Croat Med J 2005;46(4):678-684

Expression of p53 and Apoptosis in Discoid Lupus Erythematosus

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Aim To investigate whether p53 expression and apoptosis play a role in the pathogenesis of discoid lupus erythematosus (DLE) and whether they are associated with a hyperproliferative state in the epidermis of DLE lesions and epidermal atrophy.

Methods A total of 70 skin biopsy specimens, 35 DLE and 35 normal, were used. Expression of protein p53 and Ki-67 was examined immunohistochemically. Apoptotic cells were identified by terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling (TUNEL). Histopathological examination of hematoxylin-eosin-stained sections of DLE included analysis and scoring of epidermal atrophy and other histopathological parameters.

Results p53 expression was greater in DLE than in normal skin (14.5 vs 0.6%, $P<0.001$). Ki-67 expression was also greater in DLE than in normal skin (8.0 vs 3.1%, $P<0.001$). A significant positive correlation between p53 and Ki-67 expression was found in both groups (normal skin, $r=0.46$, $P<0.009$; DLE, $r=0.72$, $P<0.001$). A significant negative correlation ($r=-0.75$, $P<0.001$) between Ki-67 expression and the intensity of epidermal atrophy was found. The degree of apoptosis in the epidermis of DLE lesions with higher Ki-67 and p-53 expression was higher than when the expression of these molecules was low (3.0 ± 1.8 vs 1.6 ± 1.5 on a scale from 0-5; $P=0.048$).

Conclusion Aberrant p53 expression occurs in the keratinocytes of DLE and is associated with keratinocyte hyperproliferation and epidermal atrophy. Accumulation of p53 protein, together with an extensive apoptosis, suggests that the activation of a p53-induced apoptotic pathway may play a role in the pathogenesis of skin lesions in DLE.

Lupus erythematosus (LE) is a chronic, autoimmune, and inflammatory disease. The skin is a prominent target in LE (1), and discoid lupus erythematosus (DLE) is the most frequent form of chronic cutaneous LE (CCLE) (2,3).

Apoptosis (4) is known to be closely related to autoimmunity. Beside apoptosis, normal skin architecture is controlled by proliferation and differentiation. Cell proliferation is a way of substituting damaged cells. Ki-67 has been shown to be necessary in cell proliferation, being thus widely used as its marker (5). On the other hand, p53 protein acts as the "guardian of the genome" (6) by arresting cell proliferation when the DNA is damaged, giving the cell time to repair it (6). If the damage cannot be repaired, p53 protein can induce apoptosis (7).

The aim of our study was to investigate the possible role of p53 expression and apoptosis in the pathogenesis of the DLE, together with their

possible association with a hyperproliferative state and epidermal atrophy, in the epidermis of DLE lesions. We also analyzed the presence of various histopathological features in DLE skin samples.

Material and Methods

Skin Samples and Patients

A total of 70 skin biopsy specimens, taken from 70 randomly chosen patients treated at the Dermatovenerology Clinic, Clinical Hospital Center Rijeka, in the period from 1994-2001 were used. To make sure the selection of patients was random, the patients were coded by numbers and then randomly selected by a computer program. Thirty-five specimens were taken from the lesional skin from 35 randomly chosen patients (25 female and 10 male) out of 109 patients suffering from DLE diagnosed clinically and histopathologically and the other 35 were randomly selected from samples of healthy skin surrounding fibromas and hemangiomas totally excised for aesthetic reasons from 35 randomly chosen patients (20 female and 15 male). The median age of DLE patients was 38 years (range: 20-81), and of normal skin patients 58 years (range: 22-82).

Nineteen DLE skin specimens were taken from the head/neck region and 16 from the abdominal/thoracic wall, whereas 25 normal skin samples were taken from the head/neck region and 10 from the abdominal/thoracic wall.

Twelve (out of 35) DLE skin samples were randomly chosen for terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling (TUNEL) method, together with 12 randomly chosen samples of normal skin.

Histopathological Analysis

The samples were fixed in formalin and embedded in paraffin, and 4 μm thick sections were cut using a microtome (Microm HM340E, Walldorf, Germany) and hematoxylin-eosin stained.

Each section was coded by a number and histopathologically examined using the light microscope (Olympus, Tokyo, Japan) at 40× and 200× magnification by two independent pathologists in a blinded fashion. After histopathological examination, the two ratings were compared, and the discrepancy in scoring was resolved by consultation between the examiners.

We analyzed the presence and intensity of 10 histopathological parameters: epidermal atrophy, hyperkeratosis, orthokeratosis, parakeratosis, basal vacuolation, basement membrane thickening, subepidermal edema, type of lymphocyte infiltration (bandlike or perivascular, either in the papillary or reticular dermis). Each histopathological parameter was scored on a scale from 0 to 3 according to Jerdan's criteria (8): 0 – absence; 1 – minimum intensity; 2 – moderate intensity; and 3 – high intensity. Because of their low individual frequency and suitability for the statistical analysis, moderate and high intensity groups had to be joined into the "moderate-high intensity" group.

Immunohistochemistry

Immunostaining for Ki-67 and p53 was performed on both DLE and healthy skin samples, using the anti Ki-67 primary mouse monoclonal antibody (Clone Ki-S5, DAKO, Carpinteria, CA, USA) (1:50 dilution) and anti p53 primary mouse monoclonal antibody (Clone DO-7, isotype IgG2b-kappa, DAKO, Glostrup, Denmark) (1:50 dilution).

Immunostaining was performed using labeled streptavidin biotin (LSAB) visualization system (DAKO Chem Mate Detection Kit, No. 5001). The sections were deparaffinized with xylene and rehydrated in graded alcohols, followed by heat-induced epitope retrieval, which was achieved by immersing slides in 10 mmol/L citrate buffer (pH 6.1; Code No. S2031, DAKO, Glostrup, Denmark) and boiling for 10 minutes in a microwave. Slides were allowed to cool down for 45 minutes. Non-specific antibody binding was blocked with phosphate-buffered saline (PBS) containing normal goat serum (DAKO) for 30 minutes. The sections were incubated overnight at 4°C. After that, the slides were put in the DAKO Tech Mate Horizon automated immunostainer (LJL Biosystems Inc, Sunnyvale, CA, USA) and the Microwave Streptavidin Immunoperoxidase (MSIP) protocol was followed. 3,3-Diaminobenzidine was used as the chromogen. Substitution of the primary antibody with an isotype-matched IgG and omission of the primary antibody served as negative controls. As a positive control for the p53 staining, sections from a lung carcinoma previously shown to be strongly positive for p53 were used. With Ki-67, positive staining of chromatin in dividing cells was used as an internal positive control.

The slides were coded by numbers and evaluated in a blinded fashion by two independent pathologists, using light microscope (Olym-

pus, Tokyo, Japan), at 200× magnification. Keratinocytes displaying a strong nuclear staining were considered to be positive. The number of positive keratinocytes was expressed as a percentage of 500 counted keratinocytes.

TUNEL Staining

Apoptotic cells were identified by the TUNEL method, using the In-situ Cell Death Detection Kit-AP (Roche, Penzberg, Germany). Briefly, paraffin sections were deparaffinized in xylene and absolute ethyl alcohol and were washed and incubated sequentially according to the manufacturer's recommendations. Nuclear Fast Red tablets (Roche) were used as the chromogen. Negative controls were performed by omitting TdT from the reaction.

The degree of apoptosis was assessed on a six-degree scale, as follows: 0 – no staining, 1 – sporadic staining (<5%), 2 – minimal staining (5-25%), 3 – moderate staining (26-50%), 4 – pronounced staining (51-75%), and 5 – whole epidermis stained (>75%) (9). The number of positive keratinocytes was expressed as a percentage of the total 1,000 keratinocytes counted (apoptotic index).

Statistical Analysis

The inter rater agreement of the two pathologists was kappa=0.68. The analysis of the presence and the degree of various histopathological features was performed using Pearson, χ^2-test or Fisher exact test. The results were presented as the mean±standard deviation or median and range (5th-95th percentile boundaries), depending on the normality of distribution. When the distribution of p53 and Ki-67 expression scores did not statistically differ from the normal distribution, we used parametric tests – t test for independent samples to compare average positively stained cells number in normal skin and DLE samples, and one-way ANOVA to test the differences in p53 and ki-67 expression between gender groups and the differences in average value of the apoptotic index in normal and DLE skin. Mann-Whitney U test was used for the comparison of data that were not normally distributed. The correlation analyses were expressed by Pearson correlation coefficient or Spearman correlation coefficient, depending on the data.

All statistical values were considered significant at the P level of <0.05. Statistical analysis of data was performed using Statistica for Windows, release 6.1 (StatSoft, Inc., Tulsa, OK, USA) and MedCalc, release 7.5 (MedCalc, Maraikerke, Belgium).

Results

The average percent of p53 positive keratinocytes in the DLE epidermis was 14.5±6.1% (mean±standard deviation), compared with 0.6±0.4% in the normal skin epidermis (P<0.001, t test for independent samples). There was no statistically significant difference in the p53 expression according to the gender of the patients, in both normal (P=0.935, one-way ANOVA) and DLE skin (P=0.700, one-way ANOVA) groups. Higher levels of p53 expression were found in the samples taken from the head/neck region (Table 1). All normal skin samples containing p53 positive nuclei were taken from the head/neck region. This difference in p53 expression according to the body region, was statistically significant (P=0.001, Mann-Whitney U test). The correlation between p53 expression and the age of the patients was positive in both normal skin (Pearson's r=0.486, P=0.004) and DLE group (r=0.801, P<0.001). Elderly patients showed higher p53 expression. p53 positive keratinocytes were irregularly distributed throughout the epidermis of DLE skin, with clustering in the parts with hyper- and parakeratosis, severe vacuolation of the basal layer, and severe lymphocyte infiltration just beneath the dermo-epidermal border (Fig. 1). More p53 positive keratinocytes were found in the follicular epithelium than in the interfollicular epidermis. Considering the epidermal layer, p53 positive nuclei were mostly located in the basal layer and only few were found in the suprabasal layer.

Table 1. Ki-67 and p53 positive keratinocytes in normal and discoid lupus erythematosus (DLE) skin

	No. of samples		Percent of Ki-67 positive keratinocytes (median, 5th-95th percentile)		Percent of p53 positive keratinocytes (median, 5th-95th percentile)	
Body region	normal skin	DLE skin	normal skin	DLE skin	normal skin	DLE skin
Head/neck	25	19	6.2 (3.2-9.2)	8.8 (4.7-13.0)	0.5 (0.0-1.2)	16.6 (2.3-31.0)*
Abdominal/thoracic wall	10	16	2.6 (1.4-2.8)	3.8 (1.2-6.5)*	0 (0.0-0.0)	4.6 (1.4-7.8)†

*P<0.001 vs normal skin, Mann-Whitney U test.
†P<0.05 vs normal skin, Mann-Whitney U test.

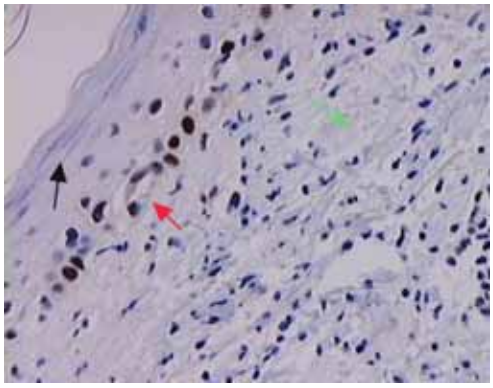

Figure 1. p53 positive keratinocytes in discoid lupus erythematosus epidermis with hyper- and parakeratosis (black arrow), vacuolation of the basal layer (red arrow), and lymphocyte infiltration with subepidermal edema (green arrow) (×200).

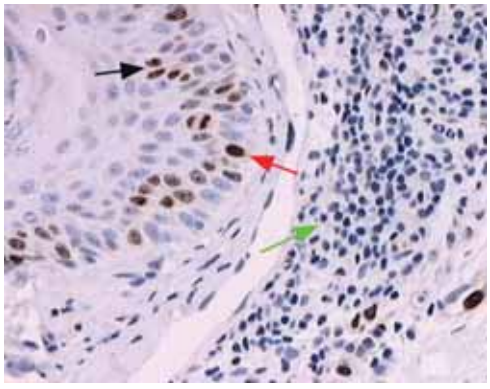

Figure 2. Ki-67 positive keratinocytes mostly in the basal layer (red arrow) and some in the suprabasal layer (black arrow) of discoid lupus erythematosus epidermis, with lymphocyte infiltration just beneath the basement membrane (green arrow) (×200).

In normal skin, p53 positive keratinocytes were scarce and present in only 3 samples.

The average percent of Ki-67 positive keratinocytes (proliferation rate) was 8.0±3.6% (mean±SD) in the DLE epidermis, compared with 3.1±1.7% in the normal skin epidermis ($P<0.001$, t test for independent samples). In both normal skin and DLE groups, Ki-67 expression did not differ significantly according to the gender of patients, but the difference according to the body region from where the skin samples had been taken was statistically significant in both groups (normal skin: $P<0.001$, DLE: $P<0.001$, Mann-Whitney U test). Higher levels of Ki-67 expression were found in the samples taken from the head/neck region (Table 1). The Ki-67 expression and age of the patients showed a positive correlation in both normal skin and DLE groups. In the normal skin group, the correlation was present but not statistically significant (Pearson's $r=0.346$, $P=0.066$), whereas it was significant in the DLE group (Pearson's $r=0.646$, $P<0.001$). Elderly DLE patients showed higher Ki-67 expression in the epidermis. Epidermal atrophy in DLE skin was negatively correlated with Ki-67 expression (Spearman's $\rho=-0.75$, $P<0.001$). Ki-67 positive nuclei in DLE skin were uniformly distributed in the basal layer of the interfollicular epidermis and follicular epithelium. Some of the Ki-67 positive nuclei were also observed in the suprabasal layer (Fig. 2).

The correlation between p53 expression and the Ki-67 expression was positive and statistically significant in both normal skin (Pearson's $r=0.46$, $P=0.009$) and DLE groups (Pearson's $r=0.72$, $P<0.001$). The significantly higher level of p53 expression ($P=0.005$, one-way ANOVA) was observed in the DLE epidermis with the proliferation rate >6% (Table 2).

Table 2. p53 positive keratinocytes according to the percentage of Ki-67 positive keratinocytes in discoid lupus erythematosus epidermis

Percent of Ki-67 positive keratinocytes	Percent of p53 positive keratinocytes (mean±SD)
<6 (n=7)	9.7±3.7
>6 (n=28)	17.0±6.2*

*P=0.005, one-way ANOVA test.

In the normal skin samples, TUNEL-positive nuclei were located mostly in the upper layers of the epidermis. The DLE samples showed an extensive apoptosis in both basal and spinous layers of the epidermis. The average value of the apoptotic index was 56.9±15.1% in the DLE and 3.0±2.2% in normal skin samples ($P<0.001$, one-way ANOVA). In normal skin epidermis, only the apoptotic scores of 0 and 1 degree were observed, whereas the DLE epidermis had the apoptotic score of 2 and above (Fig. 3). The average degree of the apoptosis in the DLE epidermis was 3.7±2.0. The value of the apoptotic index in the DLE epidermis was positively correlated with the intensity of the epidermal atrophy (Spearman's $\rho=0.56$, $P=0.04$).

The higher level of p53 expression was found to be significantly associated with the

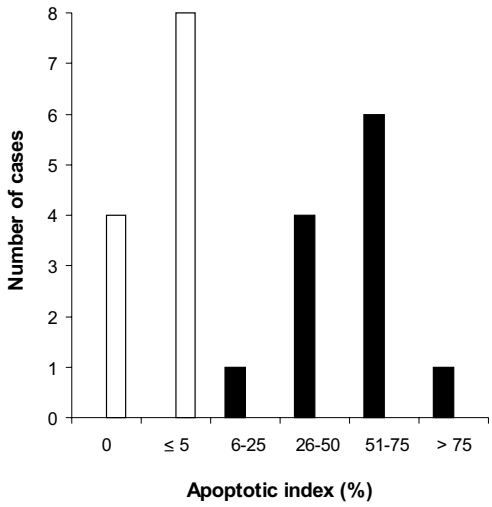

Figure 3. Apoptotic index of the normal skin and discoid lupus erythematosus samples. Open bars – normal skin; closed bars – discoid lupus erythematosus.

higher level of Ki-67 expression (P=0.04, one-way ANOVA) (Table 3). In addition, these higher levels were significantly associated with the higher degree of apoptosis (P=0.048, one-way ANOVA).

Histopathological analysis revealed that hyperkeratosis and perivascular lymphocyte infiltration in the papillary dermis were more common features (Table 4) than all other histopathological features in the DLE skin samples (P<0.05, Fisher exact test). Parakeratosis and bandlike lymphocyte infiltration were less common features (Table 4) than the epidermal atrophy, orthokeratosis, basement membrane thickening, basal vacuolation, perivascular lymphocyte infiltration in the reticular dermis and subepidermal edema ($\chi^2=44.76$, P<0.001).

Table 3. Degree of apoptosis* and the percentage of Ki-67 positive keratinocytes (mean±standard deviation) according to the percentage of p53 positive keratinocytes in discoid lupus erythematosus epidermis

Percent of p53 positive keratinocytes	Percent of Ki-67 positive keratinocytes	Degree of apoptosis
<8 (n=3)	3.2±2.9	1.6±1.5
>8 (n=9)	8.8±3.4†	3.0±1.8‡

*According to ref. 9; on a scale from 0-5.
†*P*=0.040, one-way ANOVA test.
‡*P*=0.048, one-way ANOVA test.

Discussion

We found aberrant p53 expression in DLE keratinocytes and established its positive correlation with the keratinocyte hyperproliferation and an extensive apoptosis found in the DLE epidermis.

The expression of p53 in the normal skin is, due to its short half-life, below the values detectable by immunohistochemistry in paraffin-embedded tissue samples (10). Some studies did not find a single p53 positive keratinocyte in the normal skin epidermis (11), whereas others found them to be scarce (12), as we did in our study. The presence of p53 positive keratinocytes in normal skin samples could be due to the DNA damage caused by UVB radiation, resulting in a temporary increase of the normal p53 protein (13,14) or due to the p53 mutations, resulting in a permanent expression of non-functional p53 protein (15). These hypotheses are supported by our findings that all normal skin samples containing p53 positive keratinocytes, together with the majority of DLE samples, were from the head/neck region, which is a sun exposed area, and that older age of patients, in both normal skin and DLE groups, is accompanied by higher p53 and Ki-67 expression, which is similar to the results of Liang et al (16). On the other hand, some authors found no significant correlation between p53 expression and the age and sun exposure (17). The irregular distribution pattern of the p53 positive keratinocytes, mostly in the basal and suprabasal layer of the DLE epidermis, with

Table 4. Frequency of histopathological features in discoid lupus erythematosus skin samples (n=35)

Histopathological feature	Expression		
	0 (absence)	1 (minimum intensity)	2 and 3 (moderate/high intensity)
Epidermal atrophy	7	20	8
Hyperkeratosis	1	15	19
Lymphocyte infiltration (perivascular-papillary dermis)	1	14	20
Orthokeratosis	4	18	13
Parakeratosis	21	7	7
Basal vacuolation	9	11	15
Basement membrane thickening	6	11	18
Subepidermal edema	7	18	10
Lymphocyte infiltration (bandlike)	15	13	7
Lymphocyte infiltration (perivascular-reticular dermis)	8	10	17

higher p53 expression in the follicular epithelium than in the interfollicular epidermis, is similar to the results of the other studies (11,12).

In 1998, Chung et al (11) presented the first report on p53 expression in the CLE epidermis. In their study, skin samples of various subtypes of CLE were analyzed, but the immunohistochemical results were not divided by subtypes but presented for the CLE as a single form. They found much higher p53 expression in the CLE epidermis than in normal skin. We found similar results for the p53 expression in the DLE epidermis, suggesting its role in the pathogenesis of DLE. Considering the fact that p53 protein is important in the proliferation regulation (18), its overexpression could be an attempt to down-regulate the hyperproliferation. We established a positive correlation between the hyperproliferation and p53 overexpression, similar to the results of other authors (10-12).

De Jong et al (19) found a hyperproliferative state in the DLE epidermis. Our study confirmed it on a much greater number of samples and showed significantly higher proliferation rate in DLE than in the normal skin. In normal skin, we found a low proliferative rate of 3.2%, which is similar to the other studies (10). The pattern of Ki-67 expression, mostly in the basal layer of the epidermis, as we found it, is similar to the one found in other studies (10,11,19). The presence of a few Ki-67 positive keratinocytes in the suprabasal layer could be explained as an extension of the germinative pool. The hyperproliferative state might be an attempt to replace the damaged keratinocytes from the basal layer. This reaction seems to stop ultimately and result in epidermal atrophy. This is supported by our finding of significant negative correlation between the epidermal atrophy and proliferation rate. De Jong et al (19) also found such correlation, but on a much smaller series and without stating the exact correlation r and P value, which would allow us to compare the results.

We found epidermal atrophy in 4/5 of DLE samples, mostly with the minimum intensity, similar to some authors (11), whereas others scored it absent in as much as 42% of DLE samples (20). As for other histopathological characteristics, perivascular infiltrate in the papillary dermis and basement membrane thickening were the most frequent features and parakeratosis was the rarest (Table 1), which is similar to the findings of Chung et al (11).

Overexpression of p53 could reflect activation of an apoptotic pathway, which we confirmed by the TUNEL method. The degree of apoptosis in the DLE epidermis was significantly higher than in normal skin, which is similar to the literature findings (11,12). Moreover, we found TUNEL positive keratinocytes mostly in the upper layers of the normal skin epidermis, whereas they were located basally or suprabasally in the DLE samples. The same was found by Baima et al (9).

In conclusion, basal keratinocyte proliferation may be activated to substitute damaged keratinocytes, as indicated by Ki-67 overexpression. Hyperproliferation may be followed by p53 overexpression to downregulate proliferation by activating apoptosis as a compensatory mechanism. The balance between the keratinocyte death by apoptosis and hyperproliferation might eventually be lost, resulting in an extensive apoptosis and epidermal atrophy. The relationship among the higher degree of apoptosis, p53 overexpression, hyperproliferation, and epidermal atrophy in our study suggests their possible common involvement in the pathogenesis of DLE.

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Received: November 19, 2004
Accepted: April 11, 2005

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