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Detection of cell death in human skin wounds of various ages by an in situ end labeling of nuclear DNA fragments

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Abstract The time-dependent appearance of signs of cell death was investigated in human skin wounds using in situ end labeling of DNA fragments (ISEL). In the dermal layer an average of not more than 0.3 positively stained fibroblastic cells/0.01 cm × 0.01 cm was found up to a postinfliction interval of approximately 6 h. Average numbers exceeding 1 positive cell/0.01 cm × 0.01 cm were first detectable in a skin wound after 24 h. Therefore, average numbers greater than 1 labeled cell/0.01 cm × 0.01 cm indicate a postinfliction interval of approximately 1 day. An increase in the average number of positively stained cells occurred with increasing wound age. Values exceeding 3 cells/0.01 cm × 0.01 cm were first detectable 19 days after wound infliction. Accordingly, values of more than 3 labeled cells indicate a postinfliction interval of approximately 3 weeks or more. Since low numbers of labeled fibroblastic cells or even negative results were found in wounds of advanced age, only positive results provide information which can be useful for a forensic age estimation of human skin wounds.

Key words Cell death · Apoptosis · Wound age · Immunohistochemistry

Introduction

Apoptotic cell death features can be induced by several factors such as exposure to physical or toxic conditions, cellular effects of cytokines, viral infections or immunologically relevant mechanisms [9–11]. During apoptosis, nuclear DNA is degraded and can be detected by in situ end labeling (ISEL) of fragmented DNA. Such DNA strand breaks have been shown to label apoptotic cells in a distinctive pattern prior to morphological changes [8]. Therefore, this method is useful for the identification of cell death even before routine histological parameters such as cell shrinkage, chromatin condensation or karyorhexis occur. Since nuclear DNA fragmentation is expected to appear during wound healing as a result either of direct cell injury due to mechanical tissue alteration or during "physiological" programmed cell death (for example myofibroblast apoptosis) [6], the detection of these cell death features could possibly provide information on wound age.

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Material and methods

A total of 56 human skin wounds (lacerations, stab wounds, surgical wounds) with postinfliction intervals between a few minutes and 7 months was investigated. Specimens from normal skin, normal colonic mucosa and lymph nodes served as controls. All wounds were obtained at autopsy from individuals aged between 17 and 75 years (average individual age: 52 years). The patients had not suffered from any conditions which could have influenced the course of wound healing such as severe malnutrition, malignant diseases or metabolic disorders. Furthermore, no relevant substances such as glucocorticoids or cytotoxic agents were administered during therapy. Since an unambiguous evaluation was limited in cases with advanced post-mortem intervals due to an increased unspecific background staining only specimens with a post-mortem interval of less than 3 days were investigated.

Paraffin sections (3–5 μm) were cut and stained with H&E. Nuclear DNA fragments were visualized by an enzymatic reaction according to modified protocols of Gavrieli et al. [8] and Ansari et al. [1] as previously described [3] using the ApopTag in situ apoptosis detection kit of Oncor (Gaithesburg, Md.).

For evaluation, the number of positive cells in uninjured skin was determined as an internal control. Since apoptotic cells frequently occur in the upper epidermis and in skin appendages such as hair follicles or sebaceous glands, only fibroblastic cells of the dermis were evaluated. Only cells which showed a distinct nuclear staining were regarded as positive with no significant cytoplasmatic reaction. The number of positive cells per 0.01 cm × 0.01 cm was determined in 10 randomly selected microscopic fields in at least two specimens of each patient at a magnification of 200 ×. Similarly, in skin wounds the number of positive fibroblastic cells of the dermis was estimated in the wound area or in the granula-

tion/scar tissue. For data evaluation, the average numbers of the specimens were compared.

In addition to sections from colonic mucosa and lymph nodes (positive controls), specimens without application of the in situ apoptosis detection kit served as negative controls.

Results

Colonic mucosa (Fig. 1)

In sections from colonic mucosa, few cells showing positive nuclear reaction were found mainly localized in the apical crypt. These positively labeled cells were often clustered. Diffuse staining patterns could not be observed.

Normal skin (Figs. 2 and 3)

In uninjured skin, no significant numbers of positive fibroblastic cells were detected in the dermal layers. A single positive cell could be observed in 10 evaluated microscopic fields in only 2 out of 20 investigated specimen fields leading to an average value of 0.1 labeled cell/0.01 cm × 0.01 cm. Positive cells were found in variable amounts in the epidermal layer as well as in hair follicles and sebaceous glands in accordance with the physiological apoptosis. A semiquantitative analysis (I: 0 positive cells – II: 1–5 positive cells – III: > 5 positive cells per microscopic field) revealed no relevant differences when related to the individual age.

Skin wounds (Fig. 4)

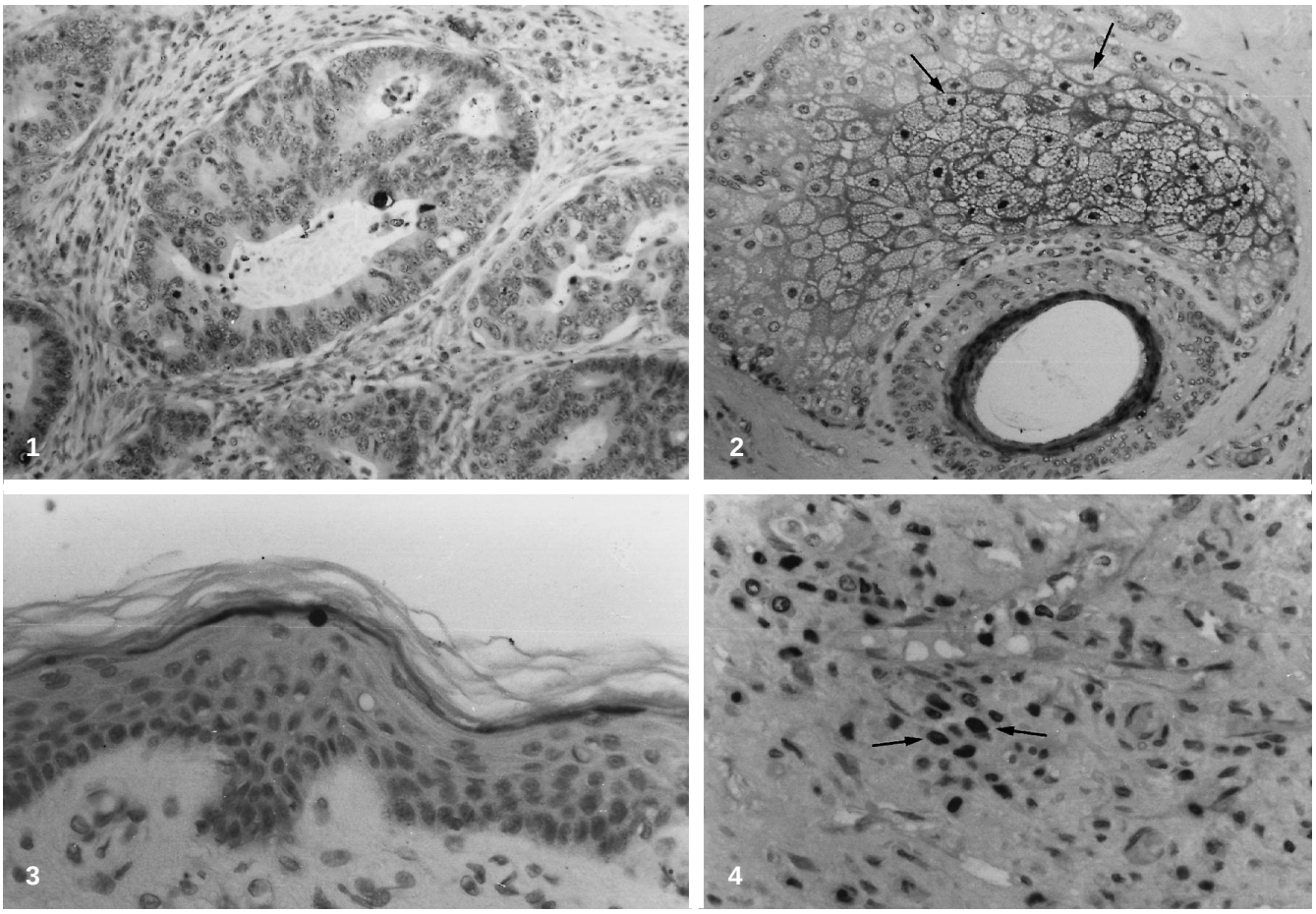
Similarly to uninjured skin, few positive cells were found in the epidermal layer and in skin appendages. Many inflammatory cells in the wound area appeared apoptotic and in some cases, an additional diffuse positive staining was observed due to tissue necrosis. Such areas were not evaluated because an exact estimation of cells showing DNA degradation was impossible due to the diffuse staining pattern. Therefore, only fibroblastic cells of the dermal layers in or adjacent to the wound area or in the granulation/scar tissue distant from diffusely stained areas were evaluated.

Fig. 1 Positive control: colonic mucosa showing single apoptotic cells (paraffin, 190 ×)

Fig. 2 Normal skin: few positively stained apoptotic cells (see arrows) of sebaceous glands (paraffin, 190 ×)

Fig. 3 Normal skin: labeled apoptotic cell in the uppermost layer of the epidermis (paraffin, 480 ×)

Fig. 4 Skin wound, postinfliction interval 1.5 months: numerous positively stained cells (see arrows) in the granulation tissue (paraffin, 480 ×)



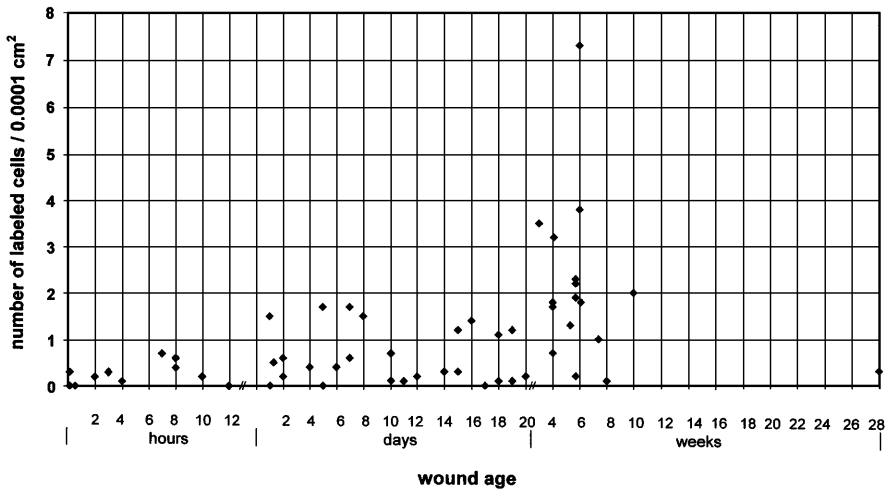


Fig. 5 Number of positively labeled fibroblastic cells/0.0001 cm² in relation to the postinfliction interval

The maximum average number of positively stained fibroblasts was 0.3 cells/0.01 cm × 0.01 cm up to a postinfliction interval of 5 h. A slight increase could be observed in wounds aged at least 6 h with maximum values of 0.6 cells/0.01 cm × 0.01 cm. In wounds with postinfliction intervals of 1 day or more, a considerable increase in the number of positive cells occurred and maximum values exceeding 1 cell/0.01 cm × 0.01 cm were found. With increasing wound age a further increase was observed and a maximum value of more than 7 labeled cells/0.01 cm × 0.01 cm was found in a wound with a postinfiction interval of 1.5 months. In skin wounds aged more than 1.5 months, a rapid decrease in the number of labeled cells occurred and in the oldest wound investigated (postinfliction interval 7 months) an average value of 0.3 cells/0.01 cm × 0.01 cm was determined. Even though increasing numbers of labeled cells in relation to wound ages up to 1.5 months occurred, several specimens with advanced postinfliction intervals revealed comparably low numbers of positive cells or even negative results.

Relevant differences in the numbers of positive cells in relation to the individual age which must be taken into consideration for a forensic wound age estimation, were not found even though a considerable intra- and interindividual variability was observed (Fig. 5).

Discussion

Cell death can occur during wound healing due to necrosis following direct mechanical tissue alteration or due to programmed cell death in apoptosis. Apoptosis especially of myofibroblasts of the granulation tissue is assumed to be responsible for the reduction in the cellularity of the granulation tissue during conversion into a scar [6]. In uninjured dermis, no relevant amounts of fibroblastic cells with apoptotic changes were seen whereas positive cells can be found in the epidermis as well as in skin appendages such as hair follicles or sebaceous glands indicating a high level of physiological cell turn-over by proliferation and apoptosis [6]. Since the number of such apoptotic cells shows a considerable interindividual variability sometimes reaching a significantly high level, the evaluation of these anatomical structures can provide only limited information for a forensic wound age estimation. In addition, necrotic parts of the tissue can be diffusely stained by in situ end labeling. Therefore, the evaluation of cells localized in such areas also seems to be of limited use for an exact numerical analysis of cell death features in skin wounds. Taking these aspects into consideration, only wound fibroblastic cells adjacent to, but distant from necrotic areas of the specimens or localized in the granulation/scar tissue can provide reliable information on the number of cells showing DNA degradation due to initial wounding. When compared to normal skin, slightly increased values of labeled cells occurred earliest after a postinfliction interval of 6 h and a considerable increase was found first in a wound aged 1 day. These results confirm the observations of other authors reporting that in most models of apoptosis cell death starts 12–24 h after an initiating trigger [9]. In this early period of wound healing, the number of labeled cells can be a result of beginning apoptosis or of cell necrosis following direct mechanical tissue alteration as recently discussed in acute myocardial infarction [2]. Since the ISEL-reaction exclusively detects nuclear DNA fragments no matter what the origin, an exact differentiation between necrosis and apoptosis cannot be performed using this method [11].

With increasing wound age apoptosis, especially of myofibroblasts of the granulation tissue, takes place leading to a reduction in wound cellularity and to a transformation of the granulation tissue into a scar as described in a rat model by Desmouliere et al. [6]. These authors found positive apoptotic staining in myofibroblasts which are assumed to be involved in wound contraction [4, 7] at the earliest 12 days after wounding. At this time, the α-smooth muscle actin expression was maximal and intensive apoptotic changes were observed between 16 and 20 days of wound age. In our series, a considerable increase in the number of labeled cells was found in wounds aged at least 19 days when compared to specimens with shorter

postinfliction intervals. This observation is in good accordance with previous studies showing numerous myofibroblasts in human skin wounds aged between 16 and 31 days [4, 5]. As suggested by Desmouliere et al. [6], the increase in labeled fibroblastic cells can easily be explained by beginning transformation of the granulation tissue into a scar which is characterized by a massive reduction in cellularity. This hypothesis was confirmed in our series, providing evidence for a rapid decrease in the number of positive cells also in human wounds with advanced postinfliction intervals of more than 1.5 months. Although a considerable intra- and interindividual variability was observed as expected in biological processes, the detection of DNA fragmentation by in situ end labeling can contribute to the estimation of postinfliction intervals. The following conclusions can be drawn:

1. Relevant numbers of fibroblastic cells with cell death-induced nuclear DNA fragments exceeding an average of 1 labeled cell/0.01 cm $\times$ 0.01 cm can be expected earliest in wounds aged approximately 1 day.
2. A rapid increase during wound healing occurs after approximately 3 weeks with average numbers of 3 positive cells or more per area.
3. Since comparably low numbers of labeled fibroblastic cells or even negative findings can also be observed in specimens with advanced postinfliction intervals, only positive results provide reliable information on wound age.

References

1. Ansari B, Coates PJ, Greenstein BD, Hall PA (1993) In situ end-labeling detects DNA strand breaks in apoptosis and other physiological and pathological states. J Pathol 170:1–8
2. Bardales RH, Hailey LS, Xie SS, Schaefer RF, Hsu SM (1996) In situ apoptosis assay for the detection of early myocardial infarction. Am J Pathol 149:821–829
3. Baretton G, Diebold J, Christoforis G, Vogt M, Müller C, Dopfer K, Schneiderbanger K, Schmidt M, Löhrs U (1996) Apoptosis and immunohistochemical bcl-2 expression in colorectal adenomas and carcinomas. Cancer 77:255–264
4. Betz P, Nerlich A, Wilske J, Tübel J, Penning R, Eisenmenger W (1992) Time-dependent appearance of myofibroblasts in granulation tissue of human skin wounds. Int J Legal Med 105:99–103
5. Betz P, Nerlich A, Wilske J, Tübel J, Penning R, Eisenmenger W (1993) The time-dependent localization of Ki 67 antigen-positive cells in human skin wounds. Int J Legal Med 106:35–40
6. Desmouliere A, Redard M, Darby I, Gabbiani G (1995) Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. Am J Pathol 146:56–66
7. Gabbiani G, Ryan GB, Majno G (1971) Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. Experientia 27:549–550
8. Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501
9. Hockenbery D (1995) Review. Defining apoptosis. Am J Pathol 146:16–19
10. Iwasa M, Maeno Y, Inoue H, Koyama Y, Matoba R (1996) Induction of apoptotic cell death in rat thymus and spleen after a bolus injection of methamphetamine. Int J Legal Med 109:23–28
11. Majno G, Joris I (1995) Review. Apoptosis, oncosis, and necrosis. Am J Pathol 146:3–15