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J. M. Suárez-Peñaranda · M. S. Rodríguez-Calvo
J. A. Ortiz-Rey · J. I. Muñoz · P. Sánchez-Pintos
E. A. Da Silva · A. De la Fuente-Buceta
L. Concheiro-Carro

Demonstration of apoptosis in human skin injuries as an indicator of vital reaction

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Abstract Demonstrating the vital character of an injury and estimation of the age are routine tasks in forensic pathology and although many different techniques have been applied to this problem none have been found to be completely satisfactory. Apoptosis, an active genetically controlled process, is the major mechanism by which homeostasis of a number of physiological systems in the body is regulated and changes in the rate following different kinds of stimuli have prompted us to test it as an indicator of vitality. We used an in situ end-labelling technique (Apop-Tag) in 30 human surgical skin injuries with age since injury ranging from 3 min to 8 h and found that apoptotic keratinocytes are found in over 50% of the cases with a post-infliction interval of at least 120 min. Apoptosis was not seen in injuries less than 120 min old or in normal skin, which was used as an external control. These results suggest that apoptosis could be a useful indicator for the intravital occurrence of injuries and could help to estimate the date of the skin injuries in some cases. The importance of strict technical control is stressed and the necessity of a complementary technique to confirm apoptosis is discussed.

Keywords Skin injuries · Apoptosis · Wound age · Vital reaction

Introduction

The process of cutaneous healing is a complex and carefully organised series of events which encompasses sequential changes involving the components of the extracellular matrix (ECM), proteins, release of growth factors and secretion of migration-stimulating cytokines (Grinnell 1992; Juhasz et al.1993). Many of these factors have been studied in order to provide information on the intravital character of an injury, but results are still under debate. Since the early work of Raekalio using histoenzymatical techniques (Raekalio 1972) up to recent investigations using monoclonal antibodies against different components of the extracellular matrix (Betz 1995; Hausmann and Betz 2001), biochemical methods (Vieira 1996), or in situ hybridisation for proinflammatory cytokines (Sato and Ohshima 2000), none has proved to be completely reliable, thus promoting the search for more positive markers. The same problem arises when investigating the inflammatory response in other tissues (Hausmann et al. 2001; Hausmann and Betz 2000).

Apoptosis is an organised, energy-dependent, process leading to cell death (Kerr et al. 1972), characterised by distinct morphological and biochemical features, which is gaining acceptance as an important contributor to different physiological and pathological processes. In the skin, apoptotic cells can be detected under a wide variety of conditions, including inflammatory dermatoses (Teraki and Shiohara 1999) and skin neoplasms (Kerr et al. 1972; Sen 1992; Thompson 1995; Weedon 1990). It is also involved in the homeostasis of healthy skin, where terminal differentiation of keratinocytes is thought to be a special form of apoptosis (McCall and Cohen 1991) and is present in the matrix and root sheath of cycling hair follicles (Norris 1995). Its role in physical and, particularly, mechanical trauma has also been demonstrated both in animal experimentation and in humans. UV radiation induces DNA fragmentation in cultured human keratinocytes through an increase in the expression of Fas and Fas-L (Gniadecki et al. 1997) and there is immunohistochemical evidence of the role of p53 genes/proteins (O'Grady et al. 1988) and bcl-2 family proteins (Sauroja et al. 1999) in this process.

The detection of an increased rate of apoptosis in the injured tissue has been considered a time-dependent

J. M. Suárez-Peñaranda (✉) · M. S. Rodríguez-Calvo
J. I. Muñoz · P. Sánchez-Pintos · L. Concheiro-Carro
Instituto de Medicina Legal Facultad de Medicina,
C/ S. Francisco s/n, 15705 Santiago de Compostela, Spain
e-mail: apimljsp@usc.es,
Tel.: +34-981-582327, Fax: +34-981-580336

J. A. Ortiz-Rey · E. A. Da Silva · A. De la Fuente-Buceta
Department of Pathology. Centro Médico POVISA, Vigo, Spain

marker of cell death in rat brain (Pravdenkova et al. 1996). To the best of our knowledge, few papers have investigated the forensic significance of apoptosis in human skin injuries (Betz et al. 1997; Sawaguchi et al. 2000). Betz et al. (1997) considered apoptosis in dermal fibroblasts as a reliable marker of the intravital character of an injury, but they failed to address the variability between different kinds of injuries, or take into account the sometimes very long post-infliction interval. The work of Sawaguchi et al. (2000) was based exclusively on skin bruises collected from autopsy cases, sometimes with a considerable post-mortem interval.

Our aim was to demonstrate the presence of apoptosis, using an in situ end-labelling technique, as an indicator of the intravital character of an injury, as well as its contribution to establishing the age of incised injuries. The use of surgical skin injuries allows precise determination of the age of the wound, and also avoids problems arising from autolysis of specimens, so common when dealing with autopsy material. This should be considered as a preliminary step towards an application of the technique to forensic casework material. We have also focused our study on wounds with a short time lapse interval, which are of particular concern in forensic practice.

Materials and methods

The study was carried out on 18 men and 12 women, all adults, with ages ranging from 28 to 76 years (average 46.7 years) and included 30 incised vital wounds inflicted with a scalpel. All were located in the abdominal skin and measured about 1.5 cm in length and 1.5 cm in depth. Transversal sections were taken from the wounds including the injury at one of the margins, the opposite margin being used as an internal control. The specimens were collected after a post-infliction interval that ranged from 3 min to 8 h and grouped into six categories of five specimens each in accordance with post-infliction intervals of <30 min, 30–60 min, 60–120 min, 120–180 min, 180–240 min and >240 min. Patients with conditions which could influence the healing process, such as metabolic disorders and corticoid or antineoplastic agent therapies, were excluded from the study. Patients consented to inclusion in the study, which was performed in accordance with ethical standards.

The specimens were fixed in 4% buffered formaldehyde, routinely processed and embedded in paraffin. Sections (4 μ thick) from each sample were placed on coated slides for in situ detection of apoptotic cells. Nuclear DNA fragments were visualised by an enzymatic reaction according to a modified protocol of Gavrieli et al. (Gavrieli et al. 1992), using the Apop-Tag in situ apoptosis detection kit (Oncor, Gaithersburg, Md.), following the manufacturer's instructions. In brief, after deparaffination and rehydration, sections were treated with proteinase K (20 μg/ml) for 5 min at room temperature and washed twice in 3% H_2O_2 for a further 5 min. The kit was then applied and the slides immersed in the TdT solution supplied for 1 h in an oven at 37°C and then developed using diaminobenzidine (6 min) and counterstained with methyl-green.

Tissue sections from tonsils were used as positive controls for DNA fragmentation. Specimens without application of the TdT enzyme served as negative controls for the technique and six specimens from non-sun exposed, normal skin, were used as external controls. Only nuclear staining was taken into account and only those cells which showed strong nuclear staining were regarded as positive.

Results

Epithelial (in the epidermis and skin adnexae) and dermal staining was evaluated and in those cases regarded as positive, only nuclear staining was noted. No significant cytoplasmatic reaction was observed. Adnexal epithelium from pilosebaceous and eccrine glands was positive in some cases, but these structures were not present in every case and, consequently, they were not taken into account.

The most consistent reaction pattern was the staining of keratinocyte nuclei (Fig. 1), which was the main parameter taken into account in the study. In the epidermis, positive results were obtained in injuries older than 120 min and 8 out of the 15 cases were clearly positive (Table 1). This was particularly common in the 120–180 min group, where 4 of the 5 cases were positive, and in the last group (older than 240 min), with 3 positive cases. In every section, labelled cells were limited to the basal and spinous layer (Fig. 2), clearly decreasing when reaching the granular cell layer, which was always negative as was the horny layer. Initially, the staining was restricted to cells close to the wound edge, mainly in the basal layer, but in some cases it had spread to affect almost the whole section length and extended through to the spinous layer.

In the dermis, convincing nuclear labelling was noted earlier than in the epidermis (Table 1) and in the 60–120 min group, 4 out of the 5 sections showed staining of

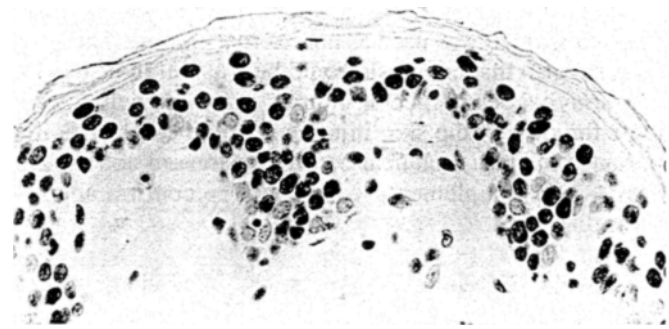

Fig. 1 Dark (*positive*) nuclei of the keratinocytes are seen interspersed with light (*negative*) nuclei in the epidermis (Apop-Tag kit ×200)

Table 1 Results of the Apop-Tag test in injuries and control cases (normal, non-sun-exposed, skin)

Postinfliction interval (min)	Epidermis	Dermis
<30	0/5	0/5
30–60	0/5	0/5
60–120	0/5	4/5
120–180	4/5	5/5
180–240	1/5	3/5
240–360	3/5	5/5
Control cases	0/6	0/6

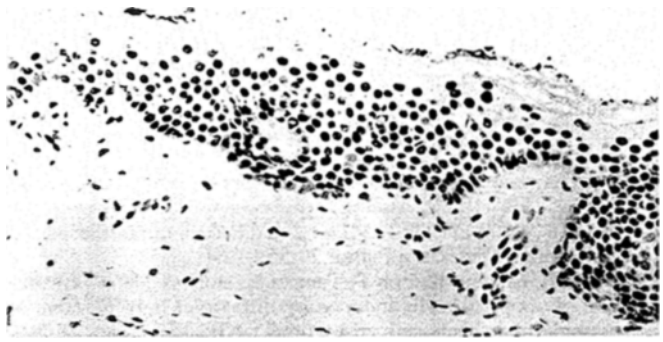

Fig. 2 Even in strong positive cases, the reaction was limited to the basal and spinous layer (Apop-Tag kit ×40)

the dermis and subcutaneous fat tissue. The degree of staining reached a maximum in the injuries in the groups 120m–180 min and 240–360 min, in which all cases were positive. These results are coincidental with epidermal positivity. Unequivocal staining was noted in the nuclei of fibroblasts and, to a lesser degree, in the nuclei of adipocytes and endothelial cells. These cells were not preferentially localised in relation to the injury margin nor to any other recognisable structure.

None of the injuries showed inflammatory responses or haemorrhagic areas, which are prone to false postive results.

All sections from normal, non-sun-exposed, skin showed no significant apoptosis, both in the epidermis and the dermis. Sections from tonsils, used as external positive controls, showed positivity restricted to the germinal centres, which was not present in sections incubated with PBS instead of TdT, which were completely negative.

Discussion

Apoptosis is involved in the physiopathology of many inflammatory dermatoses and skin neoplasms (Teraki and Shiohara 1999), in UVB-induced lesions both in animals (Okamoto et al. 1999; Ouhtit et al. 2000) and in humans (O'Grady et al. 1988) and it has been demonstrated after thermal injuries in rats (Nagata et al. 1999). Work on mechanical human skin injuries has to date received scant attention in the forensic literature, with only two previously published papers (Betz et al. 1997; Sawaguchi et al. 2000).

We have demonstrated apoptosis in epidermal keratinocytes in 8 out of the 15 injuries with a post-infliction interval of at least 2 h, but not in any of the remaining 7 cases. It is difficult to compare our results with previous work because Betz et al. (1997) did not take into account keratinocyte positivity, in orderto avoid spurious staining due to the "physiological" level of apoptosis. The demonstrable level of "physiological" apoptosis in the epidermis using the Apop-Tag kit was established by staining six control cases from normal, non-sun-exposed skin, where no significant rate of apoptosis was observed. In particular, apoptotic cells in the basal and spinous layers were never noted, which, to the best of our knowledge, is to be expected in normal skin (Teraki and Shiohara 1999). Consequently, we have taken keratinocyte positivity as the best indicator of apoptotic rate increase in the epidermis. Aditionally, the finding of positive cells far from the wound margin in some injuries with long post-infliction interval could suggest some kind of advance of the apoptotic stimulus along the epidermis. Moreover, the distribution of the apoptotic cells in the different cell layers is confined to the basal and spinous layers, similar to the distribution found in UVB radiation-induced apoptosis (Lu et al. 1999).

Contrary to Betz et al. (1997), who included several kinds of skin injuries, we limited our investigation to incised injuries to avoid the risk of divergent results arising from the heterogeneity of the lesions examined. In particular, we believe that surgical and autopsy injuries are not comparable because autolytic changes are not present in the former, but are unavoidable in the latter. Also, we have concentrated our sampling within a narrow time lapse, close to the moment of infliction, which is crucial for a differential diagnosis between vital and post-mortem injuries. Sawaguchi et al. (2000) only considered skin bruises and reported a late increase (at least 2 days) in the apoptotic rate of epidermal and dermal cells, which differs considerably from our own results. This could be due to the different nature of the injury, which, in our case implies a lesion mainly located in the epidermis, in contrast to the dermal location of bruises. While the injuries reported by Sawaguchi et al. (2000) were all bruises, they were, nevertheless, all removed after death, sometimes with a considerable delay (up to 6 days) before formalin fixation, which could have had some influence on the results. The selection of surgical material avoids this problem and, given that our samples have been taken under optimal conditions, an increase in the sensitivity of the technique could be expected.

We have confirmed the presence of apoptotic cells in the dermis and subcutaneous fat tissue, as previously noted by Betz et al. (1997) and Sawaguchi et al. (2000). The time lapse needed to detect apoptosis in dermal cells varied from 6 h to 2 days (Betz et al. 1997; Sawaguchi et al. 2000). In our study, some positive cells were found even 60 min after the infliction of the injury. The presence of some isolated positive cells in the dermis could be helpful as an indicator of early vital reaction, but since no variations with increasing wound age are apparent and they lack a defined topographic distribution this makes the validity doubtful.

Although autolysis has also been implicated in false positive results (Petito and Roberts 1995; Wolvekamp et al. 1998), in this present study it was minimal. In order to obtain reliable and reproducible results, we have carefully standardised and validated the TUNEL assay by setting up the appropriate positive (human tonsil sections) and negative controls (omission of the TdT enzyme in the TUNEL reaction mixture).

In summary, we have demonstrated that the finding of apoptosis in keratinocytes using an in situ end-labeling technique seems to be a reliable marker of vital occurrence. The fact that not all vital injuries show an increase of the apoptotic rate of keratinocytes implies that only positive results provide useful information. Consequently, when unequivocal positivity is found, the intravital character of the injury is highly probable. Strict technical control of the TUNEL assay cannot be overstated. These results should be considered as a preliminary report in order to validate the technique under optimal conditions prior to the application of the technique in daily practice.

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