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SCANNING ELECTRON MICROSCOPY OF THE IRRADIATED MOUSE BLADDER UROTHELIUM

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Abstract

The luminal surface of mouse bladder urothelium was studied by scanning microscopy 1 year after irradiation with 0, 10 and 20 Gy respectively. The controls that were anaesthetized only displayed surface characteristics indistinguishable from normal urothelium.

Irradiation with 10 Gy did not result in marked overall changes in the scanning electron microscopic features of the luminal aspect, but in some areas alterations comparable to the alterations after 20 Gy were observed. After irradiation with 20 Gy focal hyperplastic areas, superficial early ulceration and dedifferentiation of cover cells were seen. The dedifferentiation to featureless cells is probably not associated with increased proliferation, which in focally hyperplastic areas gives rise to a cobblestone or fuzzy appearance with small superficial cells and with many different surface features. The featureless cells may represent degenerative or agonal changes only, but a preneoplastic nature cannot be ruled out.

Key Words: Scanning electron microscopy, urothelium, mouse, irradiation, late effects, focal desquamation, preneoplasia

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Introduction

Delayed side effects may arise in the urinary bladder after radiotherapy for pelvic malignancies. They arise mainly in the muscular and connective tissue. However, alterations consisting of ulcera and crystal inclusions can also occur in the urothelial lining and, in rare instances, even urothelial cancer may occur (3,4,5,18,20,37). The latency of the alterations is probably due to the normally very long turnover time of the tissue (7,13, 28,32).

The rodent urothelium is a highly specialized tissue. At the lumen it consists of large cover cells, with nuclei probably having octoploid DNA content, and deeper lying, smaller tetraploid and diploid cells (10,36). The luminal surface of the cover cells is specialized with asymmetric unit membrane plaques assumed to be the basis of the osmotic resistance (10).

During experimental chemical bladder carcinogenesis, normal surface differentiation disappears. This may partly be due to loss of the cover cells. Stewart (30) irradiated bladders of CBA mice and found that the cover cells disappeared after 1/2 year. At about the same time focal hyperplasia of the urothelium appeared. Antonakopoulos et al. (2) irradiated the bladders of Fischer rats and noted an early failure of differentiation as indicated by the appearance of small superficial cells lacking the asymmetric luminal membrane. 20 months after irradiation 60% of his rats had developed well differentiated transitional cell carcinomas and another 20% multifocal hyperplasia.

Both hyperplastic and neoplastic membrane changes can be observed using scanning electron microscopy (SEM). The purpose of this paper is to report morphological changes of the surface structure of the mouse urothelium a long time after irradiation.

Materials and Methods

Female hairless (hr/hr) mice (Gamle Bomholt Gaard, Århus) aged 10-12 weeks and weighing 20-25 g were used. They were kept in groups of eight to a cage under standard conditions with free access to pelleted food (Felleskjøpet, Oslo) and tap water, and exposed to artificial light from 0630 to 1900 h.

After light intraperitoneal anaesthesia with 1 mg pentobarbital (Mebumal 5% Vet., Rikshospitalets Apotek, Oslo) in 0.125 ml solvent, the mouse bladders were irradiated with electrons from a linear accelerator. Details of the irradiation set-up and dosimetry have been published previously (28). Groups of 4 mice were irradiated with 0 Gy, 10 Gy or 20 Gy respectively and observed for 52 weeks after irradiation. They were then sacrificed together with 4 young unirradiated mice by cervical dislocation. The abdomens were opened immediately and the bladders catheterized with a galactography catheter under visual inspection. The bladders were inflated with 3% glutaraldehyde and 0.1 M sucrose in 0.1 M cacodylate buffer at pH 7.4 . The bladder necks were ligated, the bladders excised and immersed in the same fixative. After fixation for 3 days in the cold the bladders were cut in small pieces and rinsed in the same buffer as used in the fixative. 5 blocks from each bladder were chosen by a systematic random sampling procedure.

These specimens were thoroughly washed in bidistilled water, and impregnated with silver nitrate to reduce charging effects. The impregnation was the same as the silver staining method used by Thiebaut et al. (35).

After impregnation the blocks were dehydrated through graded alcohols, critical point dried by liquid CO_2 using a Balzers Critical Point Drier, mounted on specimen stubs, and covered with a 200 Å gold layer in a Balzers SCD 030.

The specimens were examined in a Philips PSEM 500 operated at 25kV and a tilt angle of 20^0. Photographs were taken using Kodak Tri-X-Pan film.

Results

All mice were healthy at the time of sacrifice except for one of the 20 Gy irradiated mice who died after 6 months of an unknown cause.

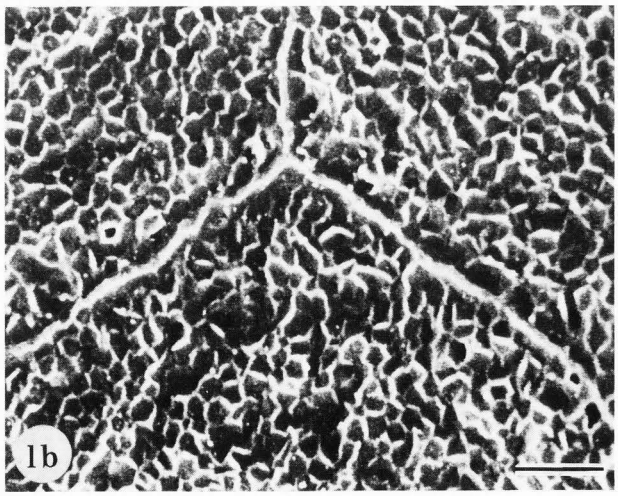
In unirradiated bladders the surface morphology of the urothelium was indistinguishable from the urothelium of healthy young mice. The cover cells were large and polygonal with a wrinkled "accordion-like" surface (Fig. 1a). At high magnification the surface consisted of hexagonal areas or plaques between microridge-like elevations, giving a honeycomb appearance to the surface (Fig. 1b). In the well expanded bladder the borders between neighbouring cells appeared as clearly raised seams, whereas in less expanded bladders the cells bulged into the lumen. The diameter of the small plaques between the microridges, however, appeared unaffected by the degree of stretching.

In some places cover cells were missing either due to physiological exfoliation or to mechanical loss during specimen processing. The underlying cells showed very few surface structures compared to superficial cells. Some cells, however, displayed microvilli-like extensions (Fig. 2 a,b).

The 10 Gy irradiated urothelium appeared to be similar to that of the unirradiated bladders. However, some areas had minor changes, such as those described for 20 Gy of irradiation.

The 20 Gy irradiated urothelium showed more pronounced alterations varying from place to place within and between the bladders. Low magnification showed that the cover cells in some areas had mostly retained their polygonal shape, but that the accordion-like appearance of the surface was missing. At high magnification these cells were seen to be nearly featureless, having only remnants of cell structures that may have been derived from microridges and plaques. This was particularly the case along the cell borders (Fig. 3). In some areas individual cover cells were lacking, revealing normal-looking underlying cells with small stubby microvilli (Fig. 4).

In the well stretched 20 Gy bladders, the borders between the cells were less marked than in the unirradiated ones. Areas with a



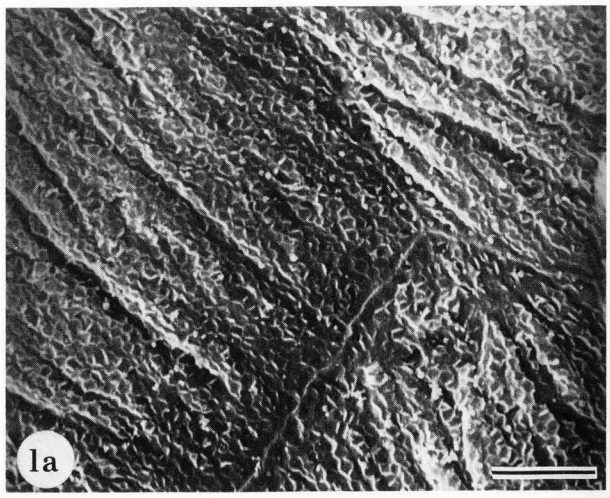
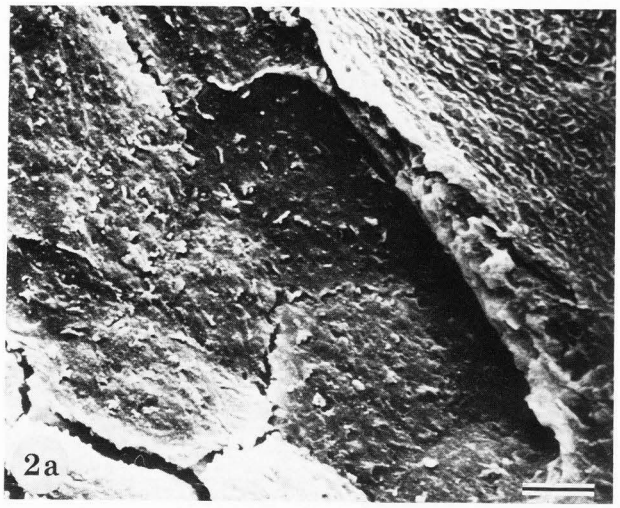


Fig. 1 Surface structure of normal unirradiated bladder epithelium. a) Survey view with wrinkled accordionlike surface. Cell borders are seen as clearly raised seams. Bar= 5µm

b) High magnification with typical microridge pattern giving rise to the honeycomb appearance of the cell surface. Bar= 2µm



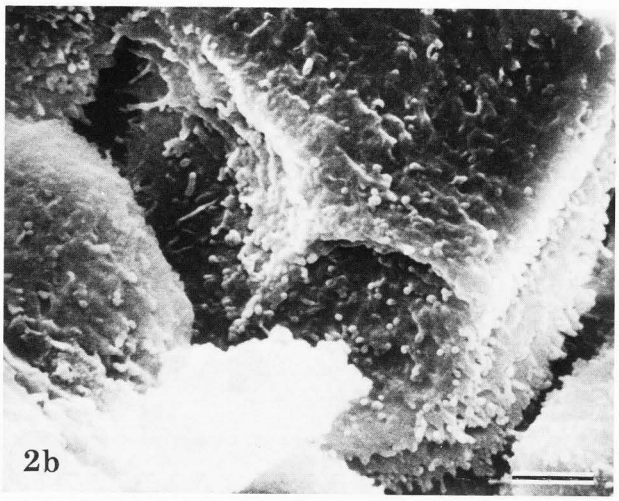
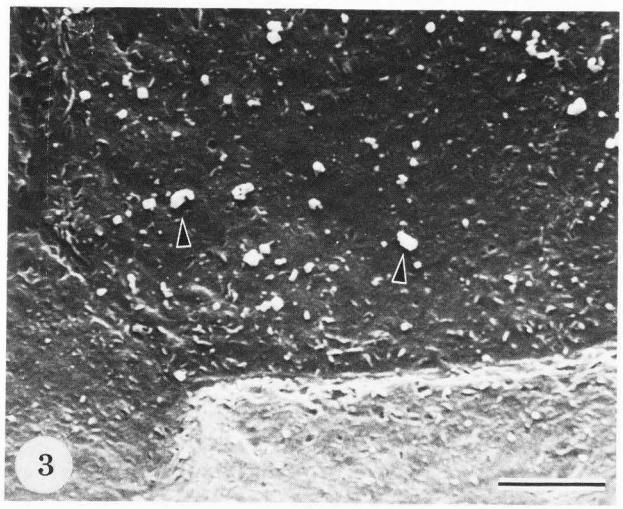
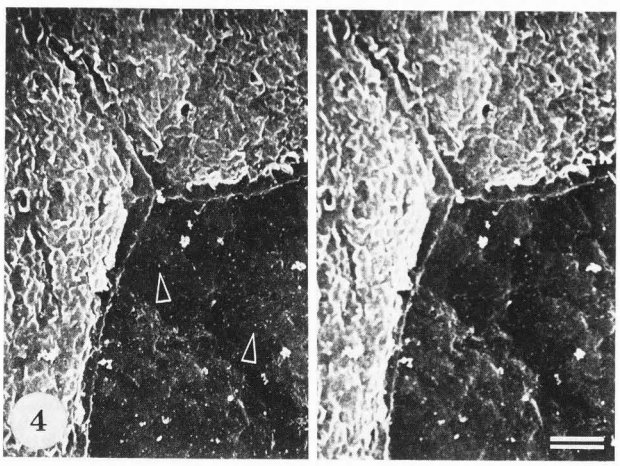


Fig. 2 Superficial and underlying cells of normal unirradiated urothelium. a) Superficial cell with typical honeycomb like pattern and the underlying cells exhibiting very few surface structures, i.e. some microvilli. Bar= 5µm b) Underlying cell from another area of the same specimen exhibiting short stubby microvilli also of the side wall. Bar= 2µm





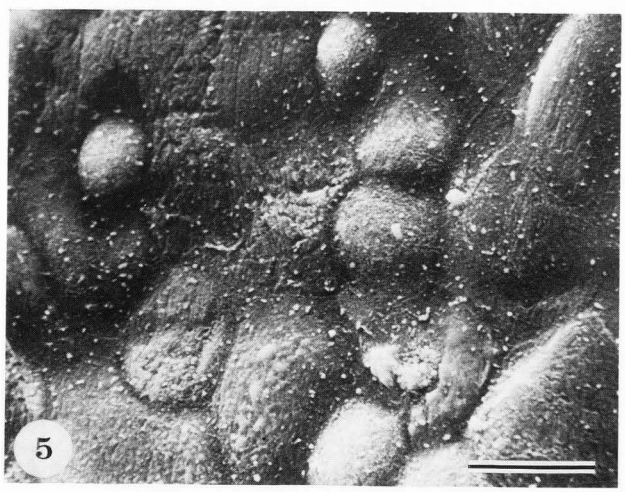


Fig. 3 Surface of epithelial cells in bladder irradiated with 20 Gy and observed after one year. Note only remnants of surface structures along the cell periphery while the central area is featureless. The small dots (arrows) are contamination from the silver staining. Bar= 2µm

Fig. 4 Stereopair of 20 Gy irradiated somewhat raised cover cells with a more normal surface structure, and an underlying cell nearly without surface structure, resembling those seen in fig.2a. Arrows point to the short microvilli found on these cells. Bar= 5µm

Fig. 5 Bulging cells with irregular size and shape giving a cobblestone appearance in a well stretched area of a bladder irradiated with 20 Gy. Bar= 20µm

cobblestone (Fig. 5) or a more fuzzy appearance were found, and in these areas a wide variance of surface SEM structures was seen (Fig. 6 a/b). The cells were irregular and lacked the polygony of the controls, and some of them bulged into the bladder lumen. These cells exhibited smaller surface areas compared to the unirradiated bladders. Many of the cells had numerous blebs and large smooth globular protrusions bulging into the lumen. Some of the cover cells contained many holes (Fig. 7 a/b). Such holes were occasionally seen in the 10 Gy irradiated bladders but never in controls. No really denuded areas were found.

Discussion

The surface appearance of the normal mammalian urothelium has been the subject of numerous investigations (14,16,17,19,23, 24,26,33,38). In most instances polygonal cover cells have been seen, but sometimes more rounded forms of cells hiding the cell contact have been reported. In the normal human bladder, bulging of cells of the dome and stretching of cells of the lateral wall has been attributed to different capability of stretching (23). The same is true for rat urothelium (26). This may indicate that the luminal cells have different capability for translocation of membrane components between positions on the surface and in intracellular pseudovesicles (21).

The mode of fixation affects the stretching of urothelial specimens. In man, transurethral biopsies may be contracted due to suburothelial muscles, and the same is true for fixation of pieces of mouse bladder. Moreover, during regeneration from mechanical ulcers, a localized muscle spasm seems to account partly for the bulging of the regenerative cells (29,39).

In this investigation the bladders were expanded during fixation. Despite this inflation fixation, some variation in the degree of the stretching occurred. This is due to individual differences in the bladders capacity and compliance, which probably have been affected at least in the heavily irradiated bladders (32).

The unirradiated bladders were unaffected compared to normal bladders of young mice, thus excluding the occurrence of any serious age changes. This is consistent with absence of cell kinetic changes due to age (7,27,31) and absence of age changes seen in tissue sections (2,27). Nor could any marked changes be detected after a dose of 10 Gy, which also corresponds fairly well with a suggested threshold dose for cell kinetic changes of 12-15 Gy (27,30).

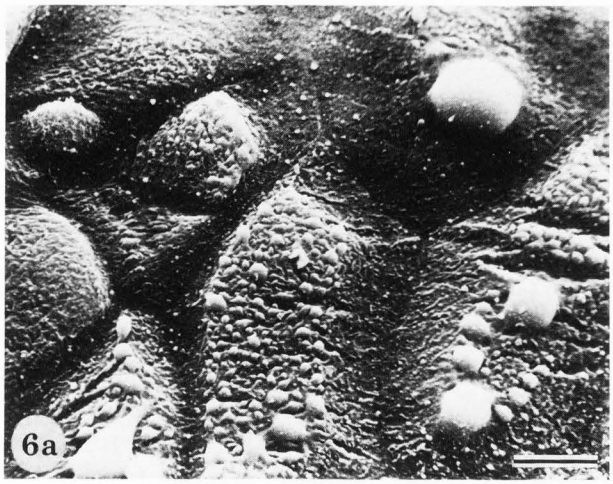
Bladders irradiated with a dose of 20 Gy showed extensive surface alterations. No really denuded areas or ulcera were registered, but focal desquamation exposing intermediate urothelial cells was seen. A measure of the tolerance level is the nominal standard dose (NSD) proposed by Ellis (6). NSD is not strictly applicable if the number of fractions given is less than 5, but indicates that 20 Gy single dose should be roughly equivalent to the generally accepted tolerance level of 1800 rets (2,22). Thus, it seems reasonable to regard the observed desquamation as an early sign of an irradiation ulcer.

Antonakopoulos et al. (2) found that an early sign of disturbance in rat urothelium after irradiation was the appearance of small superficial cells lacking the characteristic asymmetric unit membrane and fusiform vesicles of the normal cover cells, and expressing some microvilli. The same type of cells has been seen after freezing or mechanical ulceration of the urothelium (8,29,39). Pleomorphic microvilli have been shown to correlate with experimental chemical carcinogenesis and with malignancy of the human urothelium (9,15,23). In irradiated rat bladders, Antonakopoulos et al. (2) also found that cover cells developed with a symmetric luminal membrane covered with microvilli, this was associated with hyperplasia and transitional cell carcinomas. When the hyperplasia was prolonged, marked and polypoid, pleomorphic microvilli might even be seen, indicating that pleomorphic microvilli is not a specific marker of neoplasia or irreversibility (1,8). In the regenerating epithelium in our study, stubby microvilli were seen, but never pleomorphic microvilli. The normal differentiation to cover cells did not take place due either to too rapid turnover of the urothelium, or to a loss of the ability to differentiate.

In some areas we found flat hexagonal or polygonal cells resembling the normal cover cells by low magnification. However, at high magnification, lack of the normal accordion-like appearance and the hexagonal plaques was evident. Instead, only remnants of microridges were seen along the cell borders and a small number of stubby microvilli comparable to those of the normal intermediate cells could be seen, but the main characteristic was the featureless appearance of the cells.

Such cells have been observed 204 days after a dose of 30 Gy to the rabbit urothelium (12) and in irradiated tumour-bearing human urothelium (25). The significance of these cells is quite obscure but has been attributed to chemical damage by the urine (24). The reports on such featureless cells are mostly based on irradiated material. These indicate that the changes are in all likelihood a result of irradiation, but do not exclude the possibility that similar changes may be observed after treatment with other agents. Indeed, absence of surface foldings has also been reported in chemical carcinogenesis (14). The features of these cells may also represent agonal changes.

The cobblestone or fuzzy appearance of the cells in some areas strongly suggests focal hyperplasia. Focal hyperplasia of the urothelium has been observed after irradiation of the mouse (30) and rat (2). In the hyperplastic areas of the bladders in these studies normal cover cells were missing, exposing deeper cells with variable size. The reason for this focality of the



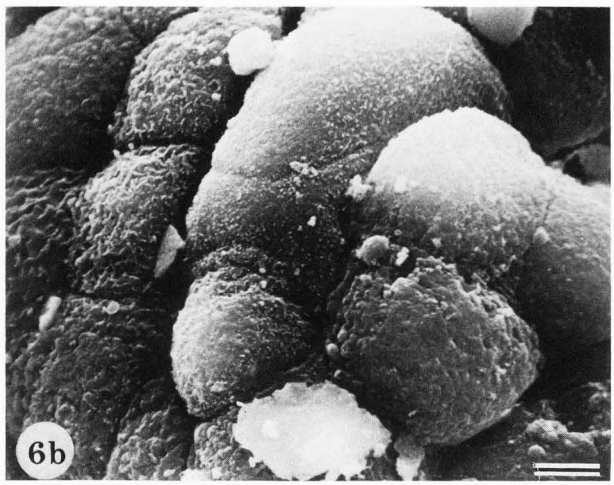
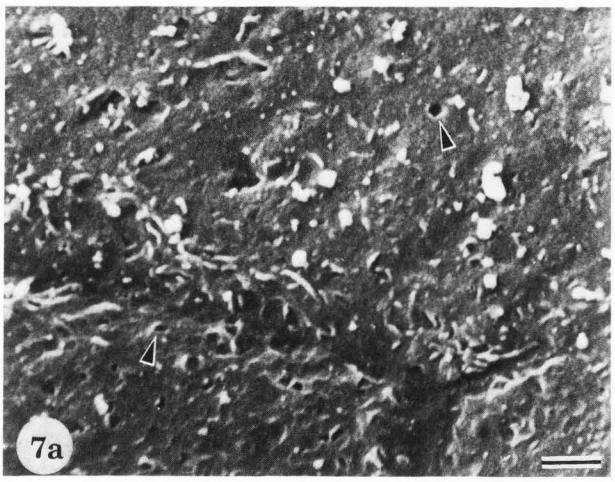


Fig. 6 Variance of surface structure in bladder irradiated with 20 Gy. a) Bulging cells showing blebs and globular protrusions. Bar= 10μm b) Bulging, rounded cells varying in surface structure, featureless cells covered with short microvilli. Microvilli on cover cells were seen only in 20 Gy irradiated bladders. Bar= 5μm



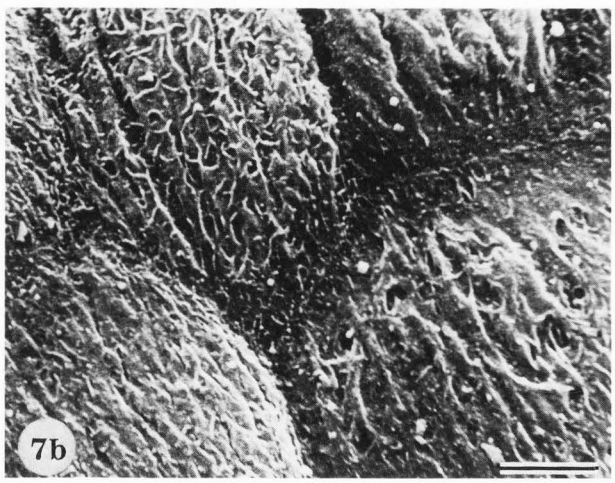


Fig. 7 20 Gy irradiated cover cells with holes. Such holes (arrows) were predominantly seen in irradiated bladders, and were seen both on cells lacking surface structures (a), bar= 2μm, and on bulging cells with many surface structures (b), bar= 5μm. The holes were checked by stereo images.

hyperplasia is unknown. In focal destruction of the epithelium, the reactive proliferation is concentrated around the lesion (8, 29,39), but even in generalized destruction, a focal reactive pattern is seen (9). In the case of irradiation, the urothelial dose is homogeneous and unaffected by regional circulatory differences. However, circulatory differences may have some impact upon nutrition and oxygenation or spatial distribution of the small amount of proliferating cells in the normal urothelium. In the focally hyperplastic rat urothelium the number of subepithelial capillaries was greatly increased (2,34). At the time of proliferation, partly irradiated rabbit bladders showed extensive hypervascularization in the irradiated area (12). In extreme depression of the circulation, however, an increase in the destructive radiation effects were seen instead of the expected protection due to hypoxia (11). Most probably regional circulatory differences control the proliferation leading to focal hyperplasia, and there is no need to assume that there are regional differences in the urothelial architecture.

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Discussion with Reviewers

GM Hodges: Why have only five small blocks been randomly sampled for SEM instead of a total survey of the bladder urothelium?
Authors: The size of the blocks was about 1 mm x 1 mm. Five blocks thus represent approximately 10% of the total bladder area. Since this study is part of a larger study including morphometry and cell kinetic studies no more material was available for SEM. In another study a total survey of normal bladder urothelium was carried out and no marked regional differences were observed.

GM Hodges: It is well established that normal bladder urothelium undergoes a characteristic progression of morphological changes as the cells mature and differentiate with expression of a unique asymmetric unit membrane (AUM) on the lumenal aspect of the terminally-differentiated superficial cells. Also, during bladder carcinogenesis both in animal models and in humans, a continuum of morphological changes from normal to varying grades of atypia has been described including the loss of AUM and, presence of pleomorphic microvilli and of a filamentous glycocalyx in place of the well-ordered normal surface configurations. In recent years a series of reviews pertinent to the aims of this study have comprehensively discussed these various features (see "Additional references"). Please comment.
Authors: We report changes observed 12 months after irradiation, and have not attempted to follow the development of these changes during this period. Further, we do not feel sure that the changes seen after irradiation are identical to those seen in chemical carcinogenesis. However, Antonakopoulos et al. (2) report loss of AUM and appearance of short microvilli as early morphological changes in irradiated rat urothelium as we have discussed in the text.

GM Hodges: There is no convincing data to substantiate the point that the observed lumenal surface desquamation in 20 Gy irradiated bladders is an early sign of irradiation ulcer - it is not clear whether desquamation is restricted to focal areas, as to the extent of such areas, nor how these desquamated areas differ from those noted in non-irradiated control bladders. Please comment.
Authors: The desquamated areas of the 20 Gy irradiated bladders were focal and involved relatively large areas. In control bladders only single cells or very few cells were desquamated or missing.

F Stewart: Abstract: On what basis do the authors conclude that cellular dedifferentiation after irradiation was not associated with increased proliferation?
Authors: Cell kinetic parameters from the same mouse strain do not indicate increased proliferation (27). These data are to be published in more detail later.

F Stewart: Is it true that the side effects of irradiation in the bladder occur mainly in the connective tissue? I thought that many complications were associated with epithelial desquamation and mucosal ulcers.
Authors: In clinical radiotherapy the main serious side effect is contracted bladder and fistulas involving wall fibrosis (18,22).

F Stewart: Do the authors believe that the featureless surface cells found after irradiation are caused by chemical damage by the urine? Perhaps it is more likely that exposure of the non specialized, intermediate cells to the urine (after loss of surface cells) leads to a persistent chemical irradiation with rapid cell production and cell loss, which prevents differentiation?
Authors: The suggestion is of course relevant, but other denuding situations such as cystitis should then also give rise to featureless cells. In addition no increase in proliferation was seen in the material.

F Stewart: I am unconvinced by the argument that regional circulatory differences in the bladder

will control proliferation and lead to focal hyperplasia. Irradiation will cause cellular death (in proportion to the dose delivered) on a random chance basis. Isolated survivors will remain and these cells will be stimulated into proliferation to regenerate the tissue, leading to hyperplasia in areas where there were surviving stem cells.
Authors: We fully agree with Dr. Stewart. However, since the number of foci is very low compared to the expected number of survivors, there must be some factor controlling which of the survivors shall give rise to focal hyperplasia.

JB Jacobs: Are the demonstrated blebs, holes etc., primary effects of irradiation or are they artefacts secondary to other membrane effects becoming visible with improper CPD?
Authors: The material from both irradiated and control bladders were fixed and processed together, and the disturbances were not seen in control bladders. Further, the alterations were focal in nature. We thus believe these features to be associated with the irradiation, but cannot conclude upon the primary or secondary nature.

JB Jacobs: Did the authors repeat this experiment, and if so, did the same changes appear throughout the 20 Gy group?
Authors: Repetition of the experiment involves a one year observation period and has not been fulfilled yet.

JB Jacobs: Light microscopy of the embedded CPD specimen would also demonstrate that the cobblestoned areas are indeed hyperplastic. Do the authors have any intention to carry out such work?
Authors: We fully agree with Dr. Jacobs that embedding and sectoning of CPD specimens will identify hyperplastic areas as well as possible critical point drying artefacts. It is, however, very time consuming first to identify areas in SEM,and secondly to embed and section the identified areas. In the present situation we do not feel that the extra information which could be obtained justifies the investment in work.

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