Int J Leg Med (1995) 107:257–262
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ORIGINAL ARTICLE

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Temporal course of intravital and postmortem proliferation of epidermal cells after mechanical injury

An immunohistochemical study using bromodeoxyuridine in rats

Received: 11 July 1994 / Received in revised form: 28 January 1995

Abstract The temporal course of epidermal basal cell proliferation in the wound of the pinna of rats was studied using bromodeoxyuridine (BrdU) immunohistochemistry. Following incisional wounding, the animals were sacrificed at intervals ranging from 0 hours to 32 days. Two biopsies were taken from each animal, one intravitally and one postmortem after 24 hours storage at 8° C. Specimens were incubated in a solution containing BrdU and embedded in paraffin. BrdU expression was demonstrated by a monoclonal antibody against BrdU. In both intravital and postmortem biopsies, the labelling indices increased significantly in the period from 32 to 60 hours post-injury. This suggests that DNS synthesis induced during life continues after death. Applied to forensic practice, the present findings point to the possibility of determining the vitality of a wound in postmortem tissue.

Key words Epidermis · Wound · Proliferation · Wound age · Intravital · Postmortem

Zusammenfassung Im Tierexperiment wurde die Zeitabhängigkeit der Proliferationsaktivität von epidermalen Basalzellen aus der Schnittwunde des Rattenohres unter Verwendung von BrdU (in vitro-Inkubation) durch Bestimmung des Markierungsindexes untersucht. Nach Schnittverletzung überlebten die Tiere unterschiedliche Zeitintervalle von 0 Stunden bis 32 Tage. Es erfolgten pro Tier je 2 Biopsieentnahmen: vital und 24 Stunden nach Eintritt des Todes (postmortal), währenddessen der Tierkörper bei 8° C im Kühlschrank gelagert wurde. Folgendes Ergebnis konnte gewonnen werden: Unter der Annahme eine $p \geq 0.01$ nahm der Markierungsindex epidermaler Basalzellen nach 32 Stunden Überlebenszeiten bis zur 60. Stunde nach Wundsetzung signifikant zu. Diese Zunahme war nach vitaler Biopsie ebenso wie nach postmortaler Biopsie nachweisbar. Es ist somit davon auszugehen, daß nach der einmal erfolgten Proliferationsinduktion sich diese auch postmortal fortsetzt. Für die forensische Praxis ist festzustellen, daß auch an postmortalem Gewebe mindestens eine Vitalitätsbestimmung vorgenommen werden dürfte.

Schlüsselwörter Epiderm · Wunde · Proliferation · Überlebenszeit · Vital · Postmortal

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Introduction

The proliferative activity of different cell types can be examined using a number of markers such as tritiated [^{3}H]-thymidine, Ki-67, proliferating cell nuclear antigen (PCNA), etc. Extensive basic research, however, has only been done on the incorporation of thymidine (Schultze 1968), which closely resembles that of the analogous molecule bromodeoxyuridine (BrdU) (Hume 1990; van Oostrum et al. 1990; Repka and Adler 1992; Lin and Allison 1993). The BrdU incorporation rate can be quantified after either parenteral administration in vivo or in vitro exposure of tissue in a solution containing BrdU (White et al. 1990). Like thymidine, BrdU is incorporated by cells in the DNA synthesising phase (S-phase) of the cell cycle. Unlike thymidine, however, BrdU can be detected by a monoclonal antibody (Gratzner 1982) that is also effective in routine paraffin sections using immunohistochemical methods (Van der Laan and Thomas 1985).

Several studies have compared the various proliferation markers: BrdU and thymidine (give refs.), BrdU and PCNA (Jones et al. 1993); BrdU and Ki-67 (Parkins et al. 1991); BrdU, Thymidine and PCNA (Zeymer et al. 1992). In addition, basic cell kinetic studies were carried out recently using BrdU (Nowakowski et al. 1989; Hardonk and Harms 1990; White et al. 1990; Wheeler et al. 1991).

Incorporation of the DNA precursor thymidine in the early postmortem interval (Oehmichen and Zilles 1984; Frasunek and Oehmichen 1986; Oehmichen et al. 1988 a, b), implies that the relevant changes in proliferative activity remain detectable long after circulatory arrest; to date,

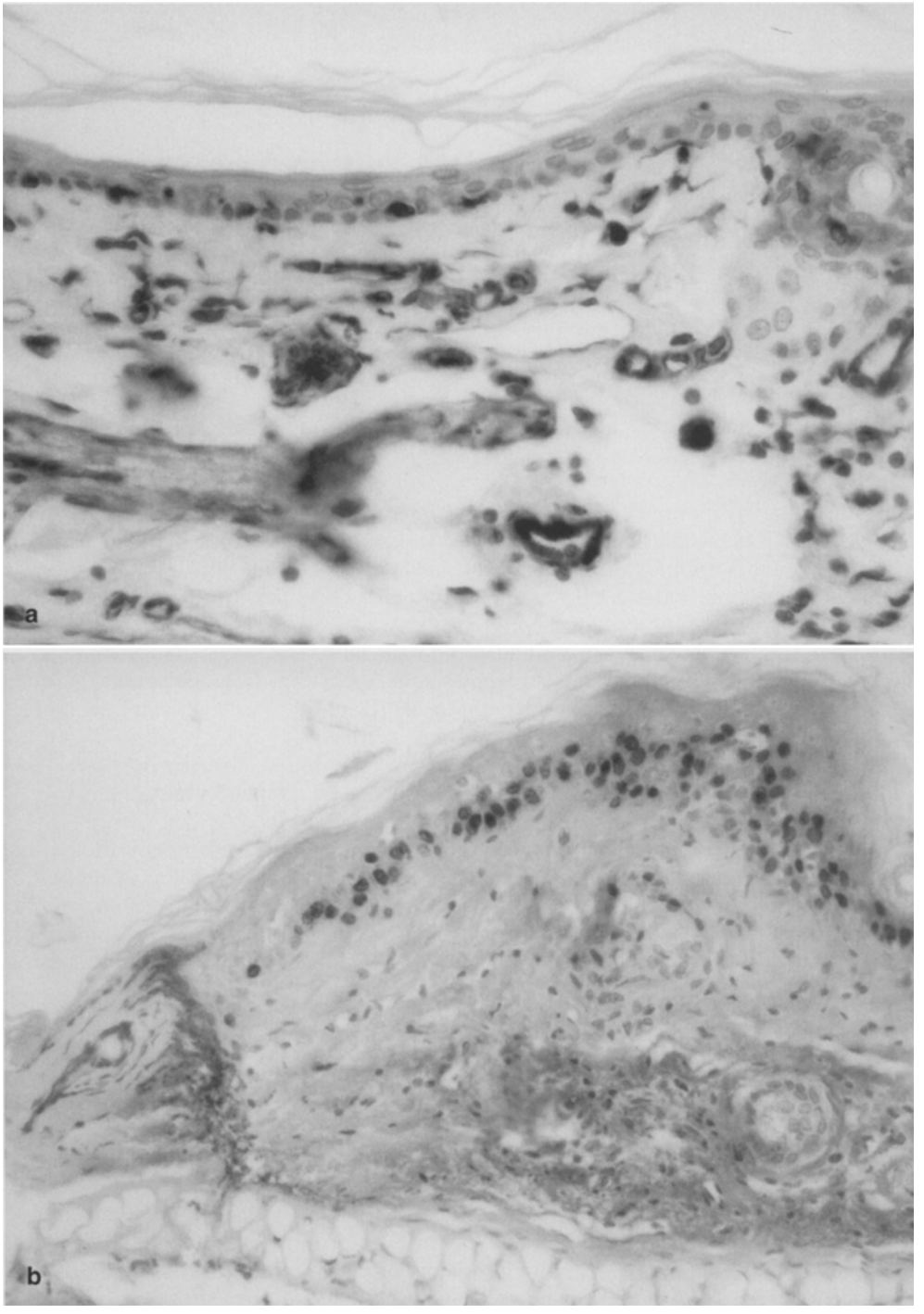

Fig. 1 BrdU-expressing cells in the basal cell layer of the epidermis: **(a)** uninjured skin of rat (anti-BrdU, nuclear fast red, × 500); **(b)** incisional wound of the rat pinna at 40 hours postwounding (anti-BrdU, nuclear fast red, × 500)

however, nothing has been published on this topic. On the other hand, an increase in in vivo proliferative activity has been reported in, for example, epidermal basal cells at the margins of mechanically induced injuries (Odland and Ross 1968; for literature see Oehmichen 1990). The present study compares the incidence of labelled epidermal basal cells in intravital and postmortem biopsies of mechanically injured skin following in vitro exposure to BrdU. A further aim of this study was based on the following consideration: proliferative activity is known to depend on the post-trauma interval in vivo. In forensic medicine, however, wound age must often be estimated in tissues after death. Therefore, we tried to ascertain whether BrdU immunohistochemistry can be useful in determining the age of wounds in postmortem tissue.

Materials and methods

Animals

A total number of 112 six-month-old female HAN-SPRD rats (Sprague Dawley) fed ad libitum on water and Altromin.

Experimental design

Under ether anaesthesia, a 6-mm-long smooth-bordered linear scissor incision was made in both pinnae of each rat. The animals were subsequently killed in groups of 7 at intervals of 0, 10, 20, 24, 28, 32, 36, 40, 48, 60, 72, 96, 120, 192, 384, and 768 hours post-wounding.

Intravital needle biopsies were taken under ether anaesthesia from one of the two wound borders (biopsy needle, Ø 3 mm, Stiefel Lab. Offenbach). The animals were then sacrificed by either intoxication with simultaneous deoxidisation and the cadavers stored at 8° C. The postmortem biopsy was taken after 24 hours from the second wound border of the same pinna.

Biopsies of non-wounded skin of the proximal part from the same ear and animal were used as an individual control to obtain information on the "normal" DNA synthesis rate of the individual rats.

Labelling of the S-phase

Intravital and postmortem biopsies were immediately (maximum interval 30 min) exposed in a BrdU solution.

The incubation medium consisted of basal medium eagle (BME Earle, code no. 47350. Serva Feinbiochemica, Heidelberg), penicillin 10,000 E/ml), streptomycin (10 mg/ml) a glutamine solution (200 ml/l), and 5-bromo-2'-deoxyuridine (code no. B5002, Sigma Deisenhoven) in a 10^{-3} M solution.

Each biopsy was incubated for 1 hour at 37° C under 2.2 atm. 0_2 pressure.

Biopsies were washed three times and fixed in a 4% paraformaldehyde solution for 24 to 48 hours. The blocks were then embedded in paraffin according to routine methods and 5 µm serial sections were cut. The sections were cut perpendicular to the incision.

Anti-BrdU antibody (code no. JO 216, Becton-Dickinson Heidelberg) diluted 1 : 100 served as the primary antibody, peroxidase-labelled, rabbit anti-mouse Ig (code no.: P 260, Dakopatts Hamburg) diluted 1 : 60 as the secondary antibody. Staining was performed with diaminobenzidine.

Nuclear fast red was used for nuclear staining.

Quantitative analysis

Only every third serial section was analysed to avoid double counting of the same nucleus. Counts of 150 basal cells of 4 different sections, each beginning at the incision and proceeding laterally, were made per specimen, for a total of 600 cells. The labelling index was obtained by calculating the percentage of cells incorporating BrdU into their nucleus. Generally, "labelled" cells could be readily distinguished from "nonlabelled" cells. Only basal cells in the epidermal layer were counted, excluding hair follicles and excretory ducts of sweat glands.

An additional classification was made for each section to find out if the DNA synthesis rate varied with lateral distance from the wound edge. Segments of 50 basal cells each were counted separately beginning at the wound edge and the average and range of the labelling index determined after counting 50 cells 4 times in different sections.

By this type of classification the calculation of the DNA synthesis rate was possible at different distances from the wound edge: the synthesis rate of nuclei near to the wound edge (cell count 1–50 cells – abt. 500 µm distance) as well as with a large distance from the wound edge (cell count 101–150 cells – abt. 130–1500 µm distance) for example.

Statistical analysis

The average, standard deviation, and range of the results for both intravital and postmortem biopsies were calculated by one-way analysis of variance, where $p \leq 0.01$ was considered significant. In addition, the multiple range test according to Scheffé (Sachs 1984) was performed, with $p \leq 0.05$ regarded as being significant.

Results

Exposure of skin biopsies in a BrdU solution revealed a clear difference in the rate of BrdU incorporation between control tissue (Fig. 1a) and wounds aged 40 hours (Fig. 1b). There were no differences between the "normal" DNA synthesis rate in the non-wounded part of the pinnae and the "time 0" at the wounded edge. The detailed quantitative results are depicted graphically in Figs. 2–4.

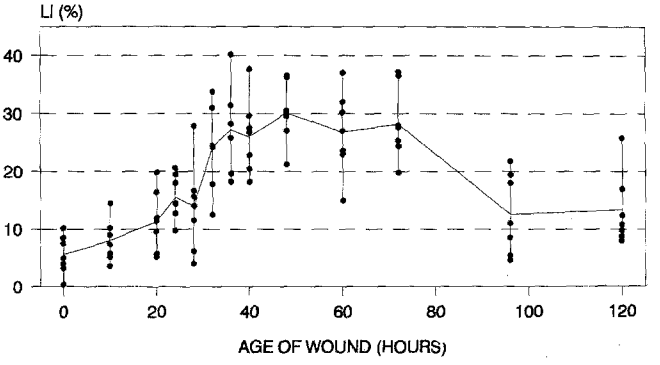

Fig. 2 Cellular kinetics of epidermal basal cells after intravital biopsy of the wound margin, demonstrated by counting 150 cells on each of 4 sections (a total of 600 cells). Experiments were continued up to a maximum of 32 days postwounding. The labelling index (LI) after intravital biopsy is shown in relation to wound age. The median and the individual measurement values are shown

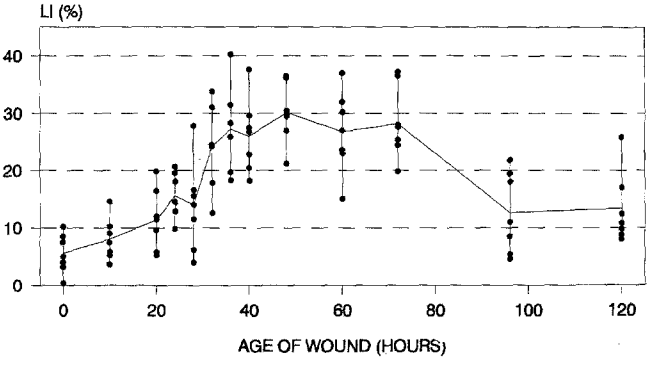

Fig. 3 Cellular kinetics of epidermal basal cells after postmortem biopsy of the wound margin, demonstrated by counting 150 cells on each of 4 sections (a total of 600 cells). Experiments were continued up to a maximum of 32 days postwounding. The labelling index (LI) after postmortem biopsy is shown in relation to wound age. The median and the individual measurement values are shown

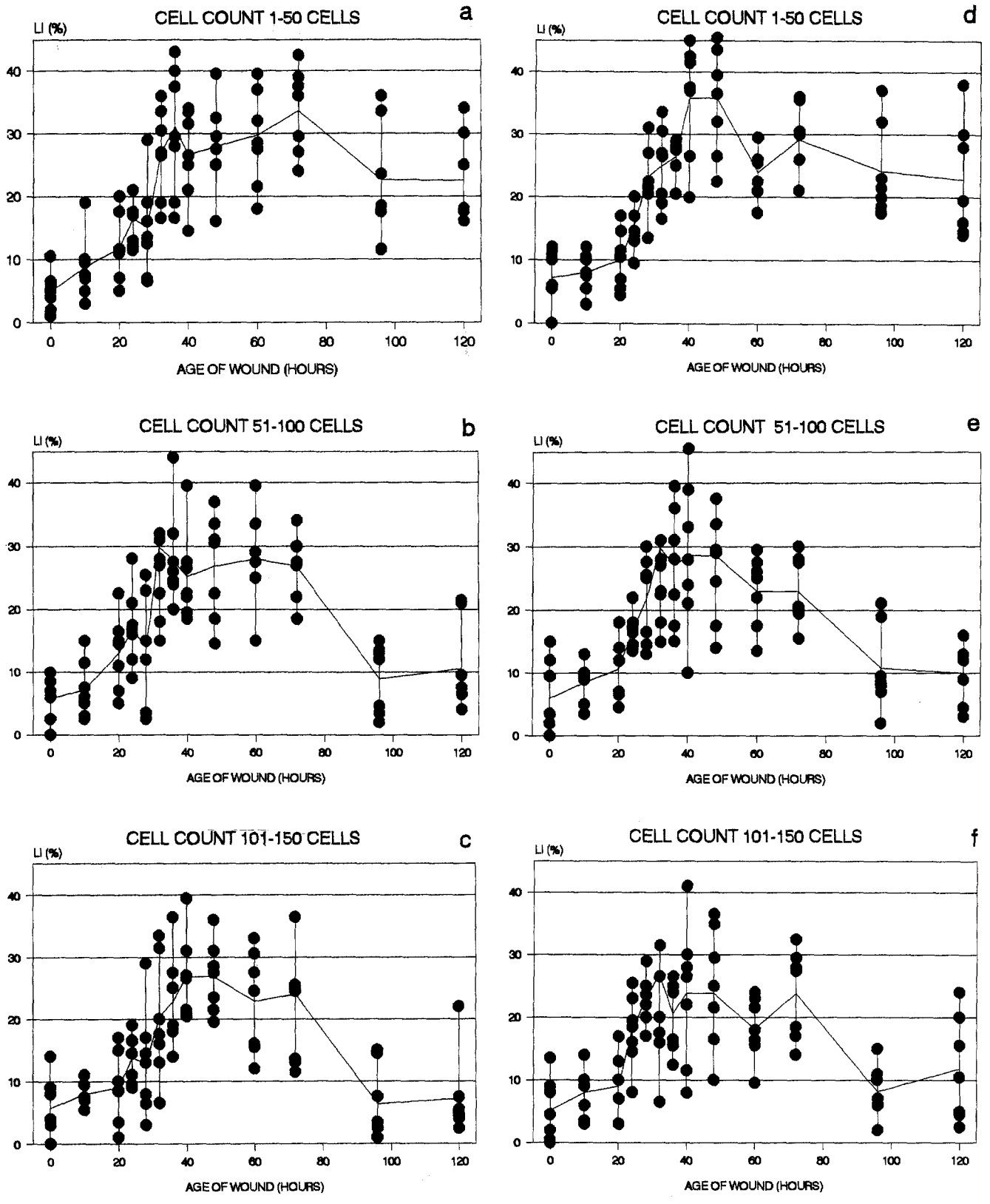

Fig. 4 Cellular kinetics of epidermal basal cells after intravital **(a–c)** and postmortem **(d–f)** biopsy of the wound margins, demonstrated by evaluating the first segment **(a and d)** of about 500 µm (counting the first 50 cells on 4 sections: a total of 200 cells), the second segment **(b and e)** of 500–1000 µm (counting the subsequent cells 51–100) and the third segment **(c and f)** of about 1000 to 1500 µm (counting the subsequent cells 101 to 150). The labelling index (LI) was measured in relation to wound age. The median and the 7 individual measurement values are shown

Proliferative activity in intravital biopsies varied as a function of wound age (cf. Fig. 2). The number of BrdU-incorporating basal cells rose significantly in the period from 32 to 60 hours post-injury, with a five-to tenfold increase in the percentage of proliferating cells as compared to controls. No difference was detected between intravital and postmortem specimens regarding either percentage of proliferating cells or the increase in their numbers in the period from 32 to 60 hours post-injury (compare Figs. 2 and 3).

Proliferative behaviour varied according to lateral distance from the wound edge (Fig. 4). The labelling index depended least on wound age in segments furthest from the incision (segments with a cell count of 101–150; cf. Fig. 4 c and f). Compared with other segments, however, the difference was not significant.

Although the time dependence of proliferation activity and postwounding interval has been demonstrated in postmortem specimens, a significant difference could be evaluated only between 32 and 60 hours postwounding interval. So we have to accept that in the case of postmortem investigation, only the vitality of wounding is demonstrated, but not the real postwounding time: a close correlation between the labeling index and the postwounding interval does not exist.

Discussion

The DNA synthesis rate of basal epidermal cells in the pinna of rats was found to change as a function of time. The percentage of BrdU-incorporating basal cells increased five- to tenfold (significant) over that of controls in the period between 32 and 60 hours post-injury. This was true regardless of whether biopsies were taken during life or 24 hours postmortem.

The intravital findings in the present study confirm those of various authors using other animal species (guinea pig, Hell and Cruickshank 1963; mouse, Bullough and Laurence 1960). Proliferation in those studies generally began 24 hours post-injury and peaked at 48 hours. Christophers and Braun-Falco (1967) reported similar findings in the pinna of guinea pigs, where the maximum rate of DNA snythesis occurred 30 to 60 hours after injury.

Previous cell kinetic studies have generally employed ^{3}H-thymidine autoradiography. In the experimental model of McDowell et al. (1990) employing BrdU immunohistochemistry to study tracheal wounds in hamsters, however, the number of BrdU-incorporating cells was observed to increase for a period of 48 to 144 hours and longer.

The one new finding of the present study is the postmortem demonstration of an increase in the number of DNA-synthesising cells. Until now it was known only that cells can synthesise DNA postmortem (Schellmann 1981; Oehmichen and Zilles 1984). Postmortem detection of DNA synthesis stimulated during life implies that the induction mechanism continues to operate after death. Growth and regeneration of skin are controlled by growth factors and interleukines on the one hand, and by the negative feedback of inhibitory factors (so-called chalones) on the other (Bullough 1973; Iversen 1981; Roels 1981).

Results in animals suggest that cell proliferation studies using BrdU may be useful et least for determining the wound vitality. So far, only one systematic study has been published on this topic (Betz et al. 1993). That study, however, employed another marker, the proliferation-associated nuclear antigen Ki-67, which was characterised by molecular biology techniques (Gerdes et al. 1991). Recently is was shown that Ki-67, previously detectable only on fresh frozen material, can also be demonstrated in formalin-fixed tissue (Gerdes et al. 1992).

Betz et al. (1993) found no correlation between the number of Ki-67-expressing basal cells and the postwounding interval in postmortem, formalin-fixed paraffin-embedded human tissue. It is surprising, however, that they did observe such a (statistically unconfirmed) correlation for fibroblast proliferation. Without casting doubt on their findings, it must be pointed out that no systematic study has been published on Ki-67 expression in relation to the length of the postmortem interval. Thus, it is not known how long this very sensitive antigen can be detected in postmortem tissue (Brown and Gatter 1990). By the same token, investigations on BrdU incorporation in basal cells in humans after injury are also lacking.

Lastly, it must be pointed out that findings obtained in animal studies, which are carried out under tightly controlled conditions, may differ markedly from those obtainable in humans, where the widely variable conditions surrounding death can lead to a corresponding disparity in results, as described for example, by Oehmichen et al. (1988 a, b). Therefore, the increase of DNA synthesis rate is to be interpretated as a vital phenomenon. In the case of investigations in human, however, it seems to be doubtful to expect a detailed temporal assignment, when there is already such a considerable range in animal experiments.

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