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Morphological changes and proliferative activity of alveolar epithelium in mouse lungs treated with urethan

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Abstract We have observed sequential cellular changes in type II pneumocytes in mouse lungs following oral administration of 0.1% urethan solution. Histologically, scatterings of swollen alveolar cells were first noticed 25 days after administration. These cells continued to swell further, after which they aggregated more compactly to form a papillary structure by day 75. After day 100, distinct tumour nodules were found with atypical large cells in some areas. Necrotic foci appeared in large tumours of more than 4 mm diameter. At day 250, nucleoli became distinct and large bizarre and multinucleated cells were intermingled with some mitotic figures. These morphologies correspond to conventional descriptions; that is, hyperplasia (day 25–75), benign neoplastic changes (day 75–100) and malignant neoplastic changes (day 100–250). The proliferating cell nuclear antigen (PCNA) and nucleolar organizer regions (AgNOR) scores in these lesions increased with time, and proliferative activities during tumour progression seemed continuous. However, proliferative activity of the cells on specific days did not differ statistically from the values on neighboring days. We speculate that the hyperplasia-like lesions seen in our mice are neoplastic in nature from their outset.

Key words Urethan · Mouse · Lung tumour
Proliferating cell nuclear antigen · Nucleolar organizer region

Introduction

The morphology of a tumour generally reflects its biological behaviour. Usually the more atypical tumour cells appear, the more aggressively they behave. Of course, there are many exceptions to this rule and small tumours may show little atypia but as tumour size increases, more atypical and bizarre cell appearances predominate. In this context, therefore, some have proposed that what is occurring is a progression from benign to malignant neoplasms. Collection to much human data has shown that hyperplasia, adenoma and adenocarcinoma coexist in some lesions and that cells in each category are difficult to differentiate from each other morphologically, implying that they may constitute a wide spectrum of the same disease process.

To determine whether such a sequence indeed exists we produced lung tumours in mice, using urethan. Urethan is known to be alkylated in vivo, to injure chromosomes, to induce mutation and to promote carcinogenesis [11]. First, we examined the lung changes produced by continuous administration of urethan between 25 and 250 days morphologically. Proliferating cells in these lesions were counted with anti-proliferating cell nuclear antigen (PCNA) antibodies and by the method of Ploton et al. [9] for argyrophilic nuclear organizer regions (AgNOR). Then, groups of mice given urethan for up to 100 days were left alive for another 100 days without further administration to see whether the lesions would grow autonomously. Our results suggest that the proliferation of alveolar cells in this experimental condition may have been neoplastic from the outset, and that morphological atypia increased with the progression of tumorigenesis.

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Materials and methods

Production of urethan-induced lung tumours

Urethan (ethyl carbamate), purchased from Kanto Chemical Inc., Tokyo, was dissolved in distilled water at a concentration of 0.1%. A total of 120 4-week-old female BALB/c mice (Clea Japan, Inc.) were utilized for this study. They were fed ad libitum and drank 0.1% urethan solution until the day of sacrifice. At 25, 50, 75, 100, 150, 200 and 250 days, 20 mice each were anaesthetized with diethyl ether (Nacalai Tesque, Inc.). Lungs were removed and canulated with a fine teflon tube, through which 10% buffered formalin was instilled. Then, they were put in 10% buffered formalin solution en bloc for 24 h. After fixation, the lungs were coronally sliced every 3–4 mm, and the number as well as the size of tumours found were measured under a dissecting microscope. For the light microscopic examination, sliced lungs were processed in

the routine manner and embedded in paraffin. Sections 4 μm thick were stained with haematoxylin and eosin.

As a control, lungs from 20 female BALB/c mice of the same age which were given distilled water instead of urethan solution for 250 days were processed and examined similarly.

Electron microscopy

Mice that received 0.1% urethan solution for 25, 100 or 200 days were examined. Lung tumours were removed from these mice and cut into small pieces of about 1 mm^3. These were fixed in 2% glutaraldehyde in 0.05 *M* cacodylate buffer solution (pH 7.2) for 2 h at 4° C, followed by post-fixation with 1% osmium tetroxide in cacodylate buffer. Blocks of tissue were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections were cut, double-stained with 1% uranyl acetate and 1% lead citrate, and examined under a Hitachi 500 electron microscope.

Proliferative activity of alveolar epithelia in the mice treated with urethan

Two serially cut 4 μm thick sections were obtained from paraffin blocks of the lungs treated with urethan for 25, 50, 75, 100, 150, 200 and 250 days. Sections were deparaffinized in xylene and hydrated in graded ethanol. Those for PCNA were immersed in methanol with 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Immunostaining was performed with the avidin-biotin-peroxidase complex technique (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif., USA) using the primary monoclonal antibody against PCNA; PC 10 (mouse IgG, 1:100 dilution, DAKO) overnight at 4° C. Diamino-benzidine-hydrogen peroxidase was used as a chromogen, and sections were counterstained by haematoxylin.

Other pair sections were deparaffinized, hydrated and stained for AgNOR according to the method of Ploton et al. [9]. Briefly, a 50% aqueous silver nitrate solution was mixed at a ratio of 2:1 with a 2% gelatin solution in 1% aqueous formic acid, and this was used as the AgNOR staining mixture. The tissue sections were covered completely with this mixture and left for 35 min at room temperature in the dark.

Counts of PCNA immunoreactivity were expressed as the percentage of positive tumour cells. For this purpose, 500 tumour cells were counted in the largest nodule using a magnification of 400. All nuclei showing a diffuse, granular and strong brown staining were considered as positive.

AgNOR were counted under an oil immersion lens at a magnification of 1000. AgNOR dots were counted in 100 selected nuclei in the largest tumour nodule. AgNOR counts were calculated as the mean number of AgNOR dots per nucleus. When the nucleolus was positive, it was also counted as one.

Growth of alveolar epithelial cells after the cessation of urethan administration

Twenty BALB/c mice each received 0.1% urethan solution for 25, 50, 75, and 100 days respectively, and then they were given distilled water. Mice were sacrificed 100 days after the cessation of urethan. Lungs were removed and examined macroscopically and microscopically as described earlier.

Results

Production of urethan-induced lung tumours

In urethan-treated mice, the formation of lung tumours was observed macroscopically from day 75, and they increased in number and size with time, as shown in Fig. 1. No tumours were found at any time in control mice.

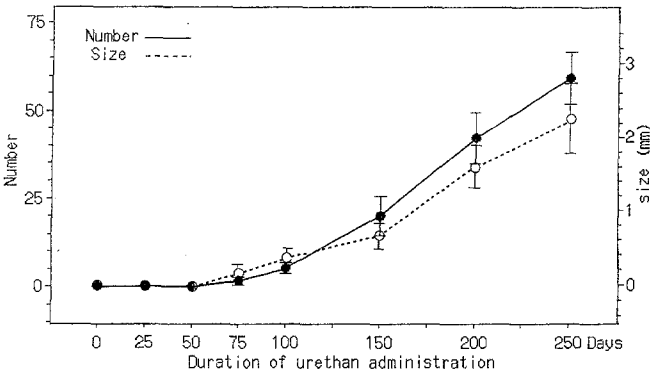

Fig. 1 Number and size of urethan-induced lung lesions

Histologically, swollen alveolar cells were already observed on day 25. They were mostly single and scattered (Fig. 2a). No such swollen alveolar epithelia were seen in any control mice. On day 50, alveolar cells were further swollen and had assumed a cuboidal shape with a round to ovoid nucleus. They tended to gather, especially near alveolar ducts (Fig. 2b). On day 75, they had aggregated more compactly to form papillary structures, but not distinct nodules (Fig. 2c). Occasional cells had hyperchromatic nuclei. The formation of distinct tumour nodules with a few atypical large cells in some areas was observed on day 100 (Fig. 2d). These cells exhibited abundant eosinophilic cytoplasm and round to oval nuclei. Nuclear cleavage and nucleoli were discernible on occasion. Mitotic figures, multinucleated cells, and necrotic foci were not present. On day 200, necrotic foci were noted in large tumours of more than 4 mm diameter (Fig. 2e), but the appearance of the epithelial cells was similar to those of day 100. On day 250, nucleoli became distinct, and large bizarre and multinucleated cells were intermingled (Fig. 2f). Mitotic figures were also scattered in places. Some proliferating cells had extended into surrounding bronchiolar lumina. At no date, were there metastatic foci or primary pathological changes in any other organs.

Electron microscopic findings

Electron microscopically, proliferating cells of 25, 100 and 200 days exhibited numerous lamellar inclusion bodies, characteristic of type II pneumocytes (Fig. 3). These bodies were composed of concentrically lamellar and/or parallel lamellar structures, enveloped by a unit membrane, but the former generally overwhelmed the latter. Although occasional lysosomal bodies were present, the small round dense bodies with some parallel lamellar structures seen in Clara cells were not observed.

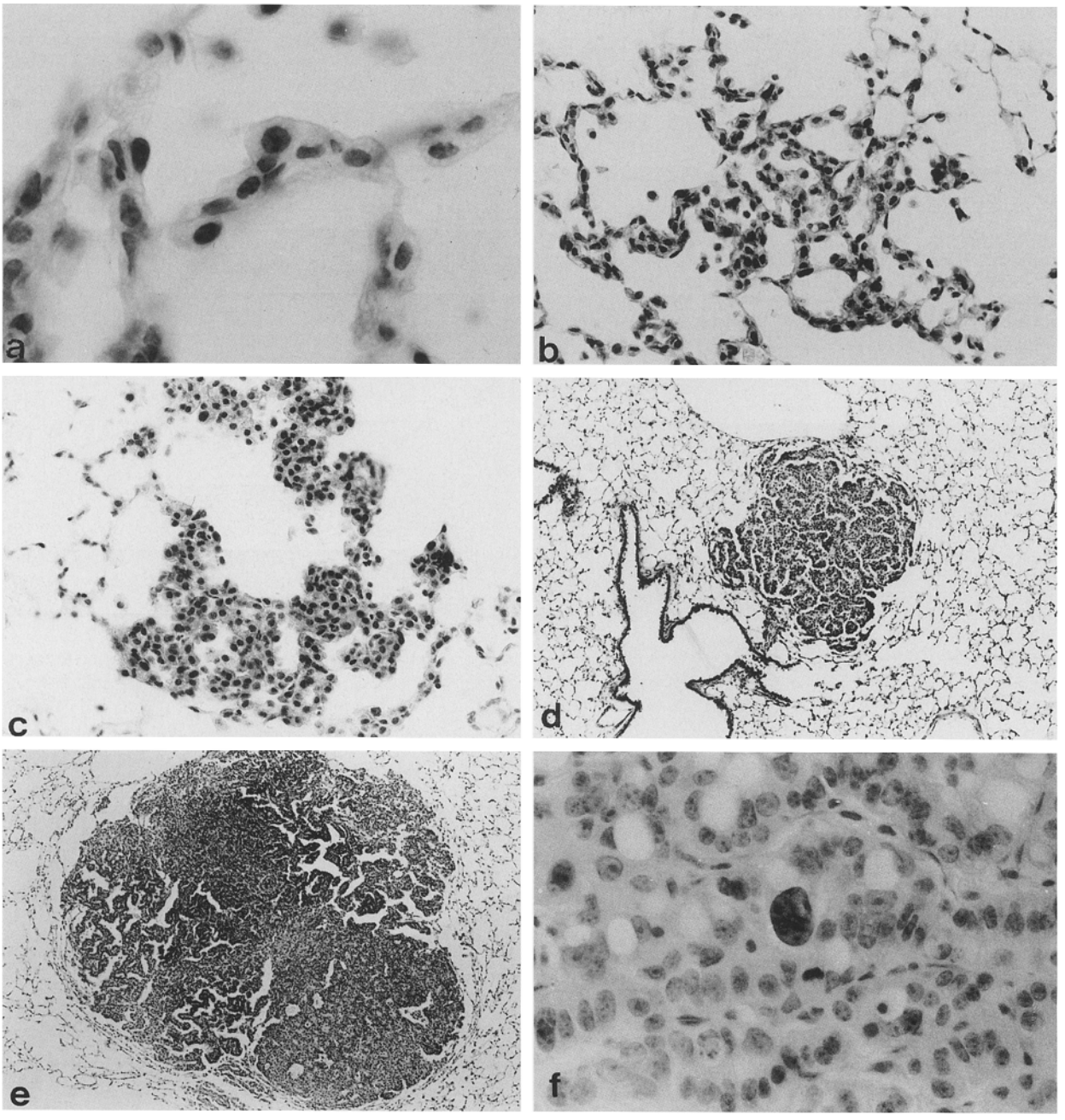

Fig. 2 Sequential change in urethan-induced lung lesions. (**a**) Day 25. Scatterings of swollen alveolar cells can be seen. ×400 (**b**) Day 50. Swollen alveolar cells line the alveolar walls. Note that the cells are plump and cuboidal. ×200 (**c**) Day 75. The cells locate near alveolar ducts and assume a papillary structure. ×200 (**d**) Day 100. The cells have proliferated and now form a distinct nodule. A few large atypical cells are seen. ×100 (**e**) Day 200. A large tumour nodule has formed. Proliferating cells grow more compactly and show more severe atypia. ×40. (**f**) Day 250. Cells have large and bizarre features. ×400

Proliferative activity in alveolar epithelia

The PCNA rates and mean AgNOR scores are shown in Fig. 4, and an example of PCNA positive cells is illustrated in Fig. 5. A few PCNA-positive cells were scattered among swollen pneumocytes from days 25 to 50, the average ranging from 3 to 5%. By day 75, the positivity of papillary lesions had risen to 10%. In contrast, by day 100, when discrete tumour nodules were observed, the positive cells had increased with average positivity of 20%. Proliferating cells with large nuclei tended to be seen more often and were strongly positive. Later, PCNA positivity continued to increase till day 250, when the positive rate reached 45%. AgNOR staining is shown in Fig. 6. AgNOR scores seemed to correlate well with the results of PCNA positivity. AgNOR scores more linearly and steeply increased from day 25 to day 100 and more gently from day 100 to day 250. Positive granules of the AgNOR varied in size, but generally became larger with time, and on day 200, and 250 positive gran-

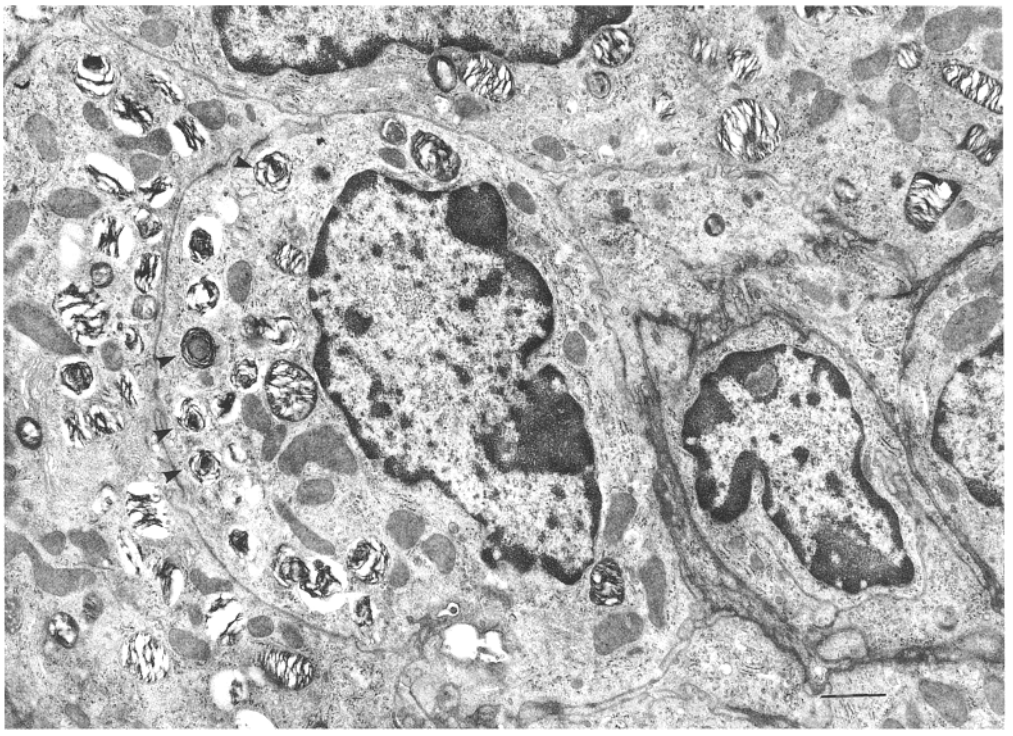

Fig. 3 Electron micrograph of proliferating cells in the urethan-induced lung lesion on day 200. Tumour cells contain numerous lamellar inclusions (*arrowheads*). ×10200. Bar=1 μm

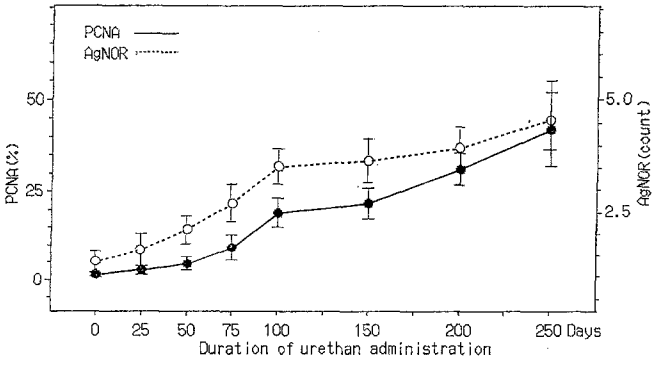

Fig. 4 PCNA and AgNOR or urethan-induced lung lesions

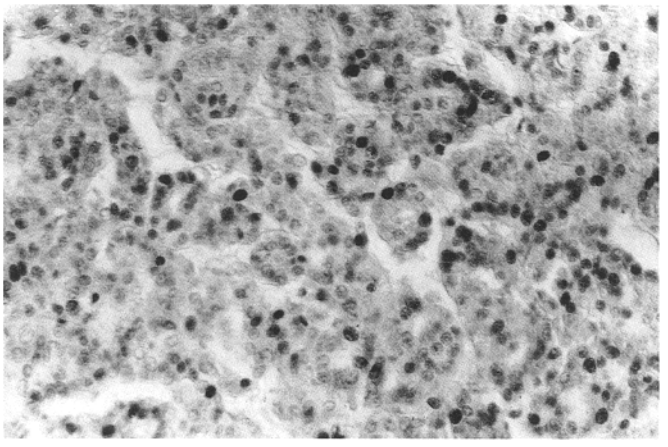

Fig. 6 Nucleolar organizer regions (AgNOR) (Day 200). There are three to seven black granules in the nuclei. ×400

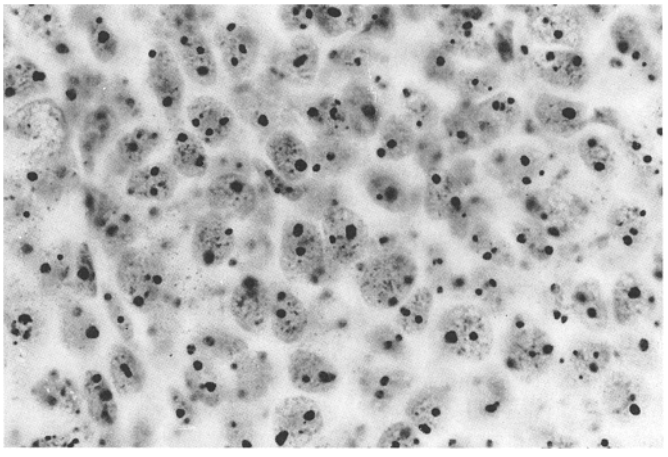

Fig. 5 Immunostaining for proliferating cell nuclear antigen (PCNA) ×200. The PCNA positive cells shown here are those of day 200. Tumour cells with large nuclei tend to be more strongly positive

ules were the largest and most conspicuous. There were no statistically significant differences between PCNA scores and AgNOR values on neighboring days ($P \geq 0.05$).

Morphological changes in alveolar epithelial cells after the cessation of urethan administration

The mean numbers and diameters of the lung nodules in mice treated with 0.1% urethan solution for 100 days and those treated with 0.1% urethan for 100 days and then with distilled water for another 100 days are presented in Fig. 7. While the mean number and diameter of the former group were 5.6 and 0.4 mm, those of the latter group

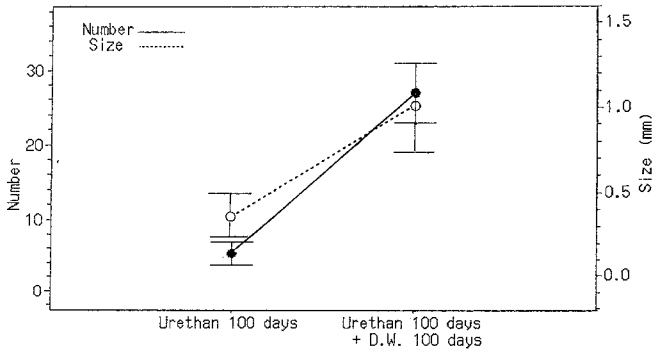

Fig. 7 Growth of alveolar epithelial lesions after cessation of urethan

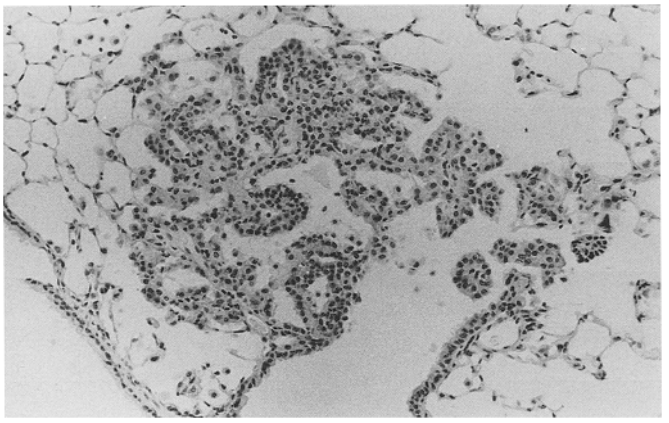

Fig. 8 Lung lesions at 75 days of water intake after 25 days of urethan administration. Swollen alveolar epithelia have aggregated to assume a papillary and nodular structure. ×100

were 27.4, and 1.0 mm respectively. The BALB/c mice, treated with 0.1% urethan solution for the first 25, 50 or 75 and then given distilled water for another 100 days, formed a few gross nodules and microscopic proliferative foci in the lungs respectively (Fig. 8).

Discussion

Long-term administration of urethan successfully produced good experimental conditions for the demonstration of sequential cellular changes in the mouse lung. First, scatterings of swollen alveolar epithelia appeared. These cells then aggregated and swelled more gradually forming papillary structures and finally nodules. In the nodular stage, the cells exhibited an increase in their morphological atypicality with time. Constituent cells in these lesions also clearly showed derivation from type II pneumocytes, as has been reported by other authors [10].

According to the conventional morphological criteria, the respective stages may be referred to as hyperplastic (day 25–75), benign neoplastic (day 75–100) and malignant neoplastic (day 100–250). One may add atypical hyperplasia (day 50–75), which may occur between hyperplasia and malignant neoplasia, as Weng et al. [12] have proposed in human cases. Some may regard a benign neoplastic conditions as a nodular hyperplasia, and their inter-relationship is not clear or are they related to each other? Our experiment was undertaken to attempt to answer this question.

Hyperplasia is a condition in which an increase in number of cells occurring in response to a stimulus returns to normal when the stimulus is eliminated. Neoplasia is a condition in which proliferation of cells continues autonomously when the initial stimulus is eliminated. Under our experimental conditions, it was clearly demonstrated that the lesions which resembled hyperplasia morphologically were in fact neoplastic because they increased in size and formed nodules after cessation of urethan administration. The implication of this result is two-fold; conditions hitherto believed to be hyperplastic might be neoplastic, without showing cellular atypia and such changes could be an early manifestation of malignancy. However, we were unable to determine whether all the hyperplastic lesions recognized were actually neoplastic. There must be examples of hyperplasia as we defined it earlier. In any case, we can say that some hyperplasia-like lesions are already neoplastic. Further study will be required to determine if the changes are actually an early manifestation of malignancy.

PCNA is a 36 kDa acidic non-histone nuclear protein which functions in virtually all phases but is strongly expressed in the late G1 through S-phases of the cell cycle [1, 3]. It is frequently used as a marker of cell proliferation and is known to correlate well with grades of malignancy [2, 7]. AgNORs are expressed as black granules identified by a one-step silver staining technique [9]. They represent loops of DNA coding for 45-S ribosomal RNA and are believed to be responsible for transcriptional activity [4, 5]. Their increase has been correlated with high proliferative activity or aneuploidy of the neoplasm [6]. For these reasons, we utilized these markers and compared their morphology with proliferative activity to see whether morphologically benign neoplasms could be separated from malignant ones on the basis of proliferative activity alone.

Both proliferative markers were well correlated and increased in a similar manner. They also correlated well with tumour size. However, statistically, the proliferative activity of the cells on specific days did not differ from that on neighboring days. Proliferative activities during tumour progression may be continuous, and do not provide a means for separating a benign condition from a malignant one. We speculate that the hyperplasia-like lesions seen in our mice are already malignant neoplasms. Recently, we have examined human cases using the same technologies, and obtained similar results for proliferative activity in the progression from hyperplasia, atypical hyperplasia to adenocarcinomas [8]. Therefore, the situation seems quite similar between animal and human cases.

In summary, we have successfully produced sequential cellular changes in type II pneumocytes in tumorigenesis, and demonstrated that the neoplasm showed a wide spectrum of morphological changes from hyperplasia to apparent malignancy.

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ANNOUNCEMENT

Sixth Dermatopathology Self-assessment Workshop September 21–23, 1995, Rome, Italy

The workshop will provide an in-depth review of difficult topics in dermatopathology: panniculitis (Daniel Su), malignant melanoma (C.J. Cockerell) and mycosis fungoides and T-cell lymphoma (P.E. LeBoit). The workshop will consist of a series of lectures and examination of slides. Microscopes will be provided for all participants. The course fee is US $200 for participation in all the course activities or US $100 for those wishing to attend the lectures only.

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