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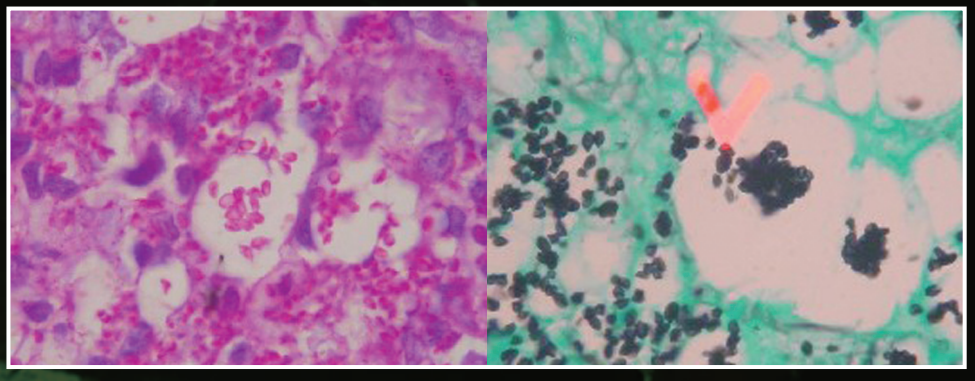

Multiple intracellular rounded yeasts surrounded with the halo in dermis in disseminated cutaneous histoplasmosis

Highlights of the issue

- Nanotechnology in medicine and relevance to dermatology
- Epidermal Fas expression in unexposed aged vs young skin
- SJS and TEN in children, causative drugs, clinical outcome
- Mucocutaneous and demographic features of systemic sclerosis
- Disseminated cutaneous histoplasmosis in an immunocompetent adult
- Terminal 4q deletion syndrome

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Expression of Apoptosis Regulatory Markers in the Skin of Advanced Hepatitis-C Virus Liver Patients

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Abstract

Background: Hepatitis-C virus (HCV) infection is considered a major worldwide public health problem with a global prevalence. Maintenance of skin homeostasis requires a delicate balance between proliferation, differentiation, and apoptosis. Meanwhile, it is unclear if there is an altered keratinocyte proliferation/apoptosis balance in advanced liver disease with HCV infection. **Aim:** This work aimed to evaluate the epidermal thickness and changes in the expression of apoptosis regulatory markers as well as apoptotic index in skin samples of advanced HCV liver patients compared to normal controls. **Materials and Methods:** Twenty biopsies were taken from apparently normal skin of advanced HCV liver disease patients, as well as five healthy control subjects. These specimens were used for histometric epidermal measurement, immunohistochemical staining of apoptosis regulatory proteins (Bax, Fas, p53, Caspase-3, Bcl-2, Bcl-xL) as well as the TUNEL technique for detection of apoptotic cells. **Results:** The mean epidermal thickness was significantly lower than the control group (P=0.000). There were significant overexpression of pro-apoptotic markers (Bax, Fas, P53, and Caspase-3) in patients (P=0.03, 0.03, 0.003, 0.003 respectively), with increased apoptotic index in HCV liver patients (P=0.002) when compared to normal controls. On the other hand, no statistically significant difference were encountered in the expression of antiapoptotic markers (Bcl-2, Bcl-xL) in HCV patients when compared to normal controls (P=0.5, 0.9, respectively). **Conclusion:** These findings suggest that an alteration in the proliferation/apoptosis balance is present in the skin of HCV liver patients.

Key Words: *Apoptosis, Bcl-2, epidermal thickness, hepatitis-C virus, liver disease*

Introduction

The liver is the largest internal organ in the body and performs many important functions, so it is not surprising that diseases of the liver are a major cause of morbidity and mortality throughout the world.[1]

Among the most common liver disorders is hepatitis-C virus (HCV) infection.[1,2] HCV infection is considered a major worldwide public health problem with a global prevalence of 3% (lower in Europe ~1.03% and Americas ~1.7% and highest in Africa ~5.3%).[3] In Egypt, about 10-13% of the population is infected with HCV,[4] meanwhile, HCV antibody prevalence among blood donors ranged from 6% to 38% with a mean of 15%.[5]

HCV, the most common cause of viral hepatitis, may present with skin manifestations in the early stages of the disease. Skin manifestations could be the first signal of HCV, even in the absence of hepatic symptoms.[6] The skin manifestations of HCV include lichen planus (LP), necrolytic acral erythema (NAE), porphyria cutanea tarda, mixed cryoglobulinemia, polyarteritis nodosa,[7] psoriasis, erythema multiforme, pyoderma gangrenosum, and dermatomyositis.[8]

Cell death can be separated into two distinct forms: Necrotic death and programmed death or apoptosis.[9,10] Necrosis results from massive cell injury and is often accompanied by inflammation. On the other hand, apoptosis is a programmed death, occurring in response to either external or internal stimuli. It plays an important role in the destruction of unwanted cells during development. It also has a role as a balancing factor in maintaining proliferative homeostasis.[11]

Increasing evidence suggests that most mammalian cells exist in a state of unstable equilibrium, poised either to proliferate or die depending on the balance between influencing factors. Thus, most if not all of the cell death machinery may be present at all times. Whether it actually gets used depends on the physiological status of one or more check points that regulate the decision whether to divide, differentiate, and initiate DNA repair, or eliminate the cells by apoptosis.[11]

Apoptosis is an essential strategy of dynamic balance in the living system to maintain homeostasis.[12] It is generally believed that the ratio of proapoptotic to antiapoptotic Bcl-2 family proteins is the critical determinant of cell fate.[13]

The Bcl-2 family of proteins contains both proapoptotic (Bax) and antiapoptotic (Bcl-2, Bcl-xL) proteins, which have special significance since they can determine if the cell commits to apoptosis or aborts the process.[14]

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The tumor suppressor protein p53 is a nuclear phosphoprotein that, in its natural form (wild-type), can bind to DNA and prevent cells from entering the S (synthesis) phase of the cell cycle until repair of DNA damage, or alternatively eliminate the cells by sending them down an irreversible apoptotic pathway.[15,16] This tumor suppressor protein p53 has a critical role in regulation of the Bcl-2 family of proteins.[17] Under normal conditions there is no or minimal p53 expression in the sun-protected skin. However, significantly higher p53 expression has been reported in sun-exposed and photo-aged skin as a result of sun-induced DNA damage.[18,19]

Fas "death receptor" is a transmembrane receptor that is a member of TNF/nerve growth factor (NGF) receptor superfamily.[20,21] Ligation of Fas by Fas-L induces apoptosis of Fas-expressing cells.[22,23] Activation of the execution caspases is considered the final pathway of apoptosis.[24] Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10).[25]

To the best of our knowledge, no previous research has investigated the effect of advanced liver disease due to HCV infection, with its biochemical, metabolic, and hormonal alterations on the skin, regarding changes in apoptosis and its regulatory proteins.

The present work aims to investigate the effect of advanced HCV liver disease on epidermal thickness, changes in the expression of various proapoptotic (Bax, Fas, p53, Caspase-3), and antiapoptotic (Bcl-2, Bcl-xL) regulatory markers as well as the TUNEL technique for identification of apoptotic cells in skin samples of liver patients in comparison with normal healthy control subjects.

Materials and Methods

The present study was conducted on 20 patients (13 males and 7 females), with advanced liver disease (HCV) attending the out-patient clinic of Dermatology, STD's, and Andrology department and both the out-patient clinic and the in-patient section of Internal Medicine Department, Al-Minya University Hospital. Age of patients ranged from 22 to 70 years with a mean and SD of 47.7±11.63.

After informed consent, 4-mm punch biopsies were obtained from apparently normal skin of the thigh of 20 HCV patients with advanced liver disease. The study was approved by the committee for postgraduate studies and research of Al-Minya University.

Control samples were obtained from the thighs of five healthy subjects (three males and two females), performing dermatosurgical procedures for another condition, and serving as a normal control group after taking an informed consent. The age of the control subjects ranged from 30 to 55 years, with a mean age of 40.4±9.4.

Biopsies were fixed in buffered 10% formalin, embedded in paraffin, sectioned into 5-µm thick sections, and stained with hematoxylin and eosin (H&E) for histometric analysis. Paraffin sections were also utilized for immunohistochemical examination of apoptosis regulatory markers (Bax, Fas, p53, Caspase-3, Bcl-2, Bcl-xL) as well as the TUNEL technique for identification of apoptotic cells.

Histological measurement of epidermal thickness (Histometry)

A computer-assisted program (analySIS® Five by Olympus Soft Imaging Solutions GmbH, Johann—Krane-Weg 39, D-48149 Münster, Germany) was employed to measure epidermal thickness in H&E stained sections of biopsy specimens. The mean distance between the outermost surfaces of the epidermis, excluding stratum corneum, and the dermo-epidermal junction through the entire length of three-examined sections was determined at multiple points.

Immunohistochemical staining of apoptosis regulatory markers (Bax, Fas, p53, Caspase-3, Bcl-2, Bcl-xL)

For evaluation of Bax and Fas expression in the keratinocytes, sections were stained by the primary antibodies [(Bax: code no.: E3381, ready-to-use rabbit antibody, SPRING BIOSCIENCE, Fermont, CA 94538, USA), (Fas: code no.: M3554, Mouse monoclonal antihuman antibody, Dako, Carpinteria, 00CA, USA: at a dilution of 1:30 with the HRP method)]. The ready-to-use LSAB2 peroxidase, DAB detection system (code no.: K0673, DAKO, Carpinteria, CA, USA) was used to demonstrate antibodies expression according to the manufacturer's instructions.

For evaluation of p53, Caspase-3, Bcl-2, and Bcl-xL expression in the keratinocytes, sections were stained by the primary antibodies [(p53: code no.: RMPD 016, ready-to-use rabbit monoclonal antibody, DBS, CA, USA), (Caspase-3: code no.: MS1770'R7, ready-to-use antibody, Thermoscientific, CA, USA), (Bcl-2: code no.: PDM 016, ready-to-use monoclonal mouse antibody, DBS, CA, USA), (Bcl-xL: code no.: #MS-1334-PO, Mouse monoclonal antihuman antibody, Thermoscientific, CA, USA: at a dilution of 1:200 with the HRP method)]. The ready-to-use detection system, universal HRP immunostaining kit (code no.: Kp-DBS, CA, USA) was used to demonstrate antibodies' expression according to the manufacturer's instructions. Negative control sections had the same blocking serum instead of primary antibody labeling. All tissue sections were stained under similar conditions to ensure equal staining quality.

The level of apoptosis regulatory markers' expression in keratinocytes was evaluated, by two blinded histopathologists, in accordance with the scoring system devised by Liang *et al*.[26] 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.

Detection of apoptotic cells by the TUNEL technique was performed using a universal apoptosis detection kit which contains equilibrium buffer, Biotin-11-Dutp, TdT Enzyme, Streptavidin-HRP, and DAB (code: KGA7031, KeyGen Biotech 3F, 15 Block No.439 Changhong Road, Nanjing, China) to detect apoptotic keratinocytes. Apoptotic index was then calculated:[27]

$$\text{Apoptotic index (\%)} = \frac{\text{No. of TUNEL positive cells}}{\text{Total No. of cells counted}} \times 100$$

Statistical analysis

Data were coded, entered, and analyzed using an SPSS Software package for statistical science (SPSS for Windows, Version 16.0.1, SPSS Inc., USA). Statistical analysis included descriptive analysis as mean value and standard deviation (SD), independent-samples *t*-test and correlation coefficient (r) for the results. Significance was expressed in terms of *P*-value and the level of significance was 0.05.

Results

Histological evaluation of epidermal thickness (Histometry)

The epidermal thickness of liver disease patients ranged from 39.6 to 55.4 µm with a mean of 46.2 µm±4.4. On the other hand, the epidermal thickness of the control group ranged from 57.4 to 75 µm with a mean of 64±7 µm. The epidermis of liver disease patients showed significantly lower thickness than the control group (*P*=0.000) [Table 1, Figure 1].

Immunohistochemical Results

Expression of proapoptotic markers in epidermal keratinocytes

1. Bax expression

Cytoplasmic staining was observed in all Bax positive specimens of both patients and controls. In liver disease patients, the stain was observed in both basal and squamous cell layers with a score ranging from 1.6 to 3.8 with a mean of 3±0.6. In controls, staining was mainly confined to the basal cell layer with a range from 0.2 to 2.5 with a mean of 2.3±0.1. The former was significantly higher than the control group (*P*=0.03) [Table 1, Figures 2 and 3]. Bax expression was inversely correlated with epidermal thickness (*P*=0.01).

2. Fas expression

Membranous staining was observed in all Fas-positive specimens. In liver patients, the stain was observed in the basal and lower 2/3 of squamous cell layers with a score ranging from 1.7 to 3.8, with a mean of 2.7±0.6. In the control group, the stain was mainly confined to the basal cell layer and it ranged from 1.5 to 2 with a mean of 1.7±0.2. Fas expression in liver patients was significantly higher than control group (*P*=0.003) [Table 1, Figures 2 and 3]. Fas was inversely correlated with epidermal thickness (*P*=0.04).

3. P53 expression

Nuclear staining was observed in all P53 positive specimens. In liver disease patients, the stain was observed

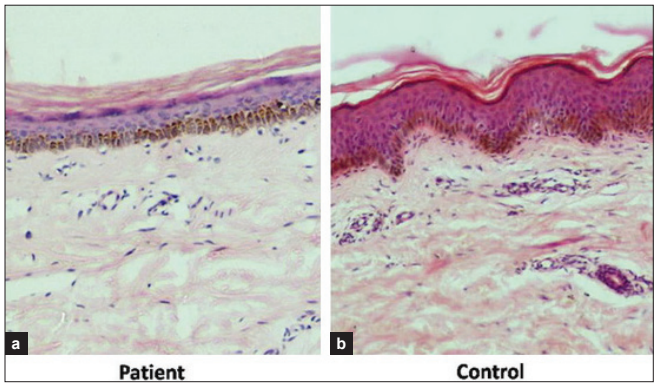

Figure 1: The epidermis of advanced HCV liver disease patients (a) is lower in thickness when compared to controls (b) (H and E, ×100)

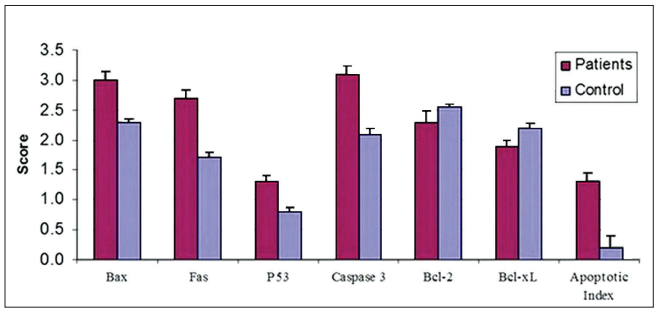

Figure 2: Expression of pro-apoptotic (Bax, Fas, P53, Caspase-3) and anti-apoptotic (Bcl-2, Bcl-xL) markers, and apoptotic index in HCV liver patients and controls

Table 1: Mean value of epidermal thickness, score of pro-apoptotic (Bax, Fas, P53 and Caspase-3) and anti-apoptotic markers (Bcl-2 and Bcl-xL) and apoptotic index in HCV liver patients and controls

Category	Epidermal thickness (µm)	Bax	Fas	P53	Caspase-3	Bcl-2	Bcl-xL	Apoptotic index
Patients	46.2±4.4	3±0.6	2.7±0.6	1.3±0.5	3.1±0.6	2.3±0.8	1.9±0.5	1.3±0.7
Controls	64±7	2.3±0.1	1.7±0.2	0.8±0.2	2.1±0.2	2.56±0.1	2.2±0.1	0.2±0.4
P-value	0.000	0.03	0.003	0.03	0.003	0.5	0.9	0.002

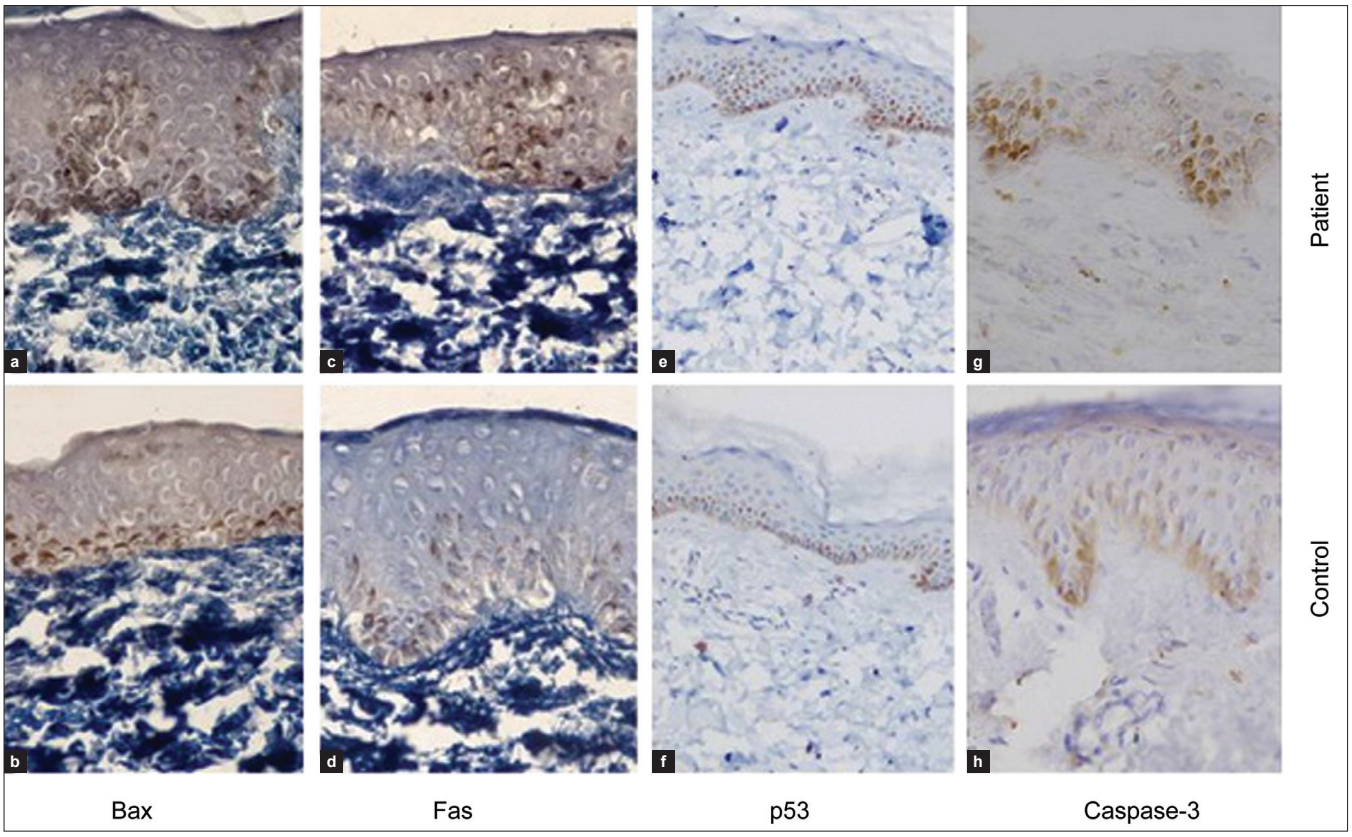

Figure 3: Expression of pro-apoptotic markers (Bax, Fas, p53, Caspase-3) in epidermal keratinocytes of advanced HCV liver disease patients (a,c,e,g) and in controls (b,d,f,h) respectively showing the following:

- **Significant increase of cytoplasmic expression of Bax staining in basal and squamous cell layers in patients (a) when compared to that confined to the basal cell layer of controls (b) (Immunohistochemical, ×400).**
- **Significant increase of membranous expression of Fas staining in basal and lower 2/3 of squamous cell layers in patients (c) when compared to that confined to the basal cell layer of controls (d) (Immunohistochemical, ×400).**
- **Significant increase of nuclear expression of P53 staining in basal and lower 2/3 of squamous cell layers in patients (e) when compared to that confined to the basal cell layer of controls (f) (Immunohistochemical, ×200).**
- **Significant increase of cytoplasmic expression of Caspase 3 staining in basal and squamous cell layers in patients (g) when compared to minimal staining confined to the basal cell layer of controls (h) (Immunohistochemical, ×400)**

in the basal and lower 2/3 of squamous cell layers with score ranging from 0.6 to 1.9 with a mean of 1.3±0.5. In control group, the positive staining was mainly seen in the basal cell layer and it showed a score ranging from 0.7 to 1.1 with a mean of 0.8±0.2. P53 expression in liver patients was significantly higher than control group (P=0.03) [Table 1, Figures 2 and 3]. P53 was positively correlated with Bax (P=0.000), Caspase-3 (P=0.04) and apoptotic index (P=0.000). While, it was inversely correlated with epidermal thickness (P=0.002).

4. Caspase-3 expression

Cytoplasmic staining was observed in all Caspase-3 positive specimens of both patients and controls. In liver disease patients the positively stained cells were observed in the basal and squamous cell layers with a score ranging from 2.3 to 4.3 with a mean of 3.1±0.6. In the control group, minimal staining was noticed in the basal cell layer with a score ranging from 1.8 to 2.4 with a mean of 2.1±0.2. The marker was significantly higher in patients than controls (P=0.003) [Table 1, Figures 2 and 3]. Caspase-3 was positively correlated with Bax (P=0.05) and P53 (P=0.04), while it was inversely correlated with epidermal thickness (P=0.002).

Expression of Antiapoptotic Markers in Epidermal Keratinocytes

1. Bcl-2 expression

Cytoplasmic staining was observed in all Bcl-2 positive specimens of both patients and controls. In the liver patients group, the positive cells were observed in basal cell layer with a score ranging from 0.5 to 3.2 with a mean of 2.3±0.8. In the control group, the stain was mainly confined to basal layer showing a score ranging from 2.4 to 2.6 with a mean of 2.56±0.1. There was no statistically significant difference between the two groups (P=0.5) [Table 1, Figures 2 and 4]. Bcl-2 expression was positively correlated with epidermal thickness (P=0.03). While, it was inversely correlated with Caspase-3 expression (P=0.001).

2. Bcl-xL expression

Perinuclear cytoplasmic staining was observed in all Bcl-xL positive specimens of patients and controls. In liver

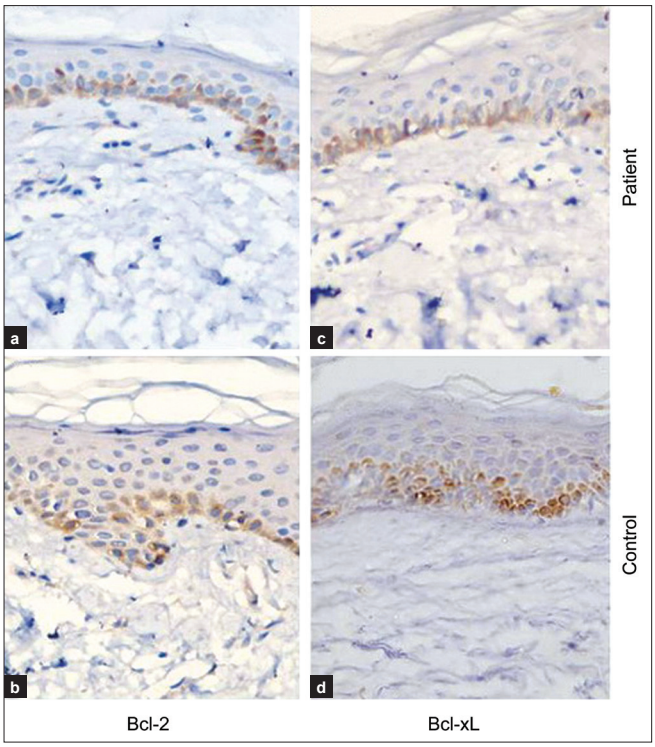

Figure 4: Expression of anti-apoptotic markers (Bcl-2, Bcl-xL) in epidermal keratinocytes of advanced HCV liver disease patients (a,c) and in controls (b,d):

- **Insiginificant lower cytoplasmic expression of Bcl-2 staining, mainly confined to the basal cell layer, in patients (a) when compared to controls (b).**
- **Insiginificant lower perinuclear cytoplasmic expression of Bcl-xL staining, mainly confined to the basal cell layer, in patients (c) when compared to that confined to the basal and supra basal cell layer of controls (d) (Immunohistochemical, ×400).**

disease patients, the positively stained cells were observed in the basal cell layer with a range from 1 to 2.5 with a mean of 1.9±0.5. In control subjects, they were also observed mainly in the basal cell layer; however, in some specimens positive suprabasal cells were also noticed. The score ranged from 2 to 2.4 with a mean of 2.2±0.1. No statistically significant difference was found between the two groups (P=0.9) [Table 1, Figures 2 and 4].

Detection of Apoptotic Cells (TUNEL)

The TUNEL method was used to detect apoptotic cells in epidermal keratinocytes. Apoptotic cells were demonstrated in basal and squamous cell layers in HCV liver patients with an apoptotic index ranging from 0 to 2 with a mean of 1.3±0.7. Control subjects showed few apoptotic cells, if present, with an apoptotic index ranging from 0-1 with a mean of 0.2±0.4. The apoptotic index in liver disease patients was significantly higher than the control group (P=0.002) [Table 1, Figures 2 and 5]. Apoptotic index was positively correlated with Bax (P=0.001) and P53 expression (P=0.000), while it was inversely correlated with epidermal thickness (P=0.001).

Discussion

Programmed cell death (apoptosis) is an essential strategy

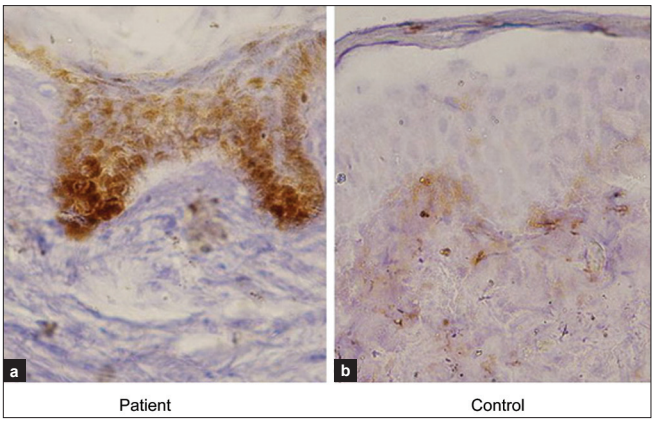

Figure 5: Many apoptotic cells present in basal and squamous cell layer in advanced HCV liver disease patients (a), compared to few apoptotic cells seen in controls (b) (TUNEL, ×400)

of dynamic balance in the living system. Homeostasis is maintained by the balance between cell proliferation and cell death.[12] Apoptosis is the major mechanism by which homeostasis of a number of physiological systems in the body is regulated. Although apoptosis is an intrinsic process present in all cells, it can be regulated by extrinsic factors including growth factors, cell surface receptors, cellular stress, and hormones.[28]

Apoptosis is morphologically and biochemically characterized by cell shrinkage, dense chromatin condensation, cellular budding, fragmentation, rapid phagocytosis by nearby cells, and DNA fragmentation. In the skin, there is considerable evidence that apoptosis plays an important role in the pathogenesis of a wide variety of skin diseases.[12]

Death of hepatocytes and other hepatic cell types is a characteristic feature of liver diseases as cholestasis, viral hepatitis, ischemia/reperfusion, liver preservation for transplantation, and drug/toxicant-induced injury. Cell death typically follows one of two patterns: oncotic necrosis and apoptosis.[29] Meanwhile, skin diseases characterized by increased apoptosis (LP) or necrosis (NAE) of keratinocytes were previously reported in HCV liver disease patients.

Overactivation of the apoptotic process can lead to significant hepatocellular damage, while inhibition of apoptosis can promote the proliferation and transformation of cells.[30] For example, apoptosis induction in infected liver cells, by HBV or HCV, is a defense mechanism to limit viral replication and promote their elimination. Various pathways are implicated in this process, including Fas and TNF systems.[31] Fas concentrations are correlated with viral hepatitis activity. Cytotoxic lymphocytes express Fas and induce hepatic apoptosis.[32] TNF can promote viral clearance *in vitro* by cytotoxic and noncytotoxic effects.[33]

Liver cell failure develops when the functional capacity of the liver can no longer maintain normal physiological

conditions. This may be manifested on the skin, and it may affect other body systems in the form of hepatic encephalopathy, cardiovascular changes, portal hypertension, hepatopulmonary syndrome, and hepatorenal syndrome.[1]

In a similar model to liver cell failure, renal failure which is a systemic disease with cutaneous manifestations.[34] The degree of renal failure in hepatorenal syndrome is a reflection of the degree of hepatocellular failure. Impaired renal function is reversed following either liver or renal transplantation.[35] Interestingly, a recent study reported over expression of epidermal P53 and Bcl-2 with increased epidermal thickness in chronic renal failure patients on maintenance hemodialysis: suggesting that an alteration in the proliferation/ apoptosis balance is most likely present in the skin of such patients.[36]

Usually, apoptosis represents a counterbalance to proliferation, and decreased apoptosis is generally thought to be associated with epidermal hyperproliferation.[12,37]

In the present work, significant overexpression of proapoptotic markers (Bax, Fas, P53, and Caspase-3) were detected in patients (P=0.03, 0.03, 0.003, and 0.003, respectively): that could explain the increased apoptotic index in HCV liver patients (0.002). Whether these changes were due to the metabolic and biochemical alterations present in such patients or to the direct effect of the virus remain to be elucidated. Especially when taking into consideration that apoptosis induction in infected liver cells, by HBV or HCV, is considered by some authors as a defense mechanism to limit viral replication and promote their elimination.[31]

Moreover, the epidermal thickness of skin biopsies from patients was inversely correlated with the increased proapoptotic markers (Bax: P=0.01, Fas: P=0.04, P53: P=0.002, and Caspase-3: P=0.002) and apoptotic index (P=0.001).

These findings suggest that an alteration in the proliferation/ apoptosis balance is present in the skin of such patients. It is still unclear whether skin diseases associated with HCV infection and characterized histopathologically by apoptosis (LP) or necrosis (NAE)[38] are due to an exaggerated response to such alteration or to another mechanism.

On the other hand, no statistically significant difference in expression of antiapoptotic markers (Bcl-2 and Bcl-xL) in epidermal keratinocytes of HCV patients and the control group (P=0.5 and P=0.9 respectively) were detected.

The result of HCV liver disease patients was different from that reported in chronic renal failure where the expression of Bcl-2 protein (antiapoptotic) was found to be higher in skin samples obtained from apparently normal skin of chronic renal failure patients on maintenance hemodialysis than in skin samples obtained from control cases. The difference between the mean values of the two groups was highly significant (P=0.0003).[36]

Meanwhile, in the present study, the epidermis of HCV liver disease patients showed significantly lower thickness than the control group (P=0.000) when measured by computer image analysis (histometry). On the contrary, in renal failure patients there were significant increase of the epidermal thickness in patients than controls (P<0.0001). However, overexpression of P53 protein was the only common finding in both chronic renal failure[36] and HCV liver disease patients, which could be attributed to significant DNA damage.

Conclusion

The results of the present study suggest an alteration in keratinocyte proliferation/apoptosis balance in advanced HCV liver patients, which are associated with an increase in the expression of skin pro-apoptotic markers (Bax, Fas, P53, and Caspase-3) with consequent increase of the apoptotic index and decreased epidermal thickness. Whether this alteration in the skin proliferation/ apoptosis balance is attributed to the metabolic and biochemical changes present in liver cell failure or to the direct effect of the virus remains to be clarified.

Future research on a larger group of patients is mandatory, and should address various apoptotic regulatory proteins in the skin as well as other organs, in HCV patients and other liver diseases, to explain how the metabolic effects of liver diseases may affect skin apoptosis.

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