was used for data management and analysis. The significance of the differences was determined using the statistical package Graph Pad Prism, version 3.02, for Windows (Graph Pad Software Inc., San Diego, California, USA). Descriptive statistics were presented as mean ± SDs for continuous variables and number and percentage for categorical variables. Comparisons between groups were performed using a nonparametric analysis of variance (ANOVA) procedure when indicated. Correlations between numeric variables were measured by Pearson’s correlation coefficients. The Student two-tailed t-test was also performed to identify the difference in the mean values. P value less than 0.05 was considered statistically significant.

Results
Clinical results
This study included 50 cases with spongiotic dermatitis (34 male and 16 female), and 10 healthy persons served as the control group (seven males and three females). The mean age of the patient group was 31.56 ± 8.089 years, whereas the mean age of the control group was 29.50 ± 8.127 years. There was no statistically significant difference between the patient group and the control group (P = 0.465). The mean ages of the studied groups were 30.4 ± 10.574, 30.79 ± 8.121, 29.799 ± 7.568, 33.799 ± 6.579 and 33.0 ± 8.027 years in AD, ACD, ICD, NE and DE groups, respectively. Using bivariate statistical analysis, there was no statistically significant difference in the age between the patient subgroups.

The mean duration of lesions was 2.400 ± 1.116, 2.600 ± 1.174, 2.200 ± 1.398, 2.900 ± 1.101 and 2.400 ± 1.265 weeks in AD, ACD, ICD, NE and DE groups, respectively.

Immunohistochemical results
The mean value of cleaved caspase-3 expression was 2.906 ± 0.855, whereas it was 0.430 ± 0.909 in the control group, (Table 1). There was a statistically significant difference between the patient group and the control group (P < 0.001). Cleaved caspase-3 was detectable in the KC of the basal layer of all skin specimens taken from lesional skin as brown nuclear/cytoplasmic staining (Fig. 1a and b). In the spinous cell layer, cleaved caspase-3 was observed in all specimens taken from lesional skin. The strongest positive staining was noticed in areas of spongiosis (Fig. 1b). In contrast, in normal skin, cleaved caspase-3 staining was rarely detectable.

Using the two-way ANOVA test, there was no statistically significant difference in the mean cleaved caspase-3 expression values between different subsets of eczematous dermatitis (P = 0.60). The cleaved caspase-3 expression values decreased in the following order: AD > ACD > ICD > DE > NE (Table 2).

The mean value of Fas expression was 2.630 ± 0.601, whereas it was 0.470 ± 1.004 in the control group (Table 1). There was a statistically significant difference between the patient group and the control group (P < 0.001) in favor of the patient group. Positive Fas expression of KC was observed in lesional skin as brown cytoplasmic/nuclear staining (Fig. 2a and b). In contrast, Fas expression on KC was almost undetectable in normal skin.

Using the two-way ANOVA test, there was no statistically significant difference in the mean Fas expression values between different subsets of eczematous dermatitis (P = 0.14). The Fas expression values decreased in the following order: ACD > AD > NE > ICD > DE (Table 2).
The mean value of Bcl-2 expression was 0.580 ± 0.945 in the patient group, whereas it was 0.470 ± 1.004 in the control group (Table 1). Bcl-2 expression was detected as basal brown nuclear/cytoplasmic staining (Fig. 3). There was no statistically significant difference between the patient group and the control group ($P = 0.740$).

Using the two-way ANOVA test, there was no statistically significant difference in the mean Bcl-2 expression values between different subsets of eczematous dermatitis ($P = 0.68$). The Bcl-2 expression values decreased in the following order: ACD > AD > DE > ICD > NE (Table 2).

### Table 1. Comparison between the patient group and the control group regarding the expression of cleaved caspase-3, Fas, Bcl-2, NF-κB and p53

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=50)</th>
<th>Controls (n=10)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved caspase-3</td>
<td>2.906 ± 0.855</td>
<td>0.430 ± 0.909</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Fas</td>
<td>2.630 ± 0.601</td>
<td>0.470 ± 1.004</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>0.580 ± 0.945</td>
<td>0.470 ± 1.004</td>
<td>0.74</td>
</tr>
<tr>
<td>NF-κB</td>
<td>0.372 ± 0.803</td>
<td>0.630 ± 1.018</td>
<td>0.38</td>
</tr>
<tr>
<td>p53</td>
<td>0.432 ± 0.878</td>
<td>0.400 ± 0.843</td>
<td>0.97</td>
</tr>
</tbody>
</table>

NF-κB, nuclear factor κB.

* $P<0.05$ is considered statistically significant.

### Table 2. Comparison between different patient subgroups regarding cleaved caspase-3, Fas, Bcl-2, NF-κB and p53

<table>
<thead>
<tr>
<th></th>
<th>AD</th>
<th>ACD</th>
<th>ICD</th>
<th>NE</th>
<th>DE</th>
<th>$P$-value</th>
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<tr>
<td>Cleaved caspase-3</td>
<td>3.360</td>
<td>3.070</td>
<td>3.040</td>
<td>2.430</td>
<td>2.630</td>
<td>0.60</td>
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<tr>
<td>Mean</td>
<td>2.560</td>
<td>2.930</td>
<td>2.560</td>
<td>2.600</td>
<td>2.500</td>
<td>0.14</td>
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<tr>
<td>SD</td>
<td>0.497</td>
<td>0.978</td>
<td>0.476</td>
<td>0.416</td>
<td>0.480</td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>0.630</td>
<td>0.670</td>
<td>0.400</td>
<td>0.200</td>
<td>0.600</td>
<td>0.68</td>
</tr>
<tr>
<td>Mean</td>
<td>1.018</td>
<td>1.095</td>
<td>0.843</td>
<td>0.632</td>
<td>0.966</td>
<td></td>
</tr>
<tr>
<td>NF-κB</td>
<td>0.700</td>
<td>0.660</td>
<td>0.200</td>
<td>0.430</td>
<td>0.470</td>
<td>0.76</td>
</tr>
<tr>
<td>Mean</td>
<td>1.139</td>
<td>1.066</td>
<td>0.632</td>
<td>0.909</td>
<td>1.004</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>0.630</td>
<td>0.700</td>
<td>0.200</td>
<td>0.430</td>
<td>0.222</td>
<td>0.30</td>
</tr>
<tr>
<td>Mean</td>
<td>1.018</td>
<td>1.139</td>
<td>0.632</td>
<td>0.909</td>
<td>0.666</td>
<td></td>
</tr>
</tbody>
</table>

ACD, allergic contact dermatitis; AD, atopic dermatitis; DE, dyshidrotic eczema; ICD, irritant contact dermatitis; NE, nummular eczema; NF-κB, nuclear factor κB.

$P > 0.05$ is considered statistically nonsignificant.

### Figures

**Figure 1.**

(a) Staining with an anticleaved caspase-3 antibody reveals (a) positive moderate brown cytoplasmic/nuclear staining of the basal and the suprabasal epidermal layers of lesional irritant contact dermatitis skin with (b) particularly intense staining in spongiotic areas and around spongiotic vesicles (streptavidin/biotin DAB, × 400).

(b) Staining with anti-Fas antibody reveals (a) positive moderate brown cytoplasmic/nuclear staining of the basal and the suprabasal epidermal layers of lesional allergic contact dermatitis skin with (b) particularly intense staining in spongiotic areas and around spongiotic vesicles (streptavidin/biotin DAB, × 400).

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The mean value of NF-κB expression was 0.372 ± 0.803 in the patient group, whereas it was 0.630 ± 1.018 in the control group (Table 1). NF-κB expression was detected as basal brown cytoplasmic staining (Fig. 4). There was no statistically significant difference between the patient group and the control group ($P = 0.379$).

Using the two-way ANOVA test, there was no statistically significant difference in the mean NF-κB expression values between different subsets of eczematous dermatitis ($P = 0.76$). The NF-κB expression values decreased in the following order: AD > ACD > DE > NE > ICD (Table 2).

The mean value of $p53$ expression was 0.432 ± 0.878 in the patient group, whereas it was 0.400 ± 0.843 in the control group (Table 1). $p53$ expression was detected as basal brown nuclear staining (Fig. 5). There was no statistically significant difference between the patient group and the control group ($P = 0.967$).

Using the two-way ANOVA test, there was no statistically significant difference in the mean $p53$ expression values between different subsets of eczematous dermatitis ($P = 0.30$). The $p53$ expression values decreased in the following order: ACD > AD > NE > DE > ICD (Table 2).

**Discussion**

Understanding the mechanisms of apoptosis and other variants of programmed cell death at the molecular level provides deeper insight into various disease processes and may thus influence therapeutic strategy [2].

As caspase-3 is the prime executioner caspase of apoptosis [11], we investigated the in-situ cleavage of caspase-3 in eczematous dermatitis using an mAb that detects cleaved caspase-3. We studied caspase-3 expression because it is a very early and highly specific marker of apoptosis.

In this study, we demonstrated that caspase-3 cleavage occurs in the KC of both the basal and the spinous layers of the epidermis in acute eczematous lesions and these particular high levels of cleaved caspase-3 are present in spongiotic areas. In cases with spongiotic vesicle formation, a single KC positive for cleaved caspase-3 was seen in the suprabasal epidermal layers close to the vesicle. These findings support the view that proapoptotic pathways are activated in the KC of patients with spongiotic disorders with predominant lymphocytes contributing to at least some of the clinical and histological features of the disease [12–14]. T-cell-mediated apoptosis of single KC is a key feature of epidermal pathology in acute eczematous dermatitis.

In contrast to lesional skin, staining of cleaved caspase-3 was weak and restricted to the basal layer in normal skin. These findings are in agreement with previously published work, which suggested that caspase-3 cleavage is limited to the basal layer in normal skin, and it is...
to protect the epidermis against apoptosis by enhancing the expression of anti-apoptotic factors [18]. The effects of NF-κB on apoptosis have far-reaching consequences for normal development and/or homeostasis in many cells and tissues, including the immune system, hair follicles and epidermal appendages [19].

In the present study, there was no statistically significant difference in the p53 expression between the patient and the control group (P = 0.97). Under normal conditions, there is no or minimal p53 expression in sun-protected skin [20]. In the present study, all skin samples were obtained from sun-protected skin. p53 plays a prominent role in the cell response to DNA damage, causing the proliferating cells to enter G1 arrest. At this pause, the cell decides whether to repair the DNA damage or commit suicide [21]. p53 expression in skin diseases has been largely evaluated in relation to UV-induced DNA damage. UV-induced DNA damage activates the mechanisms for the removal of DNA damage, DNA repair or apoptosis by transcriptional activation of p53-related genes. Mutations in the p53 gene have been detected in 50% of all human cancers and in the majority of skin carcinomas [20].

To the best of our knowledge, this study is the first one that investigates the relation between apoptosis and spongiosis in five spongiotic disorders (AD, ACD, ICD, NE and DE). This is also the first study that evaluates the expression of five apoptotic markers in spongiotic dermatitis (cleaved caspase-3, Fas, Bcl-2, NF-κB and p53). The limitation of our study was the small number of patients and controls.

Conclusion

Apoptosis of KC through an extrinsic pathway is the initiating event in the development of the epidermal pathology seen in spongiotic dermatitis. Most notably, KC apoptosis occurs in suprabasal cells, where spongiosis takes place. The knowledge of this molecular basis is pivotal in understanding the development of the pathology in spongiotic disorders, and opens a future for more focused therapeutic applications. Further genetic studies will be needed to confirm these findings and obtain benefit from both caspase-3-targeted and Fas-targeted therapies in the treatment of spongiotic dermatitis. Bcl-2, NF-κB and p53 seem to play a minor role in the KC apoptosis occurring in spongiotic dermatitis.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References
