Immunohistochemical determination of wound age in mice

Farelerde immunohistokimyasal metodlarla yara yaşı tespiti

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Abstract
Determination of wound age is one of the greatest challenges in autopsy cases. We have little knowledge on this issue despite a plethora of current studies. The purpose of this study was to shed light on wound age determination by studying the expression levels of ubiquitin and Ki-67 through immunohistochemical methods. A total of 45 (Balb/c) 6-8 week-old male mice, weighing 25-30 grams were divided into nine groups including five mice in each group. After general anesthesia, a 1.5 cm full-thickness incision was made on the dorsal skin using a scalpel. At 1, 3, 6, 12, and 24 hours and 5, 7, and 14 days, a total of 42 mice were sacrificed by cervical dislocation. An area of 1 cm surrounding the wound was excised. One mouse died in each of the Groups 6, 7, and 8, leaving 42 mice to complete the study. The mean number of fibroblasts with positive Ki-67 staining at the wound edge was significantly higher in Groups 4, 5 and 6. Groups 5 and 6 showed a statistically significant percentage of Ki-67 positive staining basal cells. Groups 7 and 8 exhibited similar features with the control group. Ubiquitin-positive fibroblast cells in Groups 1 to 4 showed similar features with the control group and the number of ubiquitin-positive fibroblast cells was statistically significant in Groups 5, 6 and 7. As a marker, ubiquitin may be useful in determining whether the wound age is older than 1 day and may be used for wounds 1 to 7 days old. We suggest that the Ki-67 marker may assist in the determination of the age of 1 to 5-day-old wounds by using immunohistochemical methods.
Keywords: Forensic pathology; immunohistochemistry; Ki-67 antigen; ubiquitin

Özet
Yara yaşı tespiti otopsi vakalarında ciddi sorunlara yol açmaktadır. Güncel çalışmalara rağmen bu konuda bilgilerimiz hala netleşmemiştir. Bu çalışmanın amacı Immunohistokimyasal yöntemlerle ubiquitin, Ki-67 belirteçlerinin ekspresyon seviyelerinin çalışılarak yara yaşı tespiti konularına ışık tutabilmektir. Ortalama 25-30 g ağırlığında ve 6-8 hafta büyüklüğünde toplam 45 adet Balb/c tipi erkek fındık faresi her bir grupta 5 fare olacak şekilde toplam 9 gruba ayrıldı. Genel anestezi sonrası 1.5 cm'lik cilt-ciltaltı kesi ile yara oluşturuldu. Fareler sırası ile 1., 3., 6., 12., 24. saatlerde, 5., 7., 14. günlerde servikal dislokasyon ile sakrifiye edilerek yaralar etrafında 1 cm'lik sağlam doku kalacak şekilde çıkarıldı. Grup 6, 7 ve 8'de birer farenin ölmesi nedeniyle 42 fare ile çalışma tamamlandı. Yara kenarında Ki-67 pozitif boyanan fibroblast ortalamasının Grup 4'ten itibaren artmaya başladığı Grup 5 ve 6'da anlamlı olduğu görülmüştür. Benzer şekilde Ki-67 pozitif boyanan bazal hücre ortalamasının Grup 5 ve 6'da anlamlı olduğu saptanmıştır. Grup 7 ve 8 kontrol grubuyla benzer özellikler göstermiştir. Grup 1'den Grup 4'e kadar kontrol grubuyla benzer özellik gösteren ubiquitin pozitif fibroblast hücrelerin Grup 5, 6, 7'de anlamlı olduğu görülmüştür. Yara yaşı tespitinde; ubiquitin belirtecinin özellikle yara yaşının bir günden fazla olup olmadığı ve 1-7 gün arasındaki yaralarda kullanılabileceği düşüncesindeyiz Ki-67 belirtecinin ise 1-5 günlük yaralarda immunohistokimyasal metodlar ile kullanılmasının yara yaşı tespitine katkı sağlayacağı düşüncesindeyiz.
Anahtar kelimeler: Adli patoloji; immunohistokimya; Ki-67 antijeni; ubiquitin

Introduction

Wound healing is a vital process that occurs as a response to tissue injury and can be defined as a series of sequential and interrelated cellular, physiological, and biochemical events. It is as old as the human history and still draws much attention due to the fact that the underlying mechanisms have not been fully enlightened (1,2).

Wound age determination has gained importance recently in the field of forensic medicine. Determination of wound vitality and wound age has been associated with many difficulties in autopsy cases. Clear information is still lacking although

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various studies have been carried out recently. Wound age is of paramount importance in forensic investigations to determine whether the wound in question incurred before or after death. Estimation of wound vitality and age is crucial for the proper course of legal proceedings (3,4).

Estimation of wound age gives important clues on how the event has actually occurred, whether the murder was made look like a suicide, whether the lesions in question are connected with the crime and for determining the wounds that occurred in the body at different times (4).

Today, wound age and whether the wound occurred before or after death can be determined using immunohistochemical, biochemical and molecular biology techniques. Ki-67 is a nucleoprotein found in proliferating cells. Primarily, it is seen in phases G1, S, M, and G2 of the cell cycle and does not exist in the G0 phase (5,6). The Ki-67 labeling index is directly correlated with other proliferation indices and several tumor stages (5,7).

Ubiquitin is a polypeptide of 76 aminoacids which are expressed in all eukaryotes (8-11). It is a member of heat shock protein family which rapidly arises as a response to various stimuli such as hyperthermia and chemical or mechanical stress. Ubiquitin mediates non-lysosomal protein degradation in eukaryotic cells via ubiquitin-protein system enzymes by covalently binding to various proteins (12).

Ubiquitin-proteasome pathway, which includes ubiquitin, is the major pathway necessary for intracellular protein catabolism. The ubiquitin-proteasome way has a critical role in the regulation of many cellular pathways, such as maintaining cellular homeostasis, cell growth and reproduction, apoptosis, DNA repair, transcription and immune response. Protein substrates are labeled with polyubiquitin chains (8), and multimeric proteases that exist in all eukaryotic cells are degraded into proteasome, peptides, and free ubiquitin. The ubiquitin proteasome system (UPS) plays an important role in various biological processes including the regulation of the cell cycle, inflammatory response, immune response, protein misfolding, and endoplasmic reticulum-associated protein degradation (9,10). In the present study we aimed to estimate wound age by determining the expression levels of ubiquitin and Ki-67 antibodies at the wound edge. We believe that qualitative and/or quantitative changes in these antibodies together or individually may elucidate wound age determination.

Materials and Methods

Forty-five Balb/c male mice, 6-8 weeks-old with a mean weight of 25-30 g were supplied from the department of physiology and divided into nine groups of five mice each. One mouse died in each of the Groups 6, 7, and 8, leaving 42 mice to complete the study. Following induction of intraperitoneal (IP) general anesthesia with 20 mg/kg ketamine and 5 mg/kg xylazine, a cutaneous-subcutaneous wound was created with a 1.5-cm cut. Mice were sacrificed with cervical dislocation at 1, 3, 6, 12 and 24 hours and on days 5, 7 and 14. Wounds were excised in such a way that 1 cm of intact tissue remained in their surrounding for study mice and 1 cm of intact tissue was excised in the control group. Approval was obtained from the Local Ethics Committee of Gaziantep University, Faculty of Medicine (Decision no. 28.09.2011/52-2).

Immunohistochemical Staining

Immunohistochemical markers used in this study were Ki-67 (rabbit polyclonal NB110-89719, Novus Biologicals) and ubiquitin antibody (EP296Y) (rabbit polyclonal NB 110-57683, Novus Biologicals). These markers were studied using the Bond Polymer Anti-Mouse Refine Detection Kit. Tissues were boiled with citrate for 30 minutes and allowed to incubation for 180 minutes with 1/40 dilution ubiquitin antibody. Tissues were boiled with citrate for 20 minutes and allowed to incubation for 20 minutes with 1/200 dilution Ki-67 antibody. Nuclear staining for Ki-67 was considered for assessment of the staining pattern. Ubiquitin is located in the cytoplasm but biochemical studies have shown that it translocates into the nucleus (12). Therefore, the nuclear reaction was analyzed in our study.

Immunohistochemical Assessment

For the tissue sections, Ki-67-positive basal cells were counted in an epidermal area of 0.2 cm and Ki-67-positive staining fibroblasts were counted in five randomly selected areas using a light microscope with x400 magnification.

Fibroblasts and inflammatory cells positively stained for ubiquitin were counted in five randomly selected areas with x400 magnification. Numerical values obtained from the sections taken from the edge of the wound and results of the control group with the intact skin were compared.

Scoring

In sections obtained from the edge of the wound, the number of basal cells and fibroblasts positively stained for Ki-67 were scored as shown in Table 1.

Table 1. Scoring of Ki-67-positive staining

Ki-67-positive Basal Cells and Fibroblasts (n)	Scoring	Staining Intensity
0-5	0	Negative
6-20	1	Slight
21-50	2	Medium
51 and over	3	Severe

In sections taken from the edge of the wound, the number of inflammatory cells and fibroblasts positively stained for ubiquitin were scored as shown in Table 2.

Table 2. Scoring of ubiquitin-positive staining

Ubiquitin-Positive Inflammatory Cells and Fibroblasts (n)	Scoring	Staining Intensity
0-2	0	Negative
3-5	1	Slight
6-10	2	Medium
11 and over	3	Severe

The reason for scoring was to obtain numerical consistency by minimizing the differences between counted cells in order to, to carry out different statistical analyses and to establish the significance more clearly.

Statistics

Kruskal-Wallis test was used to perform statistical analysis on all groups collectively. After achieving statistical significance, Mann-Whitney U-test was used prior to scoring and Kolmogorov-Smirnov test after scoring for all groups. A p value below 0.05 was considered significant.

Results

After counting positively stained cells in all groups and calculating their average numbers, statistical analyses were made in comparison to the control group (Table 3).

Statistical analyses showed that the average number of Ki-67-positive basal cells without scoring was high in Group 4, and reached statistical significance in Group 5 (P=0.009) and Group 6 (P=0.014), and it remained higher than normal despite losing statistical significance in Groups 7 and 8. The average number of Ki-67-positive fibroblasts showed statistical significance in Group 4 (P=0.015), Group 5 (P=0.009), and Group 6 (P=0.014). Group 7 and beyond exhibited similar characteristics with the control group (Table 4). Ki-67-positive staining in Group 1 and 3 were respectively shown in Figures 1 and 2.

Table 3. The average number of cells positively stained for ubiquitin and Ki-67 markers by groups

Variables	Group 1 (n=5)	Group 2 (n=5)	Group 3 (n=5)	Group 4 (n=5)	Group 5 (n=5)	Group 6 (n=4)	Group 7 (n=4)	Group 8 (n=4)	Group Cont. (n=5)
Ubiquitin-positive Inflammatory cells *	5.8 ±1.1	3.4 ±0.6	4 ±0.8	6 ±0.8	19 ±5.0	12.5 ±6.8	22.5 ±4.6	10.75 ±3.4	7.2 ±2.3
Ubiquitin-positive Fibroblasts *	2.4 ±0.8	3.4 ±1.7	3.6 ±1.3	1.4 ±0.7	13.8 ±2.0	16.75 ±5.4	16.25 ±3.1	3.5 ±1.4	1.2 ±0.4
Ki-67-positive Fibroblasts *	8.4 ±2.1	6.2 ±1.3	5.6 ±1.6	4 ±0.8	26.2 ±3.8	27.5 ±3.0	11 ±2.6	9.5 ±2.3	9.2 ±0.9
Ki-67-positive Basal cells *	2 ±0.8	13 ±4.1	4.2 ±1.3	4.4 ±2.1	112.8 ±18.1	64.25 ±17.6	56.25 ±36.1	64.25 ±24.9	8 ±1.2

*Mean±SEM

Table 4. P values for Ki-67 versus control group prior to scoring all groups-Mann-Whitney U-test

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Ki-67-positive Basal Cells	0.027	0.401	0.172	0.169	0.009	0.014	0.174	0.174
Ki-67-positive Fibroblasts	0.675	0.140	0.114	0.015	0.009	0.014	0.621	0.902

Table 5. P values for Ki-67 versus control group after scoring in all groups- Kolmogorov-Smirnov test

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Ki-67-positive Basal Cells	0.082	1.00	0.329	0.329	0.013	0.164	0.164	0.164
Ki-67-positive Fibroblasts	0.819	0.819	0.819	0.329	0.082	0.164	1.000	0.999

Table 6. P values for ubiquitin versus control group prior to scoring in all groups- Mann-Whitney U-test

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Ubiquitin-positive fibroblasts	0.237	0.237	0.125	1.000	0.008	0.012	0.012	0.128
Ubiquitin-positive inflammatory cells	0.916	0.525	0.598	0.916	0.075	0.806	0.027	0.462

Table 7. P values for ubiquitin versus control group after the scoring in all groups- Kolmogorov-Smirnov test

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Ubiquitin-positive fibroblasts	0.329	0.329	0.329	1.000	0.013	0.023	0.023	0.164
Ubiquitin-positive inflammatory cells	0.819	0.819	0.819	0.819	0.329	0.869	0.116	0.869

After completion of scoring, it was found that the average number of Ki-67-positive basal cells achieved statistical significance in Group 5 (P=0.013) and was above normal in Groups 6 (Figure 3), 7 (Figure 4) and 8. The average number of Ki-67-positive fibroblasts did not reach statistical significance in any groups and was above normal in Groups 5 and 6 (Table 5).

It was found that ubiquitin-positive fibroblasts exhibiting similar characteristics with the control group up to Group 4 before scoring increased at a significant level in Group 5 (P=0.008). This increase was also observed in Group 6 (P=0.012) and Group 7 (P=0.012) and it tended to decrease in Group 8 and beyond (Table 5). Although the number of inflammatory cells positively stained for ubiquitin increased in Group 5 (P=0.075) prior to scoring, it was low in Group 6, statistically significantly high in Group 7 (P=0.027), and decreased to normal limits in Group 8 with fluctuations (Table 6). Ubiquitin positive staining in Group 1 and 3 were respectively shown in Figures 5 and 6.

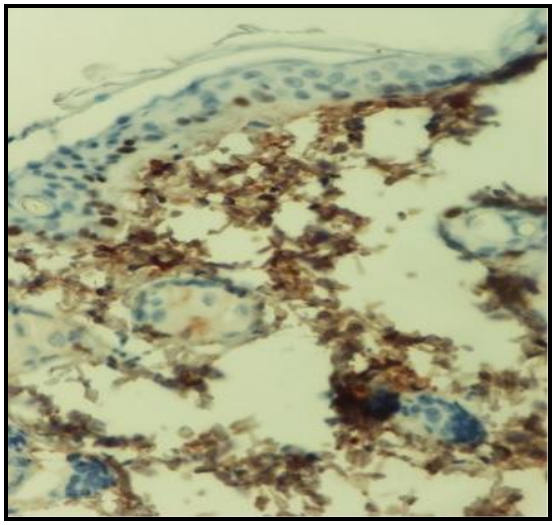

Figure 1. Ki-67-positive staining in Group 1 (x400).

Following the scoring, it was observed that the number of ubiquitin-positive fibroblasts reached greatest statistical significance in Group 5 (P=0.013). This high level significance was maintained in Group 6 (P=0.023) (Figure 7) and Group 7 (P=0.023) (Figure 8) and tended to decrease in Group 8. Ubiquitin-positive inflammatory cells did not show a statistically significant increase in any groups (Table 7).

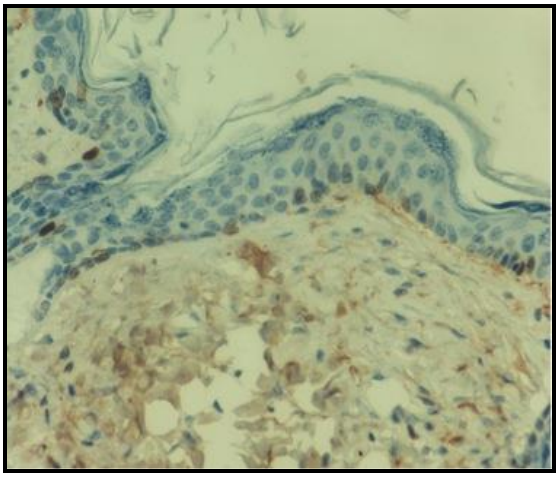

Figure 2. Ki-67-positive staining in Group 3 (x400)

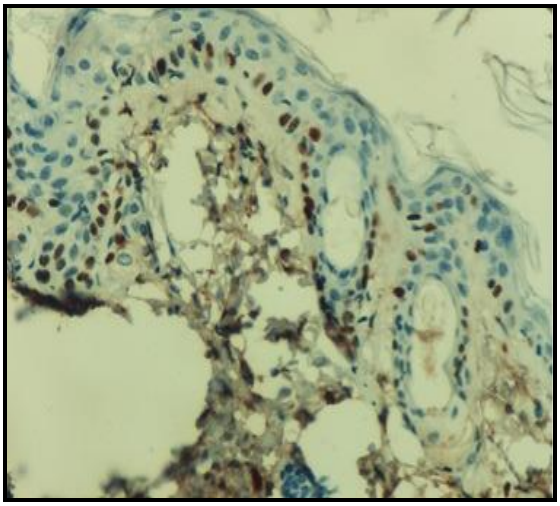

Figure 3. Ki-67-positive staining in Group 6 (x400)

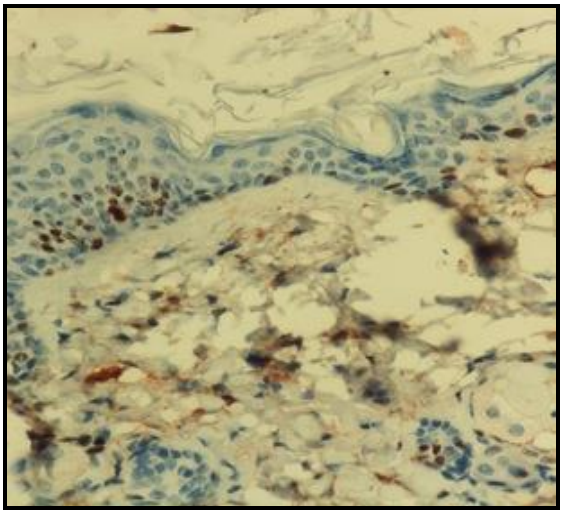

Figure 4. Ki-67-positive staining in Group 7 (x400)

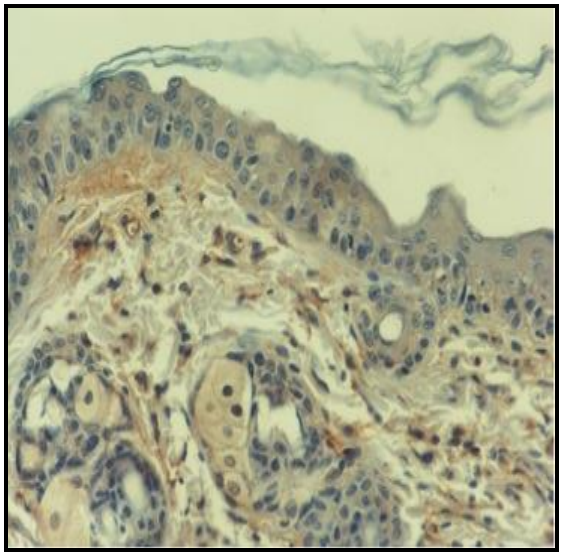

Figure 5. Ubiquitin positive staining in Group 1 (x400)

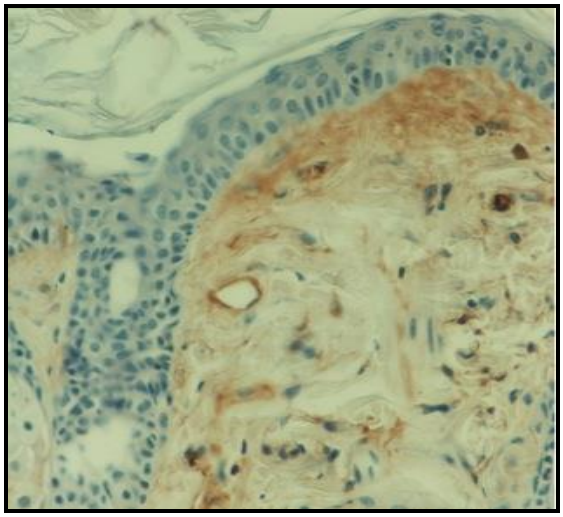

Figure 6. Ubiquitin positive staining in Group 3 (x400)

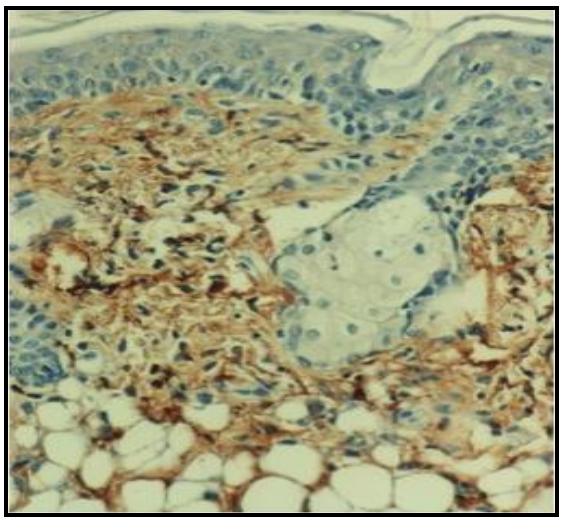

Figure 7. Ubiquitin positive staining in Group 6 (x400)

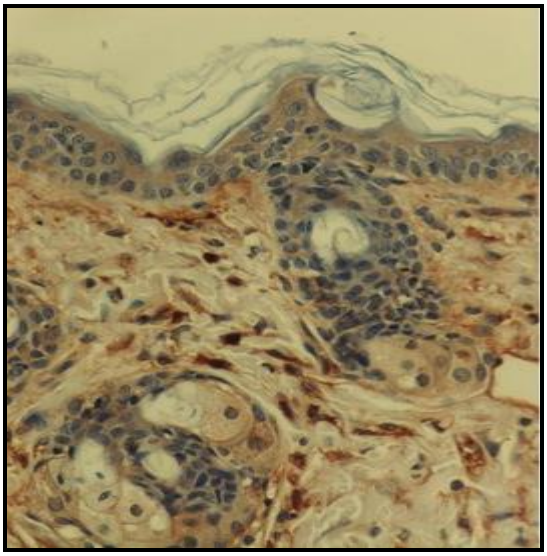

Figure 8. Ubiquitin positive staining in Group 7 (x400)

Discussion

Wound age determination is an issue that has never lost its importance in forensic medicine practices. In fact, it has become a field of extensive research due to technological and scientific developments (13). An accurate and objective description of wound characteristics is required in order to establish the exact cause of death (14). In case when there are multiple wounds, it should be determined whether they incurred at the same time (15,16).

Scientists have carried out studies to determine wound age within a complicated and complex system in an effort to find answers to the aforementioned questions. Some of these studies stood out. In their study, Dressler et al. (17,18) suggested that ICAM-1 (intercellular adhesion molecule), VCAM-1 (vascular cell adhesion molecule 1) and selectin could be used as markers of wound vitality. P-selectin was found to be expressed in the earliest stages of wound formation within a period of 3 minutes to 7 hours (17,18). Similarly, Hayashi et al. (19) suggested that VEGF-positive reaction may indicate a wound age of 7-21 days.

The Ki-67 marker which is found in all replicating cells and indicates a high rate of proliferation was investigated by Betz et al. (20) in a series of 77 cases and shown to increase 1.5 days after the wound formation.

In the current study, the statistically significant percentage of basal cells positively stained for Ki-67 observed in Group 1 where the inflicted wound was excised after 1 hour is remarkable as it contrasts with other studies. It is believed that the increase in Ki-67 basal cells seen in Group 1 may correspond to cellular changes that occur in the process of proliferative activity which is initiated by the organism as a response to a sudden cut.

Groups 5 and 6 were found to have highly significant percentages of both basal cell and fibroblast staining with Ki-67 marker. Following the scoring, this significance was only maintained in the Group 5 basal cells (P=0.013), whereas it disappeared in Group 5 fibroblasts and in Group 6. When the data were assessed collectively, it was considered that Ki-67 basal cell staining might be a very important marker for 1 to 5 day-old wounds when the average number of stained cells was calculated.

When the results of Mann-Whitney U-test were analyzed where the average numbers were assessed, it was observed that the Ki-67 fibroblasts significantly increased in a 12-hour wound, peaked in a 24-hour wound and involved basal cells, the statistical significance in the 5-day wound was maintained and it disappeared in the 7-day wound. This finding differs from the study carried out by Betz et al. (20) on 77 cases where Ki-67-positive fibroblasts began to increase 1.5 days after the wound with regard to time when Ki-67 marker began to increase. In that study, positive results were obtained in 60% of cases with a wound age between 1.5 and 5 days and 16% of cases showed ambiguous results (20). This difference can be explained by the fact that human skin wounds were examined in Betz et al.'s study (20) which included cases from different age ranges, gender differences and different localizations of the wounds. The current study was carried out on mice and the wounds were inflicted in the same anatomical locations. In other words, this was a study where groups were more standardized. Additionally, the current study used a more detailed grouping of wounds 24-hours of age and less which might be another explanation for the different findings.

The fact that statistical significance changed depending on the way of construction and detailing of the study groups suggests that wound healing is a dynamic process where not days but hours and even minutes count and that further studies are needed to fully elucidate this process.

In light of the results of all statistical analyses, we suggest that the lesion may indicate 1-5 day-old wounds at the time when Ki-67 increases in basal cells and fibroblasts without the scoring and the time period when Ki-67 specifically increases in fibroblasts but not in basal cells may indicate 12 hours old wounds.

If basal cells and fibroblasts positively stained for Ki-67 exhibit significance together before scoring and statistical significance is only found for Ki-67-positive basal cells after scoring, we suggest that this may also indicate a 1-day old wound. A wound with unknown age which exhibit a highly increased number of Ki-67-positive basal cells (although it was not statistically significant in Groups 7 and 8) versus control group and fibroblast positivity within normal limits might indicate a 7 to 14 days old wound. We suggest that, it might be more appropriate to discuss the utility of this information depending on the changes in wounds 7-14 days old detected by using other markers. From a different viewpoint, one can deduce that the age of a wound might be less than 1 day or more than 14 days when there is no increase in Ki-67 in the basal layer and fibroblasts.

Ubiquitin has become a marker of interest which has been increasingly investigated. Quan et al. (21) conducted a postmortem examination on the ubiquitin expression in the midbrains of 35 humans who died during a fire incident and found that ubiquitin immunoreactivity increased significantly in the substantia nigra of the midbrain as a result of stress in human combustion cases.

In a study carried out on mice and 55 skin wounds at different ages, Kondo et al. (12) found that ubiquitin markers increased significantly in wounds 7-14 days of age. Ishikawa et al. (22) examined ubiquitin and myoglobin levels immunohistochemically in human kidney tissues of 138 postmortem cases and determined that ubiquitin and myoglobin levels in renal tubule cells increased more in cases of fire, fatal hypothermia and sharp and blunt force injuries compared to other types of injuries. Based on these findings, they suggested that ubiquitin may be a sensitive indicator of fatal wounds (22).

In the present study, lack of statistical significance for the percentage of fibroblasts and inflammatory cells positively stained for ubiquitin in Groups 1, 2, 3 and 4 before and after, scoring might suggest that ubiquitin marker is not increased in wounds 24 hours of age and less. Guler et al. (23) grouped 170 wound samples obtained from 89 autopsy cases of known wound age and counted ubiquitin-positive inflammatory cells and fibroblasts using immunohistochemical methods. As a result, they demonstrated that the percentage of ubiquitin positivity was below 10% in wounds of 24 hours of age and less and higher than 10% in wounds of more than 24 hours of age (23). The findings of this study are consistent with ours.

Ubiquitin showed highest statistical significance in Group 5 when ubiquitin values obtained before and after scoring was examined. Thus, we believe that ubiquitin may be used primarily for determining whether the wound is greater or less than one day old. We believe that, after scoring, a prediction about the age of wounds aged between 1 day and 7 days can be made by giving particular consideration to ubiquitin-positive fibroblast cells. The time period when both fibroblasts and inflammatory cells show a statistically significant increase may indicate a wound age of 7 days without scoring.

Although lack of statistical significance in inflammatory cells and fibroblasts under any

conditions in Group 8 suggested that the use of ubiquitin may be more difficult in determining the age of 14 day-old and older wounds, further studies are needed which include interim examination periods with a wide range of wound ages.

We found that ubiquitin increased 1 day after the wound formation, started to decline on Day 7 and returned to a normal level beyond 14 days. Consistent with the resulting picture following the activation of intracellular ubiquitin–proteasome pathway in the process of wound formation and healing, it was observed that ubiquitin values tended to increase over the same period in the present study.

Kondo et al. (12) conducted morphometric analyses on mice and concluded that the rate of intranuclear ubiquitin positivity was clearly significant in wounds aged 6 days or older. As a part of the same study, 55 human skin wounds with known wound age were grouped into four groups: Group I: 0-12 hours, Group II: 1-5 days, Group III: 7-14 days and Group IV: 17-21 days. As a result, they reported that ubiquitin positivity might indicate a wound age of 7-14 days, but may not provide clear results when used alone. They stated that most importantly the rate of ubiquitin positivity was lower than 10% for wounds less than 1 day old and above 10% for wounds older than one day in all cases. Statistical significance observed in wounds of 24 hours of age and older and significant results found both in ubiquitin-positive fibroblasts and inflammatory cells in a 7-day old wound in our study are consistent with Kondo et al.'s findings (12).

The results of the current study showed that ubiquitin values cannot guide wound age determination in a 14-day old wound. In other words, in order to consider a wound age of less than 1 day and 14 days or more, ubiquitin should be used in combination with other markers after demonstration of lack of increase in the ubiquitin.

When Ki-67 and ubiquitin markers are assessed together, a wound age of 24 hours might be considered if Ki-67-positive basal cells, Ki-67-positive fibroblasts and ubiquitin-positive fibroblast cells increase concurrently. A 5-day wound age can be considered when Ki-67-positive basal cells decrease slightly and the numbers of Ki-67- and ubiquitin-positive are very high. A 7-day wound age is likely when ubiquitin-positive fibroblasts and inflammatory cells greatly increase, despite a decrease in Ki-67 basal cells. A 14-day wound age is plausible when the number of Ki-67-positive basal cells is high and Ki-67-positive fibroblasts are at a normal range.

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