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Proliferation kinetics of mouse tongue epithelium under normal conditions and following single dose irradiation

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Summary. Epithelial proliferation in the ventral surface of mouse tongue follows a pronounced circadian rhythm with a peak in mitotic activity at 10.00 a.m., preceded by a wave of DNA synthesis 8 h earlier. Nearly all cells (85%) pass through G2 and mitosis immediately after the S-phase; they subsequently divide again, usually after 2 or 3 days, indicating cohorts of cells with different G1-duration. The fraction of all nucleated cells comprised in one daily proliferation wave is about 20%, indicating a turnover time of the nucleated cell compartment of about 5 days. Cytotoxic injury by a single radiation dose of 20 Gy causes a steep decrease in cell counts, leading to complete denudation after 9–13 days. The difference between the latent period before ulceration and the tissue turnover time is explained by a marked proliferative activity of the doomed cells. The mitotic index increases steeply after day 1 to three times the control level, but most mitotic figures display gross abnormalities such as multipolar spindles or chromosome clumping. As a consequence cells with abnormal or multiple nuclei appear in the basal layers 3 days post irradiation and subsequently migrate to the upper layers. After denudation the epithelium rapidly becomes restored, with a phase of transient hyperplasia on days 13–14. Normal architecture is regained by day 15. Over the whole healing period the mitotic index remains at a high level, with most of the mitoses appearing histologically normal.

Key words: Mouse tongue mucosa – Cell kinetics – Circadian rhythm – Irradiation

Introduction

In epithelia subject to constant renewal, such as the epidermis or the epithelial lining of the digestive tract, surface cell loss is balanced by continuous cell production in the proliferating cell compartment. In stratified squamous epithelium this compartment is located in the deeper basal and suprabasal strata, which represent the germinal layer.

The stratified epithelia of the upper aerodigestive tract of rodents are known to follow a marked diurnal rhythm of proliferation. The periodicity is strictly correlated with the animals' activity and food intake, suggesting that the signal to proliferation is correlated with mechanical wear and tear.

Severe alterations occur in such epithelia during radiotherapy. Radiation-induced stomatis is an example that has considerable clinical bearing. During conventional radiotherapy for advanced tumours of the head and neck, where 5×2 Gy per week are delivered to total doses of 60–70 Gy in 6–7 weeks, the first signs of stomatitis develop within the second treatment week. They initially present as focal erythema, but progress rapidly into focal or confluent mucosal denudation, i.e. pseudomembraneous stomatis, which may necessitate an interruption of treatment for 2–4 weeks.

The biological basis of this damage is the loss of reproductive integrity in the stem cells of the germinal layer that subsequently leads to a deficit in the cellular supply of differentiated functional layers. Thus, as the natural loss of superficial material continues, hypoplasia and eventually denudation of the epithelium develop as proliferative failure is translated into a lack of functional cell replacement.

Mouse tongue mucosa was introduced as a model for radiobiological studies by Moses and Kummermehr (1986). The morphological response of this tissue, as well as the latent period before gross tissue damage becomes apparent after concentrated irradiation, is similar in the mouse (Dörr and Kummermehr 1990) and man (Van der Schueren et al. 1990). Important radiobiological principles can readily be studied in the mouse model.

The present paper reports both investigations into normal cell proliferation in this tissue and the changes occurring after single dose irradiation with 20 Gy. The diurnal rhythm, cell cycle parameters and tissue dynam-

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ics, as well as the kinetics of tissue atrophy and healing are described.

Material and methods

Female C3H mice, 10–12 weeks old, of the inbred Neuherberg colony were used in all experiments. They were housed in a specific pathogen-free environment with controlled conditions of humidity and temperature and a 12/12 h light-dark cycle changing at 6.00 a.m. and 6.00 p.m. The animals were fed a standard pellet diet and water ad libitum.

Histological processing. For histological examination, the mouse tongues were excised at their base after sacrifice, fixed in formalin-alcohol, divided along the median sulcus and embedded in methyl-methacrylate. Three sections (3 µm thick) separated by 200 µm were cut from each specimen. In a metaphase arrest pilot experiment with Vinblastine, specimens were embedded in paraffin wax and 5 µm-thick sections were cut.

Autoradiographs were prepared with Kodak NTB2 emulsion. After 3 weeks exposure at 4° C they were developed and stained with Hematoxilin and Eosin.

Microscopic examination was made of the inferior surface of the tongue where it is covered by smooth squamous epithelium, i.e. from about 1 mm posterior to the tip of the tongue.

Cell kinetic studies. Circadian fluctuations of cell influx into mitosis were assessed by the metaphase arrest method using Vinblastine (VLB; Velbe, Lilly, Bad Homburg, FRG). The drug was injected intraperitoneally at a dose of 2 mg/kg and the mice were sacrificed after arrest periods of 4 h. This dose and time interval were determined in a pilot experiment, where doses of 1 and 2 mg/kg VLB and 2, 4 and 6 h intervals were tested. Injections around the 24 h period were staggered by 2 h, thus producing two runs with a time shift of 2 h.

The stathmokinetic measurements were paralleled by the excision of control tongues between 8.00 a.m. and 6.00 p.m.

According to previous experiments (Moses and Kummermehr 1986), DNA synthetic activity in the mouse tongue epithelium is very low during the day-light hours (see Fig. 2). Starting from almost zero around 5.00 p.m., it rises to a broad peak culminating at 2.00 a.m. and then rapidly decreases. To label one entire wave of DNA-synthetizing cells in the present experiments, three injections of ^{3}H-TdR were given intraperitoneally at 9.00 p.m., 2.00 a.m. and 7.00 a.m. at a dose of 37 kBq/g body weight/injection (Amersham Buchler, Braunschweig, FRG; specific activity 185 MBq/mmol). The animals were sacrificed at different times after the first injection, ranging from 7 h to 191 h, i.e. 8 days. Vinblastine was applied 4 h before tongue excision.

In all cell kinetic experiments, three animals were used for each time point. The mean value for each animal was calculated on the basis of an examination of six sections; the mean for each time point was then calculated from three individual mean values. Results are presented as the number of nuclei/cells/mitoses/labelled mitoses per mm epithelial length.

Irradiation studies. Local irradiation of the whole inferior tongue surface by soft X-rays was performed according to a technique described previously (Dörr and Kummermehr 1990). Briefly, animals were anaesthetized with Hexobarbital (150 mg/kg i.p.) and restrained in a supine position in the central bore of a prewarmed (35° C) aluminium block. The tongue was gently pulled forward through a vertical hole in the roof of this block and the head then was supported by a wedge to avoid traction at the tongue base. The tip of the tongue was fixed by adhesive tape with the ventral surface exposed to the vertical X-ray beam at a source-to-surface distance of 15.5 cm. A Philips PW 1130 X-ray machine was used. It was operated at 25 kV and 20 mA with 0.3 mm Al filtering and a dose rate of 9.5 Gy/min. Dosimetry was performed in each session with an ionisation chamber (type M 23342, PTW Freiburg) placed close to the tongue at an identical source distance.

Several days after irradiation erythema and oedema of the tongue developed, followed by increased desquamation sometimes progressing to an erosive lesion. Maximum prevalence of these erosions was seen on day 12 and their average duration was 3 days. The incidence of these erosions or shallow ulcerations was clearly dose-dependent. In the radiobiological studies, the frequency of animals developing tongue ulcer was therefore used to measure the dose response and the curve was fitted by probit analysis. At least eight animals were used in dose groups of 12, 14, 16, 18 and 20 Gy.

To investigate radiation-induced changes in cell proliferation, 150 animals were irradiated with a single dose of 20 Gy, applied between 10.00 a.m. and noon, and two animals were sacrificed at each time point (8.00 a.m., 10.00 a.m., noon, 2.00 p.m. and 4.00 p.m.) from day 1 to 15 after irradiation.

Results

Metaphase arrest data

In a pilot stathmokinetic experiment several doses and arrest periods were tested; the resulting metaphase counts are shown in Fig. 1. For both injection times, counts increased within the first 4 h after 1 or 2 mg/kg VLB. They continued to increase beyond 4 h after 1 mg/kg of VLB. After the higher dose they decreased after 4 h, thus suggesting that cells treated at higher doses undergo degeneration earlier. Arrest periods of 2 and 4 h yielded the same counts at both doses. Thus, a dose of 2 mg/kg and an arrest period of 4 h were used in further stathmokinetic experiments.

The rate of entry into mitosis can be estimated best from the data in Fig. 1 for 2–4 h after VLB injection. Between 0 and 2 h, the increase in metaphase counts is assumed to be non-linear, as the drug needs 0.5–1 h to become fully effective (Thorud et al. 1980). At arrest times exceeding 4 h cells are lost due to degeneration in metaphase. An average mitotic influx of 12.5 cells/h and 11.7 cells/h was calculated after injections at 6.00 a.m. and 8.00 a.m., respectively. However, these values were assessed during periods of high mitotic activ-

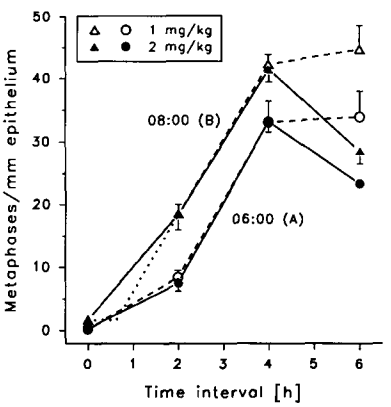

Fig. 1. Metaphase counts (±SEM) in mouse tongue epithelium at various intervals after i.p. injection of Vinblastine either at 8.00 a.m. (*B*) or at 6.00 a.m. (*A*). The doses applied were 1 mg/kg (△/○) and 2 mg/kg (▲/●). The dotted line in (*B*) is a projection of the slope encountered in (*A*) between 8.00 a.m. and 10.00 p.m.

ity and consequently do not allow an estimation of the number of daily dividing cells.

It needs to be noted that these cumulative mitotic measurements are superimposed by the dynamic changes in cell influx. The slope of curve B between 2 and 4 h, i.e. from 8.00 a.m. to 10.00 a.m. should thus be identical to that of curve A between 0 and 2 h. However, projection of the B slope on the A results in a delay in the VLB action of 0.5–1 h as expected.

Normal cell proliferation

A marked circadian rhythm of proliferation in tongue epithelium is apparent from the pronounced fluctuations in metaphases arrest counts over 24 h, as illustrated in Fig. 2. Mitotic activity is very low during the late afternoon and evening hours, but increases progressively from midnight (3.25 metaphases/mm) to 10.00 a.m. (arrest period 8.00 a.m. – noon, 37 metaphases/mm) and then falls off rapidly. A similar time pattern is seen when the number of mitotic figures (meta- and anaphases) is evaluated without VLB arrest, as also included in Fig. 2. Counts rise from 1.7 mitoses/mm at 8.00 a.m. to 7.7 at 10.00 a.m. and subsequently decrease to 0.2 at 6.00 p.m., consistent with the results obtained in the VLB study.

Fluctuations in DNA synthesis in the mouse tongue epithelium have been described previously (Moses and Kummermehr 1986). The DNA-synthetic wave precedes the mitotic wave by about 8 h, as also indicated in Fig. 2.

No significant diurnal fluctuations were detectable in the number of nucleated cells or cells in the germinal layer. Counts per mm epithelium length were 430 ± 12 and 305.7 ± 5, respectively.

An approximate value for the proportion of cells dividing per day is obtained by summing all the metaphases accumulated in sequential 4 h arrest intervals. Figure 3 shows such cumulative curves for the two runs that were started at 6.00 a.m. and 8.00 a.m., respectively.

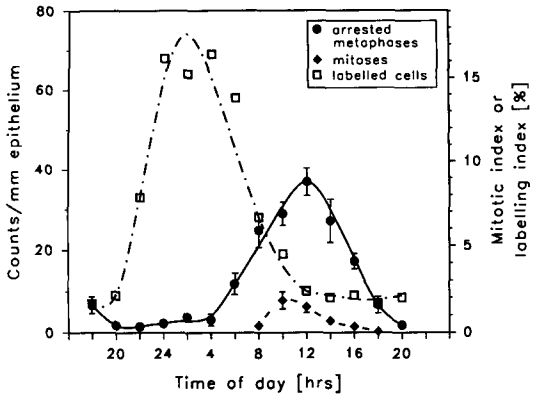

Fig. 2. Circadian fluctuations ($\pm$SD) in the number of mitoses per mm (*left axis*) and the corresponding mitotic index (*right axis*) measured spontaneously (◆) or after a 4 h VLB metaphase arrest (●). Symbols (□) and the dot-dashed line represent labelled cells after ^{3}H-TdR pulse labelling as taken from a previous study (Moses and Kummermehr 1986). The abscissa denotes the time of ^{3}H-TdR injection or the time of sacrifice

The number of cells passing through mitosis in 24 h were very similar indeed, with 84.2 and 86.6 metaphases/mm, respectively. The slope of the curves at different times correspond to the rate of entry into mitosis, also indicating diurnal variations.

When these figures are related to the total cell counts given above, it becomes apparent that 27.9% of the cells of the germinal layer, or 19.4% of the entire population, are replaced per day. From this the turnover time of the germinal layer is estimated to be about 3.6 days.

By three successive injections of tritiated thymidine at 9.00 p.m., 2.00 a.m. and 7.00 a.m., virtually all cells encompassed in a daily DNA-synthetic wave should be labelled. The subsequent mitotic activity of these cells was followed over 8 days using the metaphase arrest technique (Fig. 4).

About 85% of the metaphases were found to be labelled during the first mitotic peak after repeated labelling. In subsequent mitotic peaks during days 2 and 3,

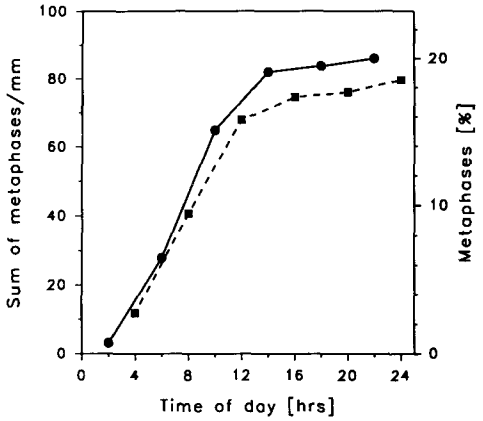

Fig. 3. Cumulative number of metaphases arrested in successive 4 h periods over 24 h. The experiment was carried out in two runs with a time shift of 2 h. Metaphase percentages relate to the total number of nucleated cells per mm epithelial length

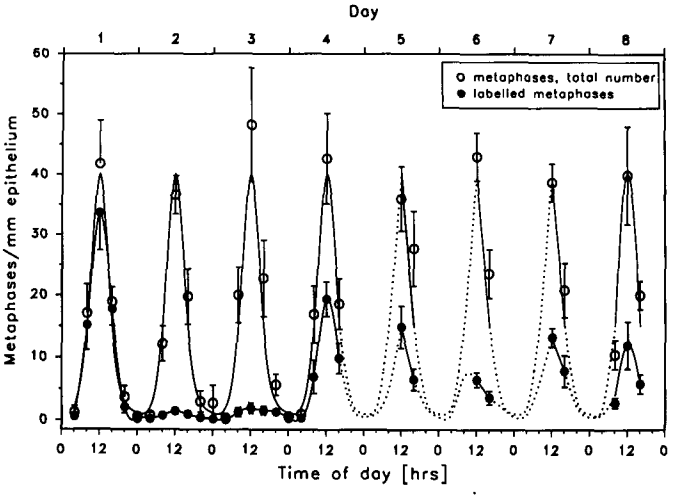

Fig. 4. Number of all (○) and of labelled (●) metaphases ($\pm$SD) over 8 days after three injections of ^{3}H-TdR at 9.00 p.m., 2.00 a.m. and 7.00 a.m. immediately prior to the first mitotic peak. Metaphases were arrested over 4 h by Vinblastine. Dotted lines from day 4 onwards represent likely reconstructions of the mitotic fluctuation

this percentage dropped to 1.4 and 6.2%, respectively, rising again to about 50% on day 4. About 40% of metaphases were labelled at the peak on day 5 and the percentage subsequently decreased to 20, 35 and 30% at the mitotic maxima on days 6, 7 and 8, respectively.

Irradiation studies

The time course of macroscopically evident damage to the tongue epithelium after single dose irradiation was the same as described in previous studies (Moses and Kummermehr 1986; Dörr and Kummermehr 1990). The first signs of inflammation, i.e. erythema and oedema, appeared after a minimum latent period of 5 days. Damage then progressed to increased desquamation, causing a rough and scaly surface, after another 2 days. At doses higher than 10 Gy changes progressed, with dose dependent frequency, to ulcerative lesions at 10–12 days post-irradiation. Where ulcers occurred, they were covered by a pseudomembrane consisting of fibrin and detritus. The individual erosions usually persisted for 2–4 days only, after which rapid regeneration occured, and every individual ulcer had healed completely by day 16 at the latest.

The incidence of ulcers in each dose group and the time course of ulcer prevalence are illustrated in Fig. 5. The frequency of animals developing ulcer, analysed by probit analysis, follows a steep sigmoid dose-response curve with an ED50 of 15.0 Gy and 95% confidence limits of 13.5 and 16.3 Gy. In contrast to this stringent dose relationship, the time course of ulcer prevalence did not depend on the radiation dose in a systematical manner. Even in dose groups where the incidence approached 100%, the prevalence did not necessarily reach a similarly high value, as not all animals responded at the same time, and in some the reaction had already healed when denudation started in others.

To investigate radiation-induced changes in the extent and time pattern of cell production, a high single dose of 20 Gy was applied between 10.00 a.m. and noon on day 0. The number of mitotic figures over the whole experimental period is given in Table 1. After irradiation, no clear circadian rhythm was observed until day 13, and hence the data are presented as mean values per day (Fig. 6). The mitotic index on day 1 post-irradiation

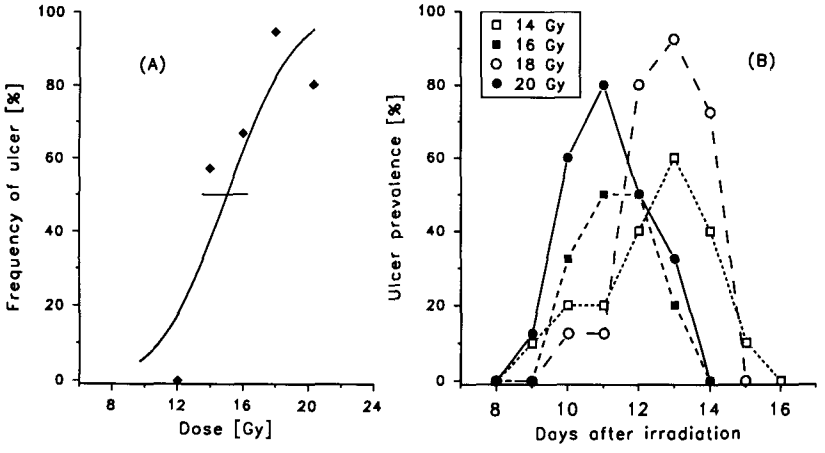

Fig. 5. Ulcer frequency (*panel A*) and time course of ulcer prevalence (*panel B*) in the mouse tongue mucosa after single dose irradiation. The dose response curve in panel A was calculated by probit analysis; error bars indicate the 95% confidence limits. Symbols in panel B represent 14 (□), 16 (■), 18 (○) or 20 (●) Gy

Table 1. Individual mitotic counts per mm epithelial length on days 1 to 15 after single dose irradiation with 20 Gy. Animals showing total denudation are indicated by stars. The mean at these days, being calculated excluding these animals, overestimates the true value. Brackets from day 13–15 reflect the reappearance of the circadian rhythm

Day	08.00		10.00		12.00		14.00		16.00		Mean	SD
1	0.00	0.13	0.10	0.08	0.08	0.09	0.29	0.17	0.04	0.00	0.10	0.09
2	0.30	1.21	1.03	0.72	0.55	0.80	0.25	0.60	0.99	1.11	0.75	0.33
3	0.89	0.51	3.92	2.73	0.56	1.86	0.77	0.35	0.78	0.87	1.32	1.16
4	4.75	6.93	3.09	2.89	1.67	0.50	0.82	0.59	0.60	0.94	2.28	2.16
5	5.24	4.21	1.44	0.94	1.72	0.92	1.68	1.00	2.14	2.11	2.14	1.45
6	0.72	0.08	1.28	0.90	1.34	1.10	0.93	1.14	1.08	0.44	0.90	0.39
7	0.30	2.30	2.37	3.77	1.88	1.67	2.48	1.63	0.89	0.74	1.80	1.01
8	2.30	2.09	0.49	5.50	1.76	0.26	2.10	0.49	0.31	3.26	1.86	1.64
9	0.50	2.07	0.22	2.90	0.69	0.53	*	0.77	0.61	0.19	0.94	0.92
10	2.77	6.22	2.13	*	*	*	3.24	*	4.24	7.21	4.30	2.02
11	4.76	6.09	*	9.25	*	2.72	*	*	7.44	4.83	5.85	2.29
12	4.83	4.59	*	6.84	2.73	5.27	*	*	3.39	3.36	4.43	1.40
13	10.16	*	11.09	10.13	7.44	6.53	4.79	0.83	1.66	1.05	(5.96	4.10)
14	8.02	7.29	11.57	10.66	11.04	5.11	3.29	3.84	1.21	0.87	(6.29	4.02)
15	6.87	5.62	11.16	6.27	6.32	2.88	2.88	4.03	1.91	3.08	(5.10	2.80)

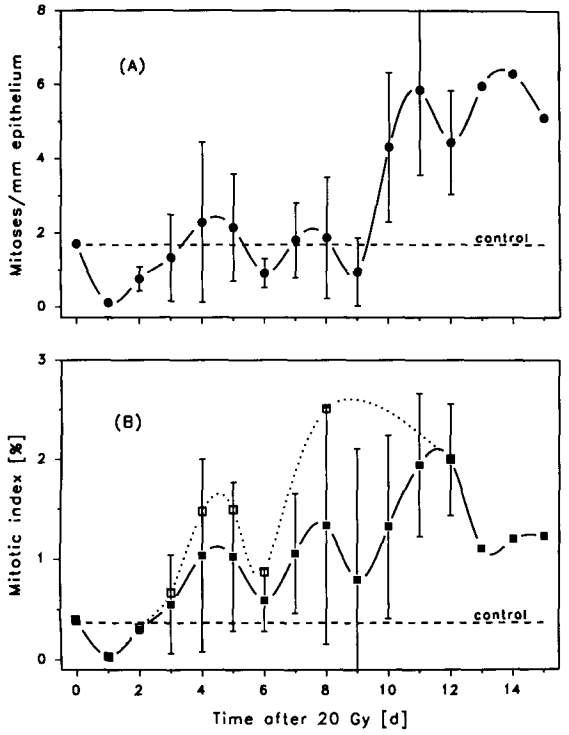

Fig. 6. *Panel A:* Absolute number of mitotic figures per mm mouse tongue epithelium averaged over 24 h after single dose irradiation with 20 Gy. *Panel B:* Time course of mitotic index (MI) after 20 Gy based on mitotic and corresponding total counts of nuclei (●) or cells (□)

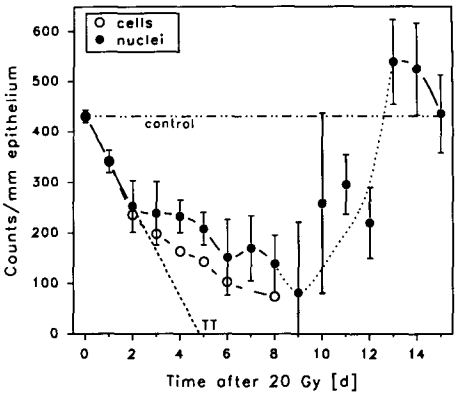

Fig. 7. Number of all nucleated cells (○) and of nuclei (●) in ventral mouse tongue epithelium after single dose irradiation with 20 Gy. The dotted curve segment covers the period where complete denudation is observed in a fraction of the animals. The dashed line is an extrapolation of the initial decline in cellularity and hits the abscissa at about 5 days, coinciding with the natural turnover time TT of the nucleated cells

was $0.03 \pm 0.03\%$, indicating a mitotic delay of at least 1 day. Average values on days 2 and 3 were only marginally different from controls. Subsequently the mitotic index increased steeply, with a peak of 1.5% on days 4–5 and a second peak on days 7–8, and then reached values that were on average three times the control with a peak value of $1.7 \pm 0.8\%$ (436% of control) on day 10.

Clear changes in the morphology of the mitotic figures were seen after day 2. Most mitoses displayed marked abnormalities, such as chromosome clumping, asymmetrical polar distribution of the chromatin or, suggesting disturbed spindle formation, mono- or tripolar figures. The number of abnormal mitoses culminated at day 6–8 and only few were seen after day 9.

The structural damage visible at mitosis was readily translated into the formation of abnormal nuclei. Binucleate cells (0.2% in controls) appeared on day 2, reached a maximum of about 20% on days 7–8 and then rapidly dropped to 5% on day 10. Cells with more than two (usually four) nuclei appeared after a delay of 1 day, thus reaching a maximum of about 20% on days 8–9. Fragmentation of nuclei was seen frequently after days 3–4. All nuclear abnormalities were first observed in the basal layer, but subsequently migrated upwards, reaching the most superficial nucleated layers by day 5, suggesting that the normal transit time was maintained.

The time course of changes in epithelial cellularity, in terms of nuclei and nucleated cells per mm epithelium, is shown in Fig. 7. Counts steeply declined from about 440 to 240 cells (260 nuclei) per mm epithelium during the first 2 days. Subsequently the decrease continued at a reduced rate, but was still prominent until day 6 when 100 cells/mm (150 nuclei/mm) were scored. Minimum values of about 75 cells (140 nuclei) per mm epithelium were seen on day 8. These figures represent average counts from ten animals per day, sacrificed at 2 h intervals between 8.00 a.m. and 4.00 p.m. The dose delivered resulted in complete denudation, i.e. 0 cells/mm, in 95% of the animals, but as denudation in the individual treatment fields was reached with a time-spread exceeding the duration of individual ulcers, complete denudation was never seen simultaneously in all animals. As a result, the number of cells averaged over ten animals remained nearly constant at about 20% of control values between days 8 and 10, followed by a rapid increase to levels above controls on day 13. The original cell counts were restored by day 16.

Discussion

The stratified epithelia of the upper aerodigestive tract of rodents are known to follow a marked circadian rhythm of proliferation. Timing factors are the light-dark cycle and the physical activity, which is usually greatest in the early morning. Mechanical wear and tear, due to food intake appears, appears to be the specific signal triggering cells into mitosis (Burholt et al. 1985; Burholt 1986). Commonly the mitotic peak occurs about 6 h after the maximum activity. The present results demonstrate similarly marked circadian fluctuations in the ventral epithelium of the mouse tongue. DNA synthesis culminates, as shown previously (Moses and Kummermehr 1986), at 2.00 a.m. with a ^{3}H-TdR pulse labelling index of 14%, and falls to a nadir of 2% around 4.00 p.m. The synthetic wave is followed by a wave of mitoses about 8 h later, with very low mitotic counts through the night. With the metaphase arrest method maximum counts (37 metaphases/mm) are observed between 8.00 a.m. and noon and minimum values (2–4 metaphases/mm) between 4.00 p.m. and 4.00 a.m.

The reported time pattern of DNA synthesis is in

good agreement with published data on the mouse tongue (Potten 1983; Scheving 1978), the rat tongue (Gasser et al. 1972), the hamster cheek pouch (Brown and Berry 1968; Izquerdo and Gibbs 1972) and the rat oesophagus (Burholt et al. 1985). The time shift of 8 h seen between maximum DNA synthesis and mitotis is in accordance with results from the hamster cheek pouch (Izquerdo and Gibbs 1972). This time interval is equivalent to the average duration of $S/2+G2+M/2$. The diurnal fluctuations in the mitotic activity described in the present study are similar to those reported for the mouse tongue (Burns et al. 1975; Potten 1983), the rat tongue (Gasser et al. 1972) and the hamster cheek pouch (Izquerdo and Gibbs 1972). A second mitotic peak has been identified in the hamster cheek pouch only (Brown and Berry 1968; Moller et al. 1985), possibly caused by a second phase of masticatory activity when stored food from the pouch is chewed.

The estimation of daily cell production in the present study is not based on measurements of T_S and LI or T_M and MI, as the marked fluctuations in LI and MI, and possibly also in T_S (Hume 1989) and T_M, make such an assessment uncertain. Instead, metaphase arrest figures from successive 4 h blocking periods over 24 h were summed to assess the cumulative number of cells dividing per day. Two runs of this experiment, staggered by a 2-h time shift, yielded an identical value of about 19% of all nucleated cells dividing in 24 h, equivalent to 27% of the germinal compartment. The slope of the two curves in Fig. 3 reflects the rate of cell flux into mitosis as a function of time of day.

From the proportions of 19% nucleated cells or 27% germinal cells dividing per day, turnover times of 5.2 and 3.7 days for the respective cell populations can be calculated. The turnover time of the tissue as a whole must be considerably longer, depending on the number of layers encompassed in the anuclear strata. Similar proportions of dividing cells per day and turnover times have been reported for the oral mucosa of the rat (Hamilton and Blackwood 1974; Cutright and Bauer 1967), whereas in the rat oesophagus (Marquez-Peireira and Leblond 1965) and the buccal mucosa of mice (Berg et al. 1981) turnover seemed to be slightly faster. In the hamster cheek pouch, strain dependence of the turnover time was demonstrated with 8.4 days in the Syrian and 3.8 days in the Chinese hamster (Izquerdo and Gibbs 1974).

The finding of a daily cohort of dividing cells can be explained by several models of proliferative organization. One extreme situation would be a growth fraction of 27% in the germinal layer and a cycle time T_C of 1 day, with an identical growth fraction dividing daily. In contradistinction, the proliferating cohort could be randomly recruited from all cells of the germinal layers each day or several defined cohorts of cells with defined T_C of several days could successively divide, constituting a growth fraction of up to 100%.

To differentiate between these possibilities all cells encompassed in one DNA-synthetic wave were labelled by three repeated injections of ^{3}H-TdR at 9.00 p.m., 2.00 a.m. and 7.00 a.m., and their subsequent passages through mitosis were followed. Assuming S-phase durations between 5 and 9 h, only cells entering S-phase prior to 4.00 p.m. could conceivable have escaped labelling; as illustrated in Fig. 2, this must be a negligible fraction. A possible problem could arise from the radiotoxicity of ^{3}H. With 3×37 kBq/g applied within 10 h most cells will incorporate an amount of activity equivalent to 74 kBq/g, which hypothetically may cause loss of cells within the first few hours after labelling, and possible reutilization of the label during the next S-phase (Franke et al. 1987). This is unlikely, however, as gross changes in proliferation kinetics due to tritiated thymidine can be excluded by the regular shapes of all metaphase waves recorded over the subsequent 4 days. The data shown in Fig. 4 therefore suggest that about 85% of the labelled cells pass through mitosis immediately, and in about equal proportions undergo their next division on day 4 (46%) and day 5 (about 40%).

The difference between total and labelled metaphase numbers in the first wave is about 15% suggesting a subpopulation of cells with a T_{G2} longer than 1 day. The difference is not significant, however, and more extended studies would be required to ascertain the existence of such a subpopulation.

The most likely explanation of the observed periodicity is the existence of different cohorts of cells undergoing division on successive days. We conclude that a wave of cells synthesizing DNA comprises at least two subpopulations with G1-phase durations (or G0+'true G1' according to Burns and Tannock 1970) of either about 2 or 3 days. This indicates non-uniform proliferation characteristics of the epithelium. Similarly long cycle times have been described for the hamster cheek pouch by Brown and Berry (1968) and more recently for the ventral tongue surface by Hume (1989).

Potten et al. (1987) described the occurrence of two peaks of labelling in the mouse and guinea-pig epidermis; these peaks appeared at the skin surface on days 4–5 and 8 (mouse) and 8 and 12–15 (guinea-pig) after intraperitoneal injection of ^{14}C-TdR. Their conclusion was that the first peak reflected rapid diffusion after cell degradation in the granular layer and therefore was not related to the cellular transit time. The second peak was thought to occur when labelled cells reached the superficial layers and their nuclei desintegrated. An alternative explanation is offered by the present findings which suggest that sequential radionuclide release is due to the periodic production and migration of daughter cells formed by one labelled cohort.

Irradiation causes a severe disturbance of epithelial cell proliferation. In the present study, a single dose of 20 Gy was delivered, i.e. a dose sufficient to sterilize the vast majority of cells. As a result of radiation injury, the normal balance of cell production and cell loss is abruptly altered and, through increasing tissue hypoplasia, complete denudation occurs after approximately 11 days. The present data show that this is by no means associated with a total block in cell production. Rather, during the latent period prior to gross damage, the doomed cells exhibit a remarkable proliferative response to the stimulus provided by increasing cell depletion.

Detailed information on this proliferative response is given by directly assessing the mitotic activity. As shown in Fig. 6, absolute mitotic counts, i.e. per mm length, drop close to zero 1 day after irradiation, but subsequently recover to regain roughly normal values at day 3. The mitotic index, however, distinctly exceeds the control rate from day 3 onwards. This difference arises from the decline in cell number per mm length that occurs between treatment and the time of complete denudation.

Both findings have interesting biological implications. The increase in mitotic index suggests that even the doomed cells enter a state of accelerated proliferation, probably responding to developing tissue hypoplasia. In the pig skin, cell depletion to 60% after single dose irradiation with 20 Gy (Morris and Hopewell 1988) or to 75% during fractionated radiotherapy (Morris and Hopewell 1987) appear to represent the critical threshold when such a response sets in. A similar threshold may exist in the mouse tongue, as suggested by the maintenance of cell numbers at this level during fractionated irradiation at various dose levels (Dörr and Kummermehr 1990).

One might expect that the return to normal mitotic counts per length unit (or per unit area) would suffice to maintain tissue cellularity at a constant, though hypoplastic, level. This conclusion is based on the two-fold assumption that the superficial loss of cellular material proceeds at its normal rate and that mitotic counts can be translated into effective cell production in the same quantitative way as in normal epithelium. The rate of attrition is difficult to assess at present. There is, however, direct evidence that, to a large extent, mitoses of doomed cells do not result in two daughter cells. The formation of a greater number of binucleate or multinucleate cells indicates a lack of cytokinesis after mitosis. On average, these cells are larger than normal prickle cells and therefore the epithelium remains thicker for a longer time than inferred by the drop in cellularity. Another factor that would lead to an overestimation of cell production rate on the basis of mitotic counts is a prolonged duration of mitosis itself. In view of the pronounced cytomorphological abnormalities, e.g. multipolar spindles or chromosome clumping, such prolongation is likely to occur. The severity of chromosomal damage even suggests that many attempts to divide may end in cell death, although the frequency of pycnotic cells is low at all times. Quite similar changes to those seen in the tongue epithelium have been reported earlier in studies on the mouse epidermis, including the persistence of DNA-synthesis in lethally irradiated, morphologically abnormal cells and their micronuclei (Devik 1961). In addition, from a differential analysis of labelled pro-, meta- and anaphase curves it was concluded that mitotic duration was subject to great variability and a proportion of cells spend several hours in mitosis (Devik 1962).

The effectiveness of cell division or production of cell mass can be deduced more quantitatively from the decline in cellularity that is recorded from day 0 to complete denudation. The initial rate over the first 2 days (20%/day) is in good agreement with a persistance of normal cell loss in the absence of significant cell production. When the initial decline is extrapolated, a hypothetical latency time to complete denudation of 5 days is derived, in accordance with the turnover time of 5 days that was estimated from the mitotic activity in normal epithelium. Between days 2 and 9 the depopulation slows to about half this rate. Again, assuming a normal number of cells being sloughed off per day, this is consistent with an effective cell production rate per mm over this period amounting to half the normal rate. Considering that virtually all cells present at this time are doomed, their abortive divisions still constitute half the normal cell supply. In fact their contribution prolongs the latent period before complete denudation to 11 days, compared with the 5 days that would be expected if cell production ceased abruptly. This is in clear contrast to the widely-held view that the latent period before gross damage in tissues undergoing constant renewal is equivalent to the post-mitotic life-span of functional cells (Michalowski 1981).

The regrowth of normal epithelium following the phase of denudation must necessarily arise from surviving stem cells. This is histologically illustrated by the return to a completely normal cellular and mitotic morphology. Proliferation of survivors is probably resumed early in the post-irradiation period, but their absolute numbers are too small after 20 Gy to make a visible contribution in the first 8 days. In some other tissues, e.g. the jejunal mucosa (Withers and Elkind 1970) or the epidermis (Withers 1967), proliferation of surviving clonogenic cells results in the formation of discrete colonies which become discernible when the doomed cells have cleared from the tissue. In the mouse tongue, even on close examination, no such colonies could be detected.

During the phase of regrowth, i.e. between days 10 and 13, the population must be assumed to consist mostly, if not entirely, of cells that have shed their radiation damage. Assessment of the epithelial regeneration dynamics is, therefore, possible with a much better reliability than before denudation. Indeed, a quantitative comparison between cell production based on the mitotic activity and tissue cellularity gives quite consistent results. The number of mitoses/mm is elevated to an average of 4.8 as opposed to 1.8 in the controls. Assuming that attrition proceeds at the normal rate, equivalent to 1.8 mitoses/mm or 84 cells/mm per day, the extra cell production is calculated to be $(4.8-1)1.8*84$ or 140 cells/mm per day. This number is entirely compatible with the rise in cellularity that is observed between day 10 and 13, as shown in Fig. 7. The rapid cell production results in transient epithelial hyperplasia between day 13 and 14 which, however, normalizes quickly. The high mitotic figures quoted in Table 1 for days 13 to 15 suggest a very high proliferation rate, but represent diurnal peak values at a time when the normal circadian rhythm has been regained.

Both during the phase of progressing tissue damage and regrowth until day 13, no distinct circadian rhythm of mitotic activity was found. This suggests that the sig-

nal triggering the cells into mitosis after irradiation is released uniformly throughout the day in the postirradiation phase, as opposed to the periodic release of the same, or even a different, stimulus in the controls. In the mouse oesophagus, irradiated with 10 Gy (Burholt 1986), hyperplasia occurs between days 4 and 7, and is characterised by persistent diurnal fluctuations, similar to the figures in the mouse tongue between days 13 and 15. This suggests that after low doses of radiation, proliferation is still dominated by the normal signal release masking the radiation-induced disturbance of cell proliferation. Following higher doses the diurnal rhythm is lost, but is restored during the phase of transient hyperplasia.

More detailed knowledge of the mechanisms and substances regulating both abortive and stem cell divisions will give a better insight into the proliferation dynamics of tissue damage and regeneration following irradiation of the oral mucosa.

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