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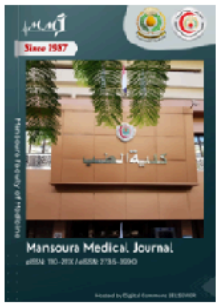

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CUTANEOUS WOUND HEALING: CORRELATION BETWEEN EPIDERMAL PROLIFERATING CELL NUCLEAR ANTIGEN AND DESMOPLAKIN EXPRESSION.

By

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ABSTRACT

Introduction & Aim of the work :

Resurfacing a wound with new epithelium involves two important processes, proliferation and migration of keratinocytes. This study aimed to investigate the correlation between epidermal proliferating cell nuclear antigen and desmoplakin expressions during normal wound healing in mice.

Material and methods :

Thirty male albino mice were divided randomly into 2 groups, control (n=6) left unwounded, and wound model group (n=24). Two full-thickness linear wounds (2 cm) were made on the shaved dorsum of the wound model group. At different time periods (1, 2, 4, 6 days) after wound, skin specimens including samples from the wounds were excised, processed as paraffin blocks, sectioned and immunostained with anti-PCNA and anti-desmoplakin (11-5F) antibodies.

Results :

During early process of wound healing, the cells at the margin of the wound proliferated and were pushed toward the surface in-order to migrate as evidenced by absence of desmoplakin expression. Later, at wound margin desmoplakin expression reappeared and proliferation of cells decreased. The new epidermis in the floor of the wound formed the source of the cells that would cover the wound. The keratinocytes in the floor

of the wound proliferated to supply the migrating and differentiating cells, after which they normalized their proliferation rate and restored their desmoplakin expression in the peripheral areas.

Conclusion :

Early during wound healing, the cells at the wound margin form the primary source of the cells that would cover the wound. Later, the new epidermis in the floor of the wound would form this source.

Keywords : wound healing, PCNA, desmoplakin.

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INTRODUCTION

Skin wound healing represents a dynamic and well ordered biological process (Clark, 1996 and Martin, 1997). The sequence of events in wound healing in skin has been intensively studied (Li et al., 2007).

Re-epithelialization is resurfacing a wound with new epithelium to restore the full thickness of the epidermis. It involves two important processes, proliferation and migration of keratinocytes (Li et al., 2007 and Javierre et al., 2009). Cell proliferation is an essential event during re-epithelization. Proliferating cell nuclear antigen (PCNA) has been considered as a well-known marker of cell proliferation (Brabo and Macdonald-Brabo, 1987).

Proliferating keratinocytes ensure an adequate supply of cells to migrate into and cover the wound (Li et al., 2007) then cellular migration plays a very important role in restoring epidermal continuity (Stenn and Malhotra 1992).

Desmosomes are the principal junctions of epidermal cells and are essential for the normal integrity and barrier function of unwounded adult skin (Vasioukhin et al., 2001 & Jonkman et al., 2005). Migrating keratinocytes undergo subcellular modifications including disassembly of hemidesmosomal links between epidermis and dissolution of most desmosomes (Singer and Clark, 1999).

The desmosomal junction comprises numerous proteins from at

least three different families: the cadherins, the armadillo proteins, and the plakins (Getsios et al., 2004). Desmoplakin is an important component of desmosomes structures that mediate intercellular adhesion in tissues subjected to mechanical stress and that serve as anchoring sites for the intermediate filament (IF) system (Getsios et al., 2004). Desmoplakin (DSP) is present in all desmosomal junctions (North et al., 1999) and exists as two isoforms, DSPI and DSPII (Green et al., 1990 and 1992).

To date, there is no study that has simultaneously investigated both proliferation and desmoplakin expression in the epidermal cells during the normal process of wound healing. The present study aimed to investigate the correlation between epidermal PCNA expression as marker for the prolifertaive state and desmoplakins expression as marker for desmosomal distribution during normal wound healing in mice.

MATERIAL & METHODS

Animals

Thirty male albino mice, 8-12weeks of age, weighting 20-22 g were obtained from experimental research center of Theodor Bilharz Institute, Cairo, Egypt. The animals had free access to water and food add libitum and were maintained in a room with controlled humidity and temperature (25 ^{o}C±2) with12 h/12 h light/dark cycle. All experiments were performed in accordance with the protocol approved by the committee on animals' experimentation of Mansoura University.

Cutaneous wound model

A model of cutaneous wound was made on the shaved backs of the animals. Under anesthesia with phentobarbital (60 mg/kg) administered intraperitoneally, two full-thickness linear wounds (2 cm) were made on the dorsum of the mice by incising the skin to the level of the subcutaneous muscle panniculus carnosus with a sterile scalpel (Tomlinson and Ferguson, 2003). Wounds were left not sutured or covered and left to heal by secondary intension. The animals were harvested at different time points (1, 2, 4 and 6days) after incision (six mice at each time point). Six mice were left unwounded and were used as control group.

Skin specimens including samples from the wounds were excised. Paraffin-embedded samples were prepared and cut into sections perpendicular to the skin surface by a microtome set and stained with haematoxylin-eosin for general morphological evaluation.

Immunocytochemical stains :

Paraffin-embedded 5 µm skin sections were mounted on glass slides coated with 0.1% poly-l-lysine. Sections were heated by microwave oven at 92°C for 10 min, and then cooled for 60 min at room temperature. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxidase in methanol for 20 min. Nonspecific reactions were blocked by 10% normal rabbit serum.

Mouse monoclonal anti-PCNA antibody (DAKO, Milan, Italy) (Fukuda et al., 2002) and monoclonal antibody to desmoplakin (11-5F) (which reacts with both DSPI and DSPII) (Parrish et al., 1987) at dilution 1:200 and 1:50 respectively were used as primary antibodies at 4°C overnight. The sections were then incubated with biotinylated rabbit anti-mouse immunoglobulin for 30 min, followed by incubation with streptavidin-peroxidase complex for 30 min and rinsed with phosphate-buffered saline. The color was developed with DAB. Finally, they were lightly counterstained with haematoxylin. Sections stained without primary antibodies served as negative controls. Sections of rat prostate were used as positive control for PCNA (Fig. AA) and sections of rat skin were used as positive control for 11-5F (Fig. BB)

Evaluation of PCNA index

The number of keratinocytes with PCNA-positive nuclei and the total number of keratinocytes were counted by two independent observers (x400 magnification). The proliferation index was calculated by dividing the number of keratinocytes with PCNA-positive nuclei by the total number of keratinocytes per section x100%.

Statistical analyses

All data were reported as mean ± standard error of mean. PCNA indices were statistically analyzed by Mann-Whitney test using SPSS software. Values of $P < 0.05$ were considered statistically significant.

RESULTS

All animals tolerated the wounding procedures without any obvious problems. Two animals developed signs of infection, manifested by purulence.

Control group :

Haematoxylin and eosin stained sections revealed normal structure of the skin. The epidermis is relatively thin and is formed of 2-3 layers of keratinocytes covered by a thin layer of keratin (Fig. 1).

Sections stained with the PCNA antibody showed positive PCNA immunoreactivity that is confined only to the nuclei of the basal keratinocyte (Fig. 2). PCNA index was 40.46% ± 5.27 (Tab.1). Positive PCNA immunoreactivity was also observed in the cells of hair follicles (Fig. 2).

Desmoplakin expression in the uninjured skin sections was observed in the whole thickness of the epidermis. Immunoreactivity has been detected in the form of brown stained lines and granules in-between the keratinocytes of all layers of epidermis (Fig. 3).

One day after the skin injury :

Haematoxylin & eosin stained sections showed increase in the number of epidermal layers at the wound margins (3-4 layers). The wound surface was covered by blood clot (Fig. 4). PCNA positive keratinocytes were observed mainly in the basal layer of the epidermis at the wound margins with few positive cells in the suprabasal layers (Fig. 5). PCNA index at wound margin was 65.28% 2.08 (Tab. 1) which was highly significant higher than that of the uninjured skin. There was partial loss of desmoplakin expression in-between cells of the superficial layers of the wound margin (Fig. 6).

A tongue of new epidermal cells (epithelial spur) had begun to invade the underlying connective tissue at each side of the wound to cover the raw surface. This tongue process was 2-3 layers thickness and was devoid of superficial keratin layer (Fig. 4). PCNA positive cells were observed in the basal layer and in some cells of the suprabasal layers of the neoepidermis at the floor of the wound with PCNA index 35.50% ± 2.85 (Fig. 5 and Tab. 2). Desmoplakin reactivity

was only restricted in-between few cells in the basal layer of the neoepidermis (Fig. 6).

Two days after the skin injury :

The epidermal layers at the wound margins were increased (4-5) layers (Fig. 7). PCNA positive keratinocytes were observed mainly in the basal layer of the epidermis at the wound margins (Fig. 8). The number of PCNA positive cells was highly significantly lower than that of the 1st day (index= 41.65% ± 6.4) (Tab. 1). The cells of the wound margin showed disappearance of the desmosoplakin expression in-between the cells of the superficial layers (Fig. 9).

The epithelial tongue increased in length, showed early evidence of keratinization and covered with a blood clot (Fig. 7). PCNA positive cells were observed in the basal and upper layers of the neoepidermis with PCNA index 44.18% ± 7.05 which was significantly higher than that of the 1st day after injury (Fig. 8 &Tab. 2). Desmoplakin reactivity was detected inside the cells (intra-cytoplasmic) of the neoepidermis (Fig. 9).

Four days after the injury :

The epidermal layers at the wound margin were still hyper-plastic and reached its maximum number (10-12 layers) (Fig. 10). The cells with positive PCNA expression were decreased in number and were coincided to the basal and suprabasal layers of the wound margin with low PCNA index (41.42% ± 9.90) (Fig. 11 & Tab. 1) which was not significantly different from that of the 2nd day. Positive desmoplakin reactivity reappeared between the keratinocytes of all layers at the wound margin (Fig. 12).

The epithelial tongue increased in length and consisted of multiple layers (3-4 layers) covered with thin layer of keratin in its peripheral part (Fig. 10).

The peripheral part of the neoepidermis close to the wound margin showed high number of PCNA positive cells seen only in basal layer. The PCNA index did not significantly differ from that of the floor of the wound in the 2nd day (Fig. 11). Some positive desmoplakin reactivity was observed in-between the cells of the peripheral part of the neoepidermis (Fig. 12).

The central part of the neoepidermis close to the wound gap showed PCNA positive cells in basal and suprabasal layers with PCNA index 43.90% ± 13.22 (Fig. 11 & Tab. 2). No desmoplakin reactivity was mainly detected inbetween the cells of the central part of the neoepidermis (Fig. 12).

Positive PCNA immunoreactivity increased in the cells of the hair follicles at different stages of wound healing (Fig. 11).

Six days after the injury :

The wound became completely healed and covered with keratinized epithelium formed of 3-4 layers (Fig. 13).

The epithelium at the wound margin was still hyper-plastic (10-12 layers) (Fig. 13). Few PCNA positive cells were detected in the basal layer of the wound margin (Fig. 14) with PCNA index 27.17% ± 2.14 which was highly significantly lower than that of the 4th day and significantly lower than that of the control (Tab. 1). Positive desmoplakin reactivity was detected between the cells of all layers of the wound margin (Fig. 15).

Few PCNA positive cells were detected in the basal layer of the floor of the wound. The PCNA index was 23.69% ± 1.63 in the peripheral part and 8.78% ± 1.87 in the central part (Fig. 14) which was highly significantly lower than those of the 4th day (Tab. 2). Positive desmoplakin reactivity appeared between cells in the floor of the wound (Fig.15).

Table (1): PCNA index of the control group and the wound margin of experimental groups at different intervals (1, 2, 4, & 6 days after the injury). Values are presented as means ± SD

	Mean	Std. Deviation	P value (vs control)	P value (vs previous group)
CONTROL	40.46	5.27		
1day after injury	65.28	2.08	.000**	
2days after injury	41.65	6.41	.649	.000**
4days after injury	41.42	9.90	.190	.069 .
6days after injury	27.17	2.14	.002*	.032*

* The mean difference is significant ($P \leq 0.05$).

** The mean difference is highly significant ($P \leq 0.001$).

Table (2): PCNA index of the floor of the wound of experimental groups at different intervals (1, 2, 4, & 6 days after the injury). Values are presented as means ± SD

	Mean	Std. Deviation	P value
1day after injury	35.50	2.85	
2days after injury	44.18	7.05	.047*
4days after injury (peripheral part)	44.16	10.47	.994**
4days after injury (central part)	43.90	13.22	
6days after injury (peripheral part)	23.69	1.63	.003♦
6day after injury (central part)	8.78	1.87	.000♦♦

The mean difference is significant ($P \leq 0.05$).

The mean difference is highly significant ($P \leq 0.001$).

* *D2 floor versus D1 floor (significant).*

** *D4 floor versus D2 floor.*

♦ *D6 floor versus D4 floor (peripheral parts).*

♦♦ *D6 floor versus D4 floor (central parts).*

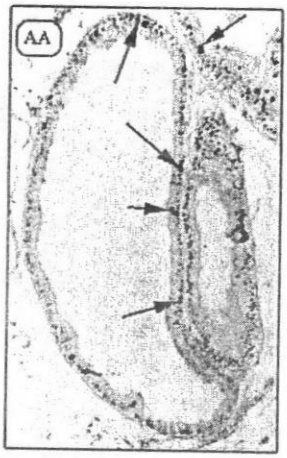

Fig. AA : A photomicrograph of a section of the rat prostate showing positive PCNA immunoreactivity (arrows). PCNA immunoperoxidase stain; X100

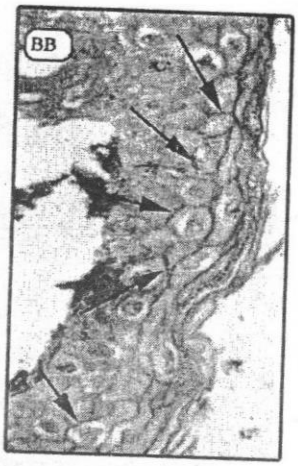

Fig. BB : A photomicrograph of a section of the rat skin showing positive immunoreactivity for desmoplakin (arrows). 11-5F immunoperoxidase stain; X400

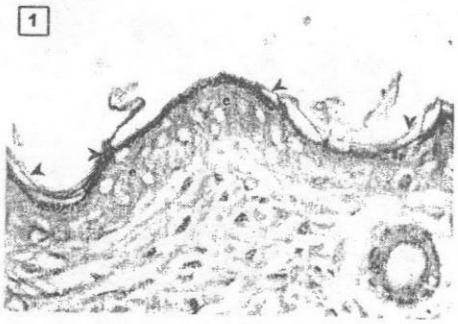

Fig. 1: A photomicrograph of a section of the mice skin of the control group showing relatively thin epidermis (e) covered by a thin layer of keratin (arrow heads). Hx. & E.; X400

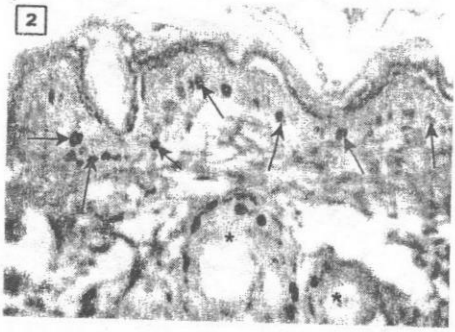

Fig. 2 : A photomicrograph a section of the mice skin of the control group showing positive PCNA immunoreactivity in the nuclei of the basal keratinocyte (arrows) and positive PCNA cells of hair follicles (*). PCNA immunoperoxidase stain; X400

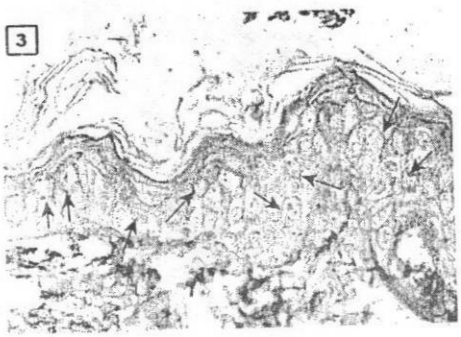

Fig. 3 : A photomicrograph of a section of the mice skin of the control group showing positive immunoreactivity in the form of brown stained lines and granules in-between the keratinocyte in all layers of epidermis (arrows). 11-5f immunoperoxidase stain; X400

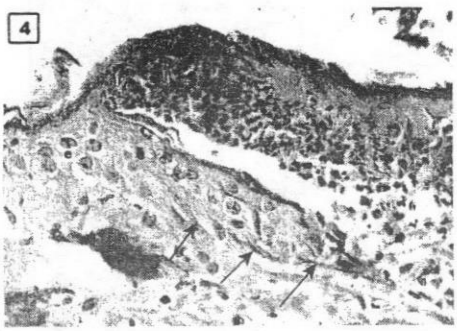

Fig. 4 : A photomicrograph of a section of the mice skin 1day after the injury showing increase in the number of epidermal layers at the wound margins (e), wound surface is covered by blood clot (*), tongue of new epidermal cells (arrows) invade the underlying connective tissue. Hx. & E.; X400

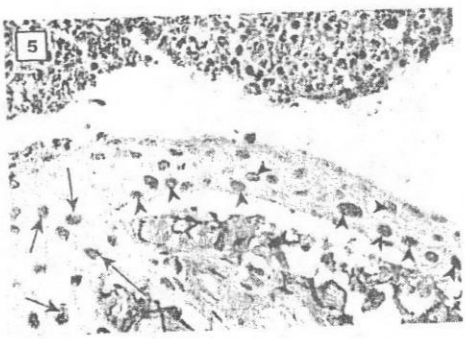

Fig. 5: A photomicrograph a section of the mice skin 1 day after the injury showing PCNA positive keratinocytes in the basal and suprabasal layers at the wound margins (arrows), PCNA positive cells can be seen in the basal layer and few suprabasal layers of the neoepidermis (arrow heads). PCNA immunoperoxidase stain; X400

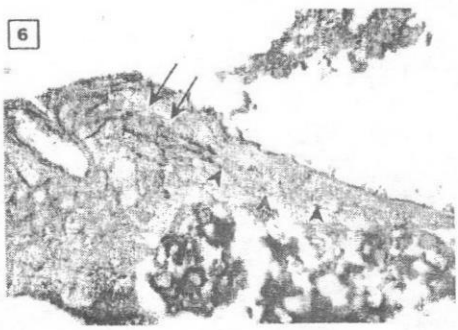

Fig. 6 : A photomicrograph of a section of the mice skin 1 day after the injury showing partial loss of desmoplakin expression in-between cells of the superficial layers of the wound margin (arrows), few desmoplakin reactivity can be seen in-between cells in the basal layer of the neoepidermis (arrow heads). 11-5F immunoperoxidase stain; X400

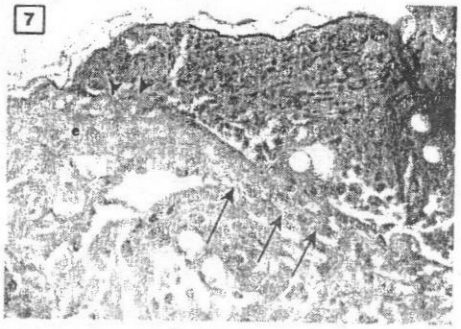

Fig. 7 : A photomicrograph of a section of the mice skin 2days after the injury showing increase number of epidermal layers (e), epithelial tongue increased in length (arrows), with early evidence of keratinization (arrow heads). Hx. & E.; X400

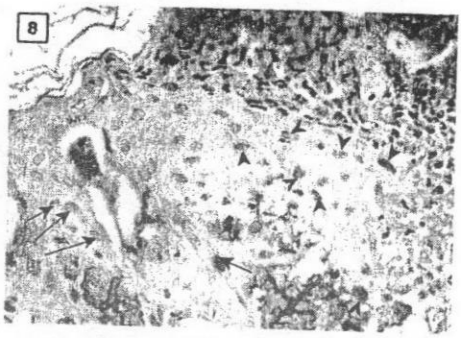

Fig. 8 : A photomicrograph a section of the mice skin 2 days after the injury showing PCNA positive keratinocytes in the basal layer of the epidermis at the wound margins (arrows), PCNA positive cells were observed in the basal and upper layers of the neoepidermis (arrow heads). PCNA immunoperoxidase stain; X400

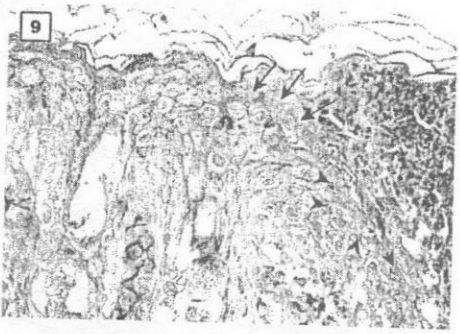

Fig. 9 : A photomicrograph of a section of the mice skin 2 days after the injury showing disappearance of the desmosoplakin expression in-between the cells of the superficial layers (arrows), intra-cytoplasmic desmoplakin reactivity was detected in the cells of the neoepidermis (arrow heads). 11-5F immunoperoxidase stain; X400

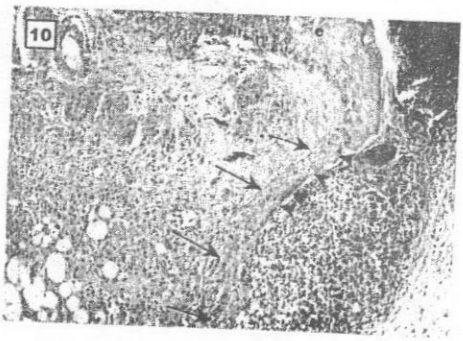

Fig. 10: A photomicrograph of a section of the mice skin 4 days after the injury showing hyperplastic epidermis (e), epithelial tongue consists of multiple layers (arrows) covered with thin layer of keratin in its peripheral part (arrow heads). Hx. & E.; X400

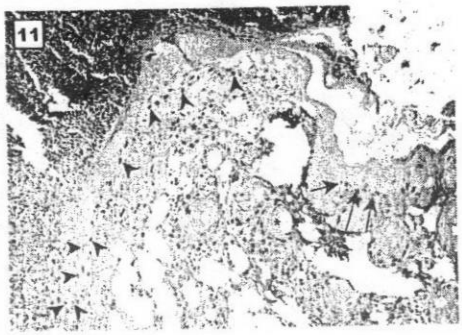

Fig. 11: A photomicrograph a section of the mice skin 4 days after the injury showing few positive PCNA cells in the basal and suprabasal layers (arrows), large number of PCNA positive cells can be seen in the peripheral part (arrow heads) and central part of the neoepidermis (blue arrow heads). PCNA immunoperoxidase stain; X100

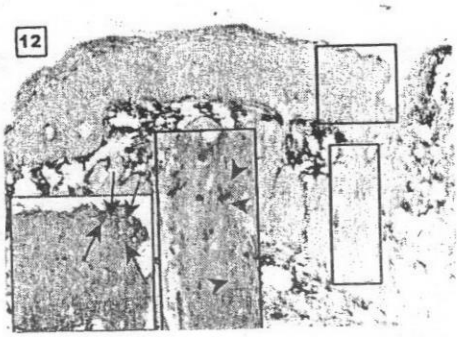

Fig. 12: A photomicrograph of a section of the mice skin 4 days after the injury positive desmoplakin reactivity between the keratinocytes of all layers at the wound margin (arrows in the highly magnified red field), few positive desmoplakin reactivity can be seen in-between the cells of the peripheral part of the neoepidermis (arrow heads in the highly magnified blue field). 11-5F immunoperoxidase stain; X100 & 400

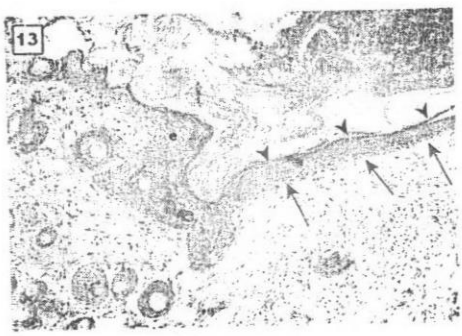

Fig. 13: A photomicrograph of a section of the mice skin 6 days after the injury showing completely healed wound (arrows) and covered with keratin (arrow heads), epithelium at the wound margin is hyper plastic (e). Hx. & E.; X400

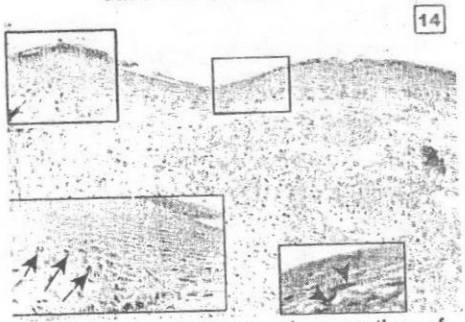

Fig. 14: A photomicrograph a section of the mice skin 6 days after the injury showing showing few PCNA positive cells in the basal layer of the wound margin (arrows in the highly magnified red field), few PCNA positive cells in the basal layer of the floor of the wound (arrow heads in the highly magnified blue fieeld) PCNA immunoperoxidase stain; X100 & 400

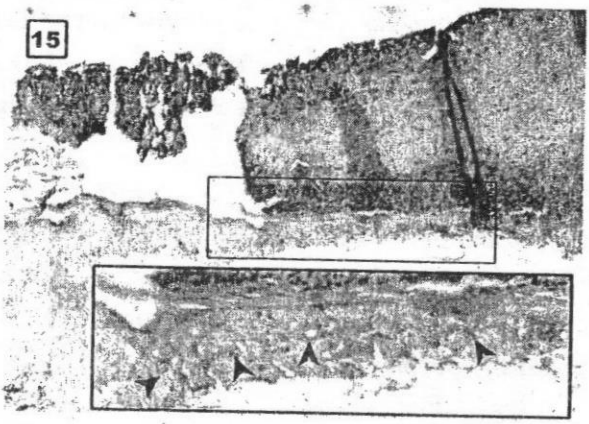

Fig. 15: A photomicrograph of a section of the mice skin 6 days after the injury showing positive desmoplakin reactivity appeared between cells in the floor of the wound (arrow heads in the highly magnified black field). 11-5F immunoperoxidase stain; X100 & 400

DISCUSSION

The mechanisms of wound reepithelialization have been debated for a long time but remain unclear. Some studies have described hyperproliferation at the wound margin (Garlick and Taichman, 1994 & Jansson et al., 1996). Contribution of the cellular proliferation to wound closure remains controversial either negligible (Marks and Nishikawa, 1973 and Pang et al., 1978) or important (Winter, 1972 and Garlick & Taichman, 1994).

The present study showed that, the cells located at the margin of the wound started to proliferate early during the 1st and 2nd day after injury. This proliferation was observed mainly in the basal layer and to less extent in the suprabasal layers. It has been previously reported that, breaking the continuity of the epidermal cell layer leads to stimulation of cell division processes in basal epidermal cells (Mcminn, 1969 and Christophers, 1996), by serum factors such as complement proteins, cytokines and growth factors, and by mediators such as serotonin and histamine (Wooseley, 1991, Flad, 1996 and Kekow and Gross, 1996). This proliferation is thought to reflect reprogramming of epidermal keratinocytes to ones dedicated to wound healing (Coulombe, 2003).

Disappearance of desmoplakin expression in-between some cells located at the superficial layers has been observed in the present study. This might indicate dissolution of the desmosomal junctions and movement of the newly formed cells to the superficial layers as they stopped the proliferation and formed the supplement of the migrating epithelial tongue. This was in agreement with the hypothesis about migration of the superficial keratinocytes to close the wound gap suggested by Ortonne et al., (1981). Also, Sun et al. (1991) noticed loss of cell-cell and cell-matrix contacts after wounding and decreased desmoplakin expression in the migrating keratinocytes at the leading edge of the wound (Garrod et al. 2005). However, adhesion remains intact between more distant cells of the epithelium, probably to allow coordinated migration of the tissue (Beaudry et al., 2010).

Early during the process of wound healing, PCNA positive cells were observed among the cells in the floor of the wound which indicated that those cells restored their ability to proliferate after their migration. This in agreement with previous observation that proliferative burst occurs after cell migration (Martin, 1997).

By the 4th day after injury, the proliferation of the cells at the wound margin has been decreased while this of cells at the floor of the wound was still increased. The cells in the floor of the wound might represent the new source of the cells that would cover the whole gap in agreement with Garlick and Taichman (1994) who reported that by the 3rd day the migrating epidermal tongue hyper-proliferates for the maintenance of the migrating cell mass. On the other hand, the desmoplakin expression started to appear in-between cells of the superficial layers of the wound margin, which could be explained by stopping of the migration of those cells in agreement with Garrod et al. (2005) who stated that desmosomal adhesion junctions are initially destabilized at the wound front to facilitate proliferation and migration, and are reassembled later during the sealing of the epithelium.

The current detailed observations along the new epidermis showed that

keratinocytes hyperproliferate in the central regions to supply migrating and differentiating cells, after which they normalize their proliferation rate in the peripheral area that had reepithelialized earlier in agreement with (Laplante et al., 2001). This could explain the current observation about positive desmoplakin reactivity in-between cells of the peripheral part of the neoepidermis as they will stop migration and start the process of differentiation and provide adhesive strength to the migrating epithelium (Beaudry et al., 2010). However, the desmoplakin reactivity was intracytoplasmic in the cells of the central part of the neoepidermis as those cells will continue the process of proliferation and migration.

By the 6th day, the wound was completely closed. Proliferation of the cells at the wound margin decreased to reach a level below the normal level. This is might be due to inhibition of the process of proliferation and increase in the process of remodeling and apoptosis (Kane and Greenhalgh, 2000).

Minimal proliferation was observed in the floor of the wound. This was expected to increase to the normal level by day 10 to restore the whole epidermis at the floor of the wound according to Beaudry et al. (2010).

Desmoplakin expression appeared between the cells of the floor of the wound and was expected to be increased as Beaudry et al. (2010) reported that by day 10, expression of all desmosomal components will be restored to levels similar to those observed in uninjured epidermis.

It has been observed that during the process of wound healing the proliferating cells in the hair follicles were markedly increased in number. Those cells might be a source of the cells that would close the wound gap (Sun et al., 1991). Hair follicle epithelial stem cells have been demonstrated to be critical for re-epithelialization during wound healing in mouse skin (Brouard and Barrandon, 2003).

In conclusion, the cells at the wound margin underwent proliferation, pushed toward the surface, lost their desmoplakin expression then they underwent migration to cover the

wound. Later, the new epidermis in the floor of the wound would form the source of the cells that would cover the wound. The keratinocytes hyperproliferated in the wound floor to supply the migrating and differentiating cells, after which they normalize their proliferation rate and restore their desmoplakin expression starting from the peripheral area toward the central region. Further studied are needed to elucidate the mechanism of keratinocyte migration and the apoptotic process that occurs during the process of remodeling and the actual role of hair follicles during the process of wound healing.

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الملخص العربى

التئام الجروح الجلدية: الارتباط بين ابانة المستضد النووى للخلية المتكاثرة و الديسموبلاكين

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المقدمة و الهدف من البحث :

تنطوى عملية تغطية الجرح بنسيج ظهارى جديد على اثنين من العمليات الهامة هما تكاثر و هجرة الخلايا القرنية. و كان الهدف من هذه الدراسة هو دراسة الارتباط بين ابانة المستضد النووى للخلية المتكاثرة و الديسموبلاكين أثناء التئام الجروح العادى فى الفئران.

المواد و الطرق المستخدمة :

تم تقسيم ثلاثون من الفئران البيضاء الذكور عشوائيا الى مجموعتين: مجموعة ضابطة (عدد=٦) و مجموعة نموذج الجرح الإختبارية (عدد= ٢٤). وقد تم عمل جرحين كاملى السمك بطول ٢ سم على الظهر الحليق لمجموعة نموذج الجرح. وفى فترات زمنية مختلفة (١ ، ٢ ، ٤ ، ٦ أيام) بعد الجرح، تم أخذ عينات الجلد بما فى ذلك عينات من الجروح ومعالجتها وصباغتها بمضادات المستضد النووى للخلية المتكاثرة و الديسموبلاكين .

نتيجة البحث :

وجد أنه خلال وقت مبكر من عملية التئام الجروح، تتكاثر الخلايا على هامش الجرح و تدفع باتجاه السطح تمهيدا لهجرتها كما يتضح من غياب الابانة للديسموبلاكين. وفى وقت لاحق يرجع ظهور الديسموبلاكين على هامش الجرح كما يقل تكاثر الخلايا و أصبح نسيج الأدمه الجديد فى أرضية الجرح مصدر الخلايا التى تغطى الجرح. فقد تكاثرت الخلايا القرنية فى أرضية الجرح لتزويد الخلايا المهاجرة ، وبعد ذلك يرجع معدل تكاثرها الى المستوى الطبيعى و تستعيد ابانتها للديسموبلاكين فى المناطق الطرفية.

الخلاصة : فى وقت مبكر خلال التئام الجروح، تشكل الخلايا على هامش الجرح المصدر الرئيسى للخلايا التى من شأنها تغطية الجرح. اما فى وقت لاحق، فإن البشرة الجديدة فى ارضية الجرح تشكل هذا المصدر.