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Short communication

Determination of colony numbers in pig epidermis as an estimate for radiosensitivity. A rapid assay based on *in vitro* BrdU-labelling

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Abstract. A rapid assay has been developed for the quantitation of colonies arising from surviving clonogenic cells in pig epidermis after irradiation. The number of surviving clonogenic cells per unit area was related to the epidermal *in vivo* response of moist desquamation. After irradiation with single doses, ranging from 20 to 36 Gy, skin biopsies were taken and incubated in dispase for enzymatic separation of the epidermis and dermis. Full thickness epidermal sheets were labelled with bromodeoxyuridine (BrdU) *in vitro*. Proliferating cells were visualized using standard immunohistochemical procedures. Cell groups containing $\geqslant 16$ cells were counted as colonies. These colonies were first seen on day 14/15 after irradiation. The number of colonies per cm^2, as a function of skin surface dose, yielded a cell survival curve with a D_0 ($\pm$SE) of 3.87 ± 0.57 Gy. The ED_{50} for the epidermal *in vivo* reaction of moist desquamation corresponded with a colony density of 2.7 colonies per cm^2. After higher doses, abundant smaller colonies of 4–8 BrdU-positive cells were seen and these were more radioresistant, as represented by higher D_0 values.

Radiation-induced epidermal injury of the skin is often assessed with the use of macroscopic endpoints such as erythema and moist desquamation. In pig skin these endpoints can be evaluated at 4–6 weeks after irradiation. However, the severity and incidence of this damage may be influenced by environmental factors, such as infections, partially obscuring the dose–effect relationship. To avoid these possible confounding factors a more direct approach was used, by studying colony formation from surviving epidermal clonogenic cells. There is likely to be an inverse relationship between the incidence of moist desquamation and the survival of clonogenic cells. Colony formation can be used as an estimate of radiosensitivity [1–3]. In the past, several groups have attempted to determine radiation damage (more directly) using the number of colonies arising from surviving clonogenic cells as a parameter for epidermal radiosensitivity [4–7]. Various types of assays were used, such as (i) a macroscopic *in vivo* colony assay for rodent skin [5–7], (ii) autoradiographs of whole epidermal sheets for the detection of proliferating cells in mouse skin [4], (iii) cross-sections of pig skin viewed by light microscopy [8], or (iv) blocking cells in mitosis followed by staining with Schiff's reagents using whole epidermal sheets from biopsies of pig skin [9]. A drawback of the macroscopic colony assay is the fact that smaller colonies are easily missed, while the use of autoradiographs and cross-sections is laborious and time consuming. The assay introduced by Chen et al [9] involved three injections of vincristine, resulting in the induction of a metaphase arrest, which allowed recognition of proliferating cells by the presence of metaphases. In this assay, separation of the epidermis from the dermis was accomplished by hydrolysis with 5 N HC1 followed by teasing apart with forceps.

The objective of this paper is to describe a relatively easy and rapid method for the detection of colonies in pig epidermis at around 2 weeks following irradiation using *in vitro* labelling with bromodeoxyuridine (BrdU). The epidermis and dermis of small skin biopsies, obtained from Yorkshire pigs, were separated enzymatically. Proliferating cells in these whole epidermal sheets were labelled *in vitro* with BrdU followed by an immunohistochemical procedure to visualize these cells. An immunohistochemical procedure for cell cultures was adapted and altered according to the specific requirements for whole epidermal sheets. Screening of whole epidermal sheets with light microscopy was quick

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as the colonies, consisting of distinct clusters of tightly packed, labelled cells, were easily recognized. Cell survival curves were obtained by expressing the number of colonies per unit area as function of the skin surface dose. A correlation was made between the number of colonies per unit area and the *in vivo* response of the epidermis, *i.e.* moist desquamation.

Materials and methods

Irradiation

Young, Yorkshire strain, female pigs weighing ≈25 kg, purchased from a commercial supplier, were used for this study. After hair clipping, a total of 15 fields, 3 cm × 3 cm and 3 cm apart, were tattooed on each flank. Prior to irradiation the animals were sedated with ketamine and stresnil to enable endotracheal intubation. During irradiation an anaesthetic gas mixture of ≈70% oxygen: ≈30% nitrous oxide and 1–2% halothane was used [10]. Skin fields were irradiated with a ^{192}Ir stepping source from an HDR-microSelectron (Nucletron). The microSelectron was connected to an array of four parallel, equidistant catheters in a 5 cm × 5 cm silicon flexible template, which was taped to the skin. The template contained four catheters, 1 cm apart, which covered the 3 cm × 3 cm field. The distance between the axis of the catheters and the skin surface was 5 mm. A stepping source was programmed, using the Nucletron Planning System (NPS), so that each catheter had seven dwell positions at a distance of 5 mm, *i.e.* a total of 28 positions over the field. Dwell times for each source position were optimized using the NPS to minimize dose variations at a distance of 5 mm in a plane field. The 95% isodose was at the level of the basal layer, while the 80% isodose was at the dermis–fat interface. Dosimetry was carried out in three different ways: (a) accurate calibration of the source activity, according to the NCS protocol [11, 12]; (b) accurate computer planning for brachytherapy [13]; and (c) thermoluminescent dosimetry (TLD) measurements. Source calibration and computer planning allowed dose calculations to be made with errors of <3%. The doses delivered were checked with TLD measurements, which gave, after correction for TLD thickness, deviations of <3%. The skin fields were irradiated with skin surface doses of either 20, 24, 27, 30, 32 or 36 Gy.

Immunohistochemistry

At days 10 and 14/15 following irradiation, biopsies of 4 mm in diameter were taken from three pigs. A total of 16 biopsies was collected per field, avoiding the edges of the field. Each data point represented the results from 16–96 biopsies. Biopsies were incubated in 0.5% dispase (Boehringer, Mannheim, Germany) in phosphate buffered saline (PBS) at 37 °C in order to separate the epidermis from the dermis. Dispase is a protease that preferentially dissolves connective tissue of the basal membrane [14, 15]. After 2 h incubation the epidermis and dermis could be gently separated with forceps leaving a full thickness epidermal sheet. After separation each epidermal sheet was incubated in 1 ml DMem/HamF12 medium with 30 µl BrdU added for 24 h at 37 °C. This was followed by immersion in 1 ml 70% ethanol for 1 h, 1 ml 1 N HCl for 15 min at 60 °C, a 1 ml borax solution (0.1 M Na-tetraborate+0.2 M boric acid) for 30 min, and finally 1 ml of 2% H_2O_2 in PBS for 30 min. This procedure was followed by incubation overnight at 4 °C with a monoclonal antibody specific for BrdU (Mab-BrdU) (ICN Pharmaceuticals, Aurora, Ohio, USA) in 100 µl of a 1% bovine serum albumin (BSA) solution in PBS. Previous tests indicated that a Mab-BrdU dilution of 1:50 (v/v) yielded good results. Overnight incubation was followed by a rabbit anti-mouse conjugated peroxidase (Ram-Po) (DAKO a/s, Glostrup, Denmark) using a 1:80 (v/v) dilution in 100 µl of 1% BSA in PBS together with 2% human AB serum. BrdU-labelled cells were stained with a 100 µl solution of 0.05% 3,3′-diaminobenzidine tetrahydrochloride dihydrate (DAB) (Fluka Chemika, Zwijndrecht, The Netherlands) in PBS, in the presence of 0.03% H_2O_2. Between each step the epidermal sheets were washed three times with 1 ml of 1% BSA in PBS. After staining with DAB the epidermal sheets were dehydrated through graded ethanol solutions, followed by xylol/100% ethanol (1:1) for 20 min and xylol for 20 min. The epidermal sheets were covered by a standard glass coverslip and examined microscopically. Omission of Mab-Brdu, Ram-Po or DAB constituted negative controls. Preparation of skin samples for light microscopy took less than 3 days.

Analysis

The epidermal sheets were examined at a magnification of 200×. Distinct clusters of tightly packed cells containing at least 16 brown nuclei indicative of proliferating cells were counted. The total area screened in each biopsy was converted to metrical units using the Vernier scales. The number of colonies counted in 16 biopsies obtained from one field was expressed per cm^2. Each data point contained 16–96 biopsies from one to six fields and the mean number of colonies per cm^2 over these fields was calculated. The

relationships between the mean number of colonies per cm^2 and dose were analysed using linear regression analysis.

Approval

This study was approved by the animal ethical committee of the Medical Faculty, Eramus University, Rotterdam (protocol number 605.93.03). All procedures with animals were carried out according to the guidelines set by this committee.

Results and discussion

After 2 h incubation with the enzyme dispase a full thickness epidermal sheet was obtained with normal morphology (an intact basal layer and all other keratinocyte layers) (Figure 1a). Occasionally, at the edges of the biopsy, the basal layer became detached from the cell layers above and tended to curl. Incubation of biopsies with dispase for periods up to 6 h disrupted the basal layer so that accurate counts were impossible.

Since full thickness epidermal sheets were used in this study, a labelling period of 24 h was used to ensure that BrdU penetrated the epidermis sufficiently. In unirradiated skin, BrdU-labelled cells were seen as solitary cells (Figure 1b) and as pairs (Figure 1c). In irradiated skin, colonies with $\geqslant 16$ BrdU-positive cells, *i.e.* cells that had undergone at least four cell divisions, were counted. Although the limit of $\geqslant 16$ cells per colony was arbitrary, it did exclude very small colonies where the number of divisions was more limited as these smaller colonies were unlikely to contribute significantly to re-epithelialization [8].

At 10 days following a single dose of 20 Gy, no clusters of BrdU-labelled cells containing $\geqslant 16$ cells could be found. Occasionally, solitary BrdU-labelled cells were seen. At days 14/15 following irradiation, the first colonies containing at least 16 BrdU-positive cells were seen. In nearly all colonies the BrdU-labelled cells were tightly packed together as a cluster (Figure 1d). Even if the clustering of BrdU-labelled cells was less tight they could still be recognized as belonging together in one colony, since the surrounding area showed only a few scattered BrdU-positive cells. After lower radiation doses these solitary cells were more common than after higher doses. The number of colonies per cm^2, expressed as a function of skin surface dose, is presented in Figure 2a. The slope of the cell survival curve indicated a D_0 ($\pm$SE) of 3.87 ± 0.57 Gy and an extrapolation number ($\pm$SE) of $(15.1 \pm 1.7) \times 10^3$. A similar D_0 of 3.05 ± 0.38 Gy was obtained by Chen et al [9] for a $4\ cm \times 4\ cm$ skin field. Although not significant this slightly lower D_0 value could be the result of a more uniform depth dose distribution using 3 MeV electrons, and hence a smaller contribution from cells migrating from the follicular epithelium. In the present study the depth dose distribution was not uniform, with the 80% isodose at the dermal fat layer allowing the much more radioresistant epithelium of the hair follicles to contribute substantially to re-epithelialization [3, 16]. Although not quantified, colonies were often seen in the vicinity of hair follicles. However, other variables in the experimental set-up of Chen et al [9] could have influenced this small difference in D_0 values. For example, they counted colonies with $\geqslant 32$ cells, and biopsies were taken at days 19–21 following irradiation. In addition, the difference in the strain of pig (Landrace) and in anaesthesia might also have been a contributing factor [17].

Three different levels for the incidence of moist desquamation, the ED_{20}, ED_{50} and ED_{80}, obtained from a separate *in vivo* study carried out with the same strain of pig using the same irradiation set-up with the HDR-microSelectron, the same flexible silicon template and the same anaesthetic procedures [18], are indicated for comparison. The ED_{50} value (95% confidence interval) at the skin surface was 33.6 (32.0–35.1) Gy, which corresponded to 2.7 (4.0–1.8) colonies per cm^2, while the ED_{20} and ED_{80} levels corresponded with 10.4 and 0.7 colonies per cm^2, respectively (Table 1). In the study by Chen et al [9] no data for macroscopic endpoints were presented and hence no comparison between colony numbers and *in vivo* responses was possible. Comparison of gross skin responses with cell survival data in mouse epidermis gave similar results [5].

In addition to the large colonies containing $\geqslant 16$ BrdU-labelled cells, other colonies of different sizes were observed. These macrocolonies and microcolonies were arbitrarily divided into colonies consisting of 4–8 cells, 9–15 cells, 16–31 cells and $\geqslant 32$ cells. At day 10 after a single dose of 20 Gy, only colonies with 4–8 BrdU-positive cells were seen, with a total of 23.53 ± 0.21 colonies per cm^2 (mean $\pm$ SE). The number of colonies in relation to colony size is expressed as function of skin surface dose at day 14/15 in Figure 2b. Large colonies with $\geqslant 32$ BrdU-labelled cells were extremely rare at this stage (in one skin field after 27 Gy there was one such colony), while after 30 Gy three colonies with $\geqslant 32$ cells were seen. Smaller colonies were abundant, particularly the very small ones. Smaller colonies were also seen in the vicinity of larger colonies, suggesting that clonogenic cells may have migrated from these initial colonies, contributing to rapid re-epithelialization.

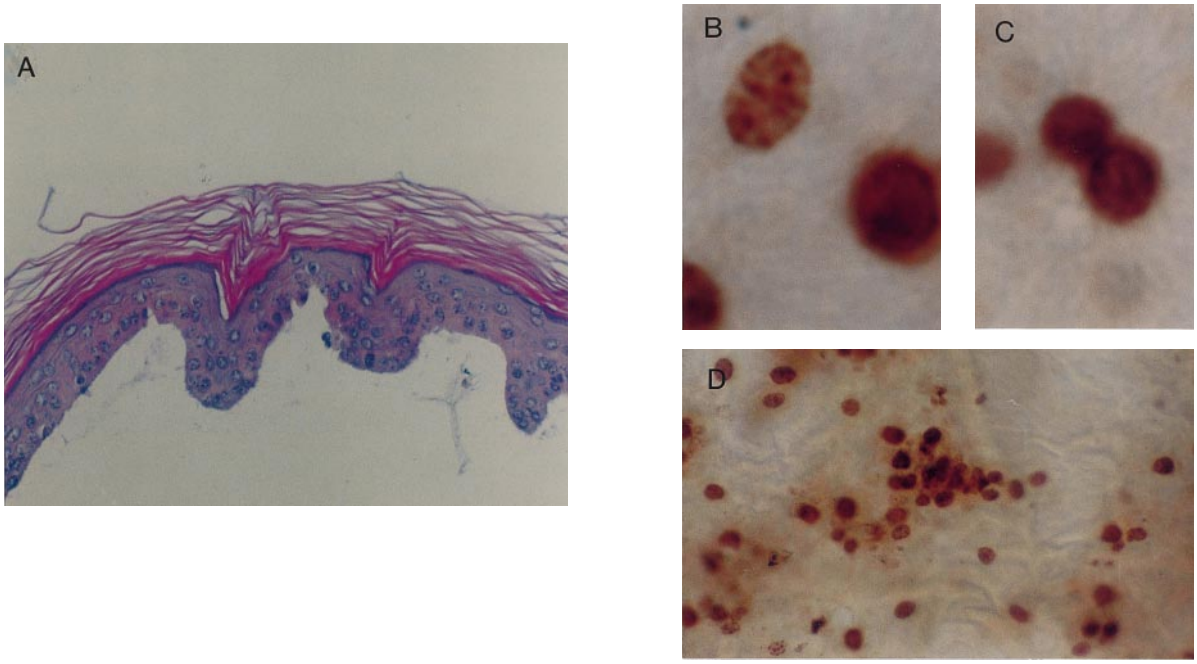

Figure 1. (A) Histological cross-section of pig epidermis after enzymatic separation with dispase for ≈2 h. All epidermal cell layers and rete pegs are intact. Only at the edge of the biopsy did the basal layer occasionally become detached from the layers above and tend to curl. HE staining, original magnification 100×. (B) Solitary BrdU-labelled cells in the dorsal area of unirradiated pig epidermis. Original magnification 1000×. (C) BrdU-labelled cells as pairs in the dorsal area of unirradiated pig epidermis. Original magnification 1000×. (D) Compact cluster of ⩾16 BrdU-labelled cells indicating a colony, 14 days following a single dose of 27 Gy. Solitary cells are sparsely scattered.

Alternatively, not all surviving clonogenic cells may begin proliferation at the same time and/or the growth rate of these colonies may differ, resulting in colonies of different size.

Surprisingly, the D_0 value increased for smaller colonies (Table 1). Apparently, higher doses resulted in the production of large numbers of small colonies and thus a high D_0 value. This is in agreement with cell density data in pig epidermis where a faster proliferation rate was noticed for higher doses [19]. Large numbers of small colonies containing <16 cells were also counted in other studies on pig skin [16, 19], as well as rodent skin [4, 20] and mouse lip mucosa [21]. It was proposed that these small colonies represent transit cells with a limited proliferative capacity entering post-mitotic maturation [22, 23]. It was found that these microcolonies were also more radioresistant, as observed in this study by higher D_0 values. In an extensive cell kinetic study of mouse tongue epithelium [24], it was concluded that sterilized clonogenic cells started proliferation, but the number of these cell divisions turned out to be very limited; they were called abortive

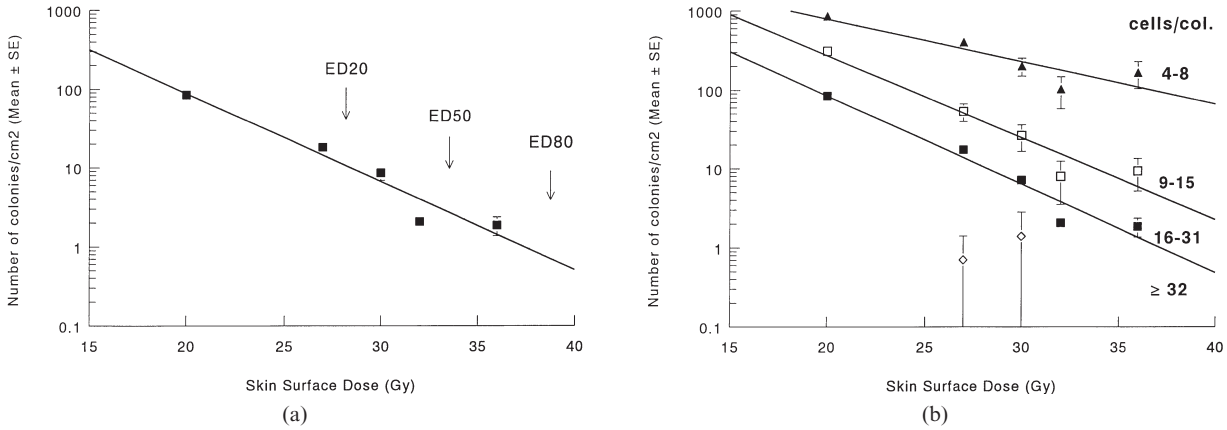

Figure 2. (a) Cell survival curve in pig epidermis for colonies containing ⩾16 BrdU-labelled cells at 14/15 days following irradiation. Three different levels for the incidence of moist desquamation, ED_{20}, ED_{50} and ED_{80} value, obtained *in vivo*, representing 28.3 Gy, 33.6 Gy and 38.8 Gy skin surface dose, respectively, are indicated for comparison. (b) Cell survival curves in pig epidermis for colonies containing different numbers of BrdU-labelled cells at 14/15 days following irradiation. Subdivision into colony size was arbitrary.

Table 1. D_0 values ($\pm$SE), extrapolation number ($\pm$SE) and number of colonies per cm^2 at ED_{20}, ED_{50} and ED_{80} level for moist desquamation, obtained *in vivo*, in relation to colony size

Colony size (No. of cells)	D_0 value (Gy)	Extrapolation number ($\times 10^{-3}$)	No. of colonies		
			ED_{20}	ED_{50}	ED_{80}
4–8	8.01 ± 2.28	9.5 ± 1.1	282	146	77
9–15	4.15 ± 0.68	32.9 ± 3.7	37	10.5	3.0
16–31	3.86 ± 0.53	14.7 ± 1.6	10.0	2.5	0.7
$\geqslant 16$	3.87 ± 0.57	15.1 ± 1.7	10.4	2.7	0.7

divisions. The result was a large number of small colonies, as also observed in this study.

Conclusions

This study demonstrated that the use of full thickness epidermal sheets is suitable for investigation of colony formation after irradiation and that colony numbers can be used as an estimate for radiosensitivity. Small skin biopsies were used for enzymatic separation of epidermis and dermis followed by *in vitro* BrdU-labelling and immunohistochemistry, allowing quantitative assessment of proliferating cells. Processing of biopsies for light microscopy took less than 3 days. Colonies containing $\geqslant 16$ cells were first seen at 14/15 days after single irradiation doses of 20–36 Gy. The number of colonies per cm^2 decreased with increasing dose, giving a D_0 of 3.87 Gy. The ED_{50} value for the *in vivo* epidermal response of moist desquamation corresponded with 2.7 colonies per cm^2. However, further studies are required to determine interanimal and intraanimal variability and to optimize various immunohistochemical procedures. Further studies are also needed to obtain cell survival parameters for colonies containing $\geqslant 32$ cells at later stages after irradiation.

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