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Original Paper

p53 Expression in Skin Carcinogenesis and its Relationship to Cell Proliferation and Tumour Growth

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The immunoreactivity of p53 protein was studied in relation to tumour development, histopathological characteristics, cell proliferation, and basement membrane organisation following the induction of skin carcinogenesis in tumour-sensitive and -resistant mouse strains by ultraviolet (UV) irradiation or 7,12-dimethylbenz(a)anthracene (DMBA). In non-neoplastic skin exposed to UV irradiation or DMBA, p53 immunoreactivity was observed in nearly 50% of the basal layer cells. These cells were morphologically and histochemically indistinguishable from the p53-negative cells, occurring similarly in the tumour-producing and the tumour-negative mouse strains and regardless of subsequent tumour formation. In induced epidermal hyperplasia and in benign tumours, p53-positive and proliferating cells constituted 40–50% of all cells in the basal layer, while superficial cells were p53 negative. In dysplastic epidermis, p53-positive cells and proliferating cells were seen in all cell layers. In the case of squamous cell carcinomas, p53-positive proliferating cells in differentiated neoplasms were localised close to the basement membrane and, more frequently, in border areas showing invasion and basement membrane destruction. In horn cysts, centrally located cells were non-proliferating and p53 negative. In moderately differentiated neoplasms, proliferating cells were located closer to the basement membrane, while p53-positive cells were distributed diffusely in the neoplasm. In poorly differentiated neoplasms, p53-positive cells were more common than proliferating cells and were arranged in a diffuse pattern. The results showed that the number and location of p53-positive cells depended upon histology, with a close relationship to tumour type and degree of malignancy, but not on the mode of induction, nor on the animal strain or the relationship to subsequent tumour formation. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: UV, DMBA, mouse, strain, p53, PCNA, laminin
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INTRODUCTION

MUTATION OF the *p53* gene is the most frequent genetic change detected in human cancers [1, 2]. Mutant *p53* genes have been noted in 60% of colon and lung cancers and in 25% of breast cancers [3–6]. Human non-melanoma skin cancers arising in body areas exposed to solar radiation have exhibited mutations in the *p53* gene at locations opposite the potential pyrimidine dimer sites induced by ultraviolet (UV) irradiation [7–13]. A relationship between exogenous tumour induction and p53 expression has been reported in man [14–18] and in chemically induced skin papillomas and carcinomas of the skin [19, 20]. Stephenson and colleagues [21] reported that, while there was a highly significant trend in the proportions of p53 oncoprotein-positive lesions evolving from keratoacanthomas to poorly differentiated squamous cell carcinomas, p53 expression was inadequate for distinguishing between benign and malignant neoplasms.

The *p53* mutations are diverse, but most occur in highly conserved regions of the gene that are presumably of functional importance. Since the mutant *p53* gene encodes proteins with a prolonged half-life [22, 23], these mutations usually lead to a relative overexpression of p53 protein. In neoplasia, the immunohistochemical detection of p53 proteins has in some studies been considered to be synonymous with *p53* mutations [22, 24, 25]. Thus, immunohistochemical staining methods, although not able to pinpoint the

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mutations in the gene, may allow the detection of early changes, and the determination of the localisation of cellular changes in the tissue.

During the development of squamous neoplasia of the skin, alterations in the processes of cell proliferation, differentiation and maturation result in changes in the distribution of keratin [26, 27]. The most distinct characteristics of malignant neoplastic behaviour are invasion and an immunohistochemically observable destruction of the basement membrane [28, 29]. Normal cellular proliferation is thought to be regulated by growth and suppressor signals mediated by proto-oncogenes and tumour suppressor genes [30, 31]. Alterations in these signalling pathways could allow the neoplastic progression of the cell. Thus, *p53* could play a role in the regulation of the cell cycle and the rate of cell proliferation [31, 32]. Recent studies have shown that antibodies against the proliferating cell nuclear antigen (PCNA) are useful tools in assaying cell proliferation in cutaneous neoplasms [33, 34], as well as in other neoplastic and preneoplastic conditions [35]. It is crucial to develop assays that will assess biological activities of altered p53 proteins found in clinical specimens. This will allow probably irrelevant or silent mutations that are acquired by the malignant cell to be distinguished from mutations that truly contribute to the malignant phenotype [36].

Skin tumours are the most commonly induced neoplasms in man, resulting mostly from exposure to UV radiation, but also from chemical carcinogens. Experimental skin carcinogenesis offers a model for studying different stages of the process, using different mouse strains, which have a widely varying sensitivity to tumour formation [29, 37–39].

The purpose of this study was to determine the occurrence of mutated p53 expression in UV- and carcinogen-exposed skin of tumour-sensitive and -resistant animal strains, and to determine whether the mutated p53 protein-positive cells were morphologically and biologically different from those of p53-negative cells.

MATERIALS AND METHODS

Animals

A total of 210 NMRI, C57BL/6 and DBA/2 female mice (see Table 1), were obtained from the University of Kuopio and University of Oulu breeding facilities and kept in the air-conditioned animal facility of the University of Oulu, which is supervised by an animal control committee. Their age at the beginning of the experiments was 10–12 weeks. They were maintained on a 12/12 h light/dark cycle and received water and powdered animal fodder *ad libitum*. The mice were monitored for 50 weeks, during which time they were photographed and their lesions were recorded at regular intervals. A complete autopsy was performed at the termination of the experiment.

Experiment procedures

Chemical exposure. Groups 1, 2 and 3 were untreated control groups of NMRI, C57BL/6 and DBA/2 mice, respectively. In group 4, the shaved back skin of the mice was exposed to 100 μg 7,12-dimethylbenz(a)anthracene (DMBA) in 50 μl of acetone, once a week for 10 weeks.

UV exposure. Four Philips TL40W/12® UV lamps were used as the light source; the light tubes were located 22 cm above the surface level. The UV exposure times for groups 5, 6 and 7 were determined according to preliminary results, and a single dose, equivalent to two minimal erythemal doses for NMRI mice, was administered. The mice were exposed for 20 min, three times a week for 30 weeks, receiving a total dose of 11.1 J/cm^2 UVA and 18.0 J/cm^2 UVB. Skin specimens and specimens from any abnormal organs were taken for histological and immunohistochemical examination, both during and at the end of the experiment when the animals were moribund.

Specimens

The exposed skin was fixed in 10% phosphate-buffered formalin, embedded in paraffin and stained with haematoxylin–eosin, or other stains when required. Selected specimens were rapidly frozen in liquid nitrogen and stored either in liquid nitrogen, or at -70°C, and were subsequently sectioned using a cryostat microtome. The specimens selected for electron microscopy were fixed in glutaraldehyde and osmium tetroxide.

Immunoperoxidase staining

Antibodies. The different antibodies were used according to the manufacturers' specifications. Laminin antibody was a gift from Drs Juha and Leila Risteli (Department of Clinical Chemistry, University of Oulu, Finland). The P1 fragment of laminin was purified from human placenta as described previously [40]. Antisera to these proteins were raised in rabbits. The antibodies were purified by immuno-absorption with the relevant antigen coupled to Sepharose 4B. The laminin antibodies were cross absorbed with 7-S collagen and vice versa. There was no cross-reaction between the two antibodies in the radioimmunoassay. The p53 antibody, CM5, was obtained from Novocastra (Novocastra Laboratories Ltd, Newcastle, U.K.) and the monoclonal PCNA antibody, PC10, was obtained commercially (Dako Inc., Copenhagen, Denmark). Different antibodies for various cytokeratins were

Table 1. *Groups, experimental procedures and neoplastic response*

Group	Strain	*n*	Exposure	TBA	Pap.	K-ac.	SCC	Undiff.	Fibromas
1	NMRI	30	Untreated	0	0	0	0	0	0
2	C57BL/6	30	Untreated	0	0	0	0	0	0
3	DBA/2	30	Untreated	0	0	0	0	0	0
4	NMRI	30	DMBA	20	4	1	12	3	6
5	NMRI	30	UVB	13	3	1	1	8	1
6	C57BL/6	30	UVB	5	1	0	0	3	1
7	DBA/2	30	UVB	1	2	0	0	0	0

TBA, tumour-bearing animals; Pap, papillomas; K-ac, keratoacanthomas; SCC, squamous cell carcinomas; Undiff, undifferentiated carcinomas; DMBA, 7,12-dimethylbenz(a)anthracene. See Materials and Methods for further details.

used as markers of epithelial differentiation according to manufacturers' instructions.

Immunohistochemical methods

The avidin–biotin modification of the peroxidase–anti-peroxidase method, the streptavidin method, or the immunofluorescence method, were used for the immunohistochemical studies of histological sections.

Avidin–biotin method. Five micrometre sections were cut, placed on slides coated with 3-triethoxysilylpropylamine (Sigma, St. Louis, Missouri, U.S.A.) and fixed at 37°C overnight. Slides were deparaffinised in a graded series of xylene and ethanol. Endogenous peroxidase activity was quenched in 0.6% H_2O_2 methanol solution for 30 min. Slides were then rehydrated by consecutive washes with 100% ethanol (twice), 95% ethanol and 70% ethanol, and were placed in phosphate-buffered saline (PBS). Sections were subsequently equilibrated with 0.1% Triton X-100 in PBS for 1 h, and rinsed in PBS. Selected specimens were then submitted to pretreatment using either (a) microwave apparatus in citrate solution, regulated at +95°C for 4 min; (b) 0.4% pepsin (Merck Darmstadt, Germany) at 37°C for 2 h; (c) hyaluronidase (Sigma), 1 mg/ml in 0.5% mol/l sodium acetate, pH 5.0, with 0.85% NaCl, for 1 h at 37°C; or (d) a pressure cooker for 5 min at boiling temperature. The slides were then rinsed with PBS and blocked with 2% normal swine serum and 1% bovine serum albumin (BSA) in PBS, for 20 min. The specimens were exposed to the primary antibody (5 μg/ml in PBS, containing 2% normal swine serum and 1% BSA) overnight at +4°C. Subsequently, the tissue samples were submitted to 30 min incubations with 2% normal goat serum, followed by biotinylated antirabbit immunoglobulin (dilution 1:400, Vector Laboratories, Burlingame, California, U.S.A.), and finally avidin (Vector). The peroxidase reaction was then performed using 3,3′-diamino-benzidine (Sigma). The slides were counter-stained with haematoxylin for 15 sec, dehydrated with increasing concentrations of ethanol, placed in PBS and xylene, and mounted.

Streptavidin method. Formalin-fixed, paraffin-embedded, 5-μm-thick sections were dehydrated in xylene and alcohol and washed. Endogenous peroxidase was blocked with 0.6% H_2O_2 for 20 min at room temperature. The sections were incubated overnight with the primary antibody in PBS containing 2% normal swine serum and 1% BSA. Control sections were incubated with PBS alone, or with non-reactive serum. Biotinylated anti-immunoglobulin in PBS, with carrier protein and preservative (Multilink, Bio-Genex, San Ramos, California, U.S.A.), was then applied for 20 min at room temperature. Incubation in the labelling solution (enzyme-labelled streptavidin in PBS) was performed for 20 min at room temperature. The peroxidase reaction was carried out with 3,3′ diamino-benzidine (Sigma), for 20 min at room temperature. The samples were then counter-stained with haematoxylin for 15 sec, dehydrated in increasing concentrations of ethanol and xylene, and mounted.

Immunofluorescence. Five micrometre thick sections, fixed in cold methanol–acetone, were washed and treated with hyaluronidase for 60 min. Endogenous peroxide activity blocking was carried out as previously described. The sections were exposed overnight to the primary antibody and to the secondary fluorescein- or rhodamine-conjugated antibody for 60 min. The sections were then washed, mounted with an antifading compound, and examined under a Leitz fluorescence microscope.

Controls. Several staining methods were compared in preliminary studies. Frozen sections, the avidin–biotin method and the streptavidin method on paraffin sections all gave similar results. The best morphological preservation and clearest images were obtained with the streptavidin method. Endogenous peroxidase was blocked to remove non-specific reactivity, and control specimens showed no reduction in sensitivity. Primary antibody staining times varying from 30 min to 24 h, at both room temperature and +4°C, were tested during the preliminary studies. The best results were obtained using an overnight exposure to the primary antibody at +4°C. Permeabilisation was carried out using different enzyme pretreatment protocols, in which various concentrations, durations and temperatures were tested, as well as microwave pretreatment utilising different temperatures and varying durations. Boiling pretreatment was also analysed. 3,3′ diamino-benzidine was chosen for the staining reaction rather than 3-aminoethyl-carbazole. To exclude non-specific staining, the following controls were performed: (a) second antibody only; (b) replacing the first antibody with saline, or non-reactive serum; and (c) replacing the reactive compound in the various steps with a non-reactive one, or omitting the step completely. Specimens from human skin and skin tumours, as well as from cultures of human and animal epidermal cells, were also used as controls.

Image cytometry

Image cytometry was performed for the analysis of staining results using a CAS 200 image analysis system (Becton-Dickinson, Belgium). Approximately 500 cells in each specimen were analysed and classified based on the analysis of the absorbance measured by two simultaneously recorded video-camera images, processed with a personal computer using standard software. Quantitation of the immunoreactivity was based on the assumption that the total absorbance was directly proportional to the immunoreactivity. Nuclear and antibody threshold levels were determined by visual analysis of appropriate specimens. The image analysis system standard quantitative software program was employed for the determination of the percentage of positive cells, with the adjacent total epidermis or the total number of tumour cells being used as a reference. The percentage of the surface area staining positive was recorded similarly. The percentage of positive nuclei was used for the determination of cell proliferation and p53 immunoreactivity with 1–25% p53-positive nuclei or cells being classified as grade 1, from 26 to 50% positive cells as grade 2, and more than 50% as grade 3. Laminin immunoreactivity was classified based on the extent of abnormality, with 0 = no abnormality with a regular, thin, continuous basement membrane; 1 = slight abnormalities with a focally thickened, or absent basement membrane in small areas; 2 = moderate abnormalities with a thickened, patchy, or absent basement membrane in large areas; and 3 = distinct abnormalities with a largely absent basement membrane.

RESULTS

A number of tumours and preneoplastic conditions were seen in sensitive NMRI mice exposed to a high dose of DMBA (group 4, Table 1), while skin tumours were totally absent in unexposed animals (groups 1–3). However, several

exposed animals were also devoid of preneoplastic changes and tumours. Repeated DMBA exposures resulted in a slight hyperkeratosis in the skin in 25% of animals, without any other diagnostic morphological changes. Epidermal hyperplasia, consisting of a proliferation of regular cells, was seen in parts of the exposed areas of the remaining animals at the end of the study. Extended exposure to DMBA resulted in intraepithelial atypia (Figure 1) of the epidermis, with an increase in histological disorganisation and cytological atypia. A number of papillomas, consisting of large numbers of regular cells with extensive keratinisation and different extents of stromal connective tissue participation, were seen at termination (Table 1), as were keratoacanthomas, consisting of proliferation of squamous epithelium on a cup-shaped base (Figure 2). A large number of squamous cell carcinomas were also found in animals exposed to DMBA. Well-differentiated carcinomas (grade 1) showed cellular atypia and extensive keratinisation with multiple horn cysts (Figure 3). Moderately differentiated carcinomas (grade 2) consisted of tightly arranged tumour cells with hyperchromatic nuclei and scanty cytoplasm (Figure 4). Undifferentiated spindle cell neoplasms rarely exhibited individual cell keratinisation.

Similar preneoplastic alterations and tumours were seen in sensitive NMRI mice following UVB exposure (group 5) although the number of undifferentiated neoplasms was relatively much higher (Table 1). Among the other UVB-exposed mouse strains, heavily pigmented C57BL/6 mice (group 6) showed epidermal alterations, dysplasia (Figure 5) and neoplasms similar to those observed in the UV-exposed albino NMRI mice, but they were less extensive. Tumour-resistant,

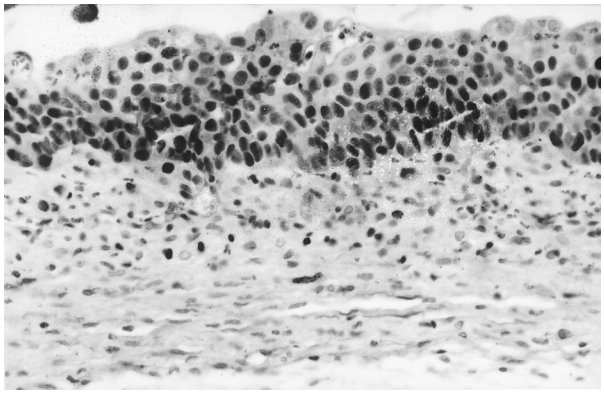

Figure 1. Proliferating cell nuclear antigen (PCNA)-positive cells in 7,12-dimethylbenz(a)anthracene (DMBA)-exposed dyplastic epidermis of an NMRI mouse. PCNA; original magnification×145.

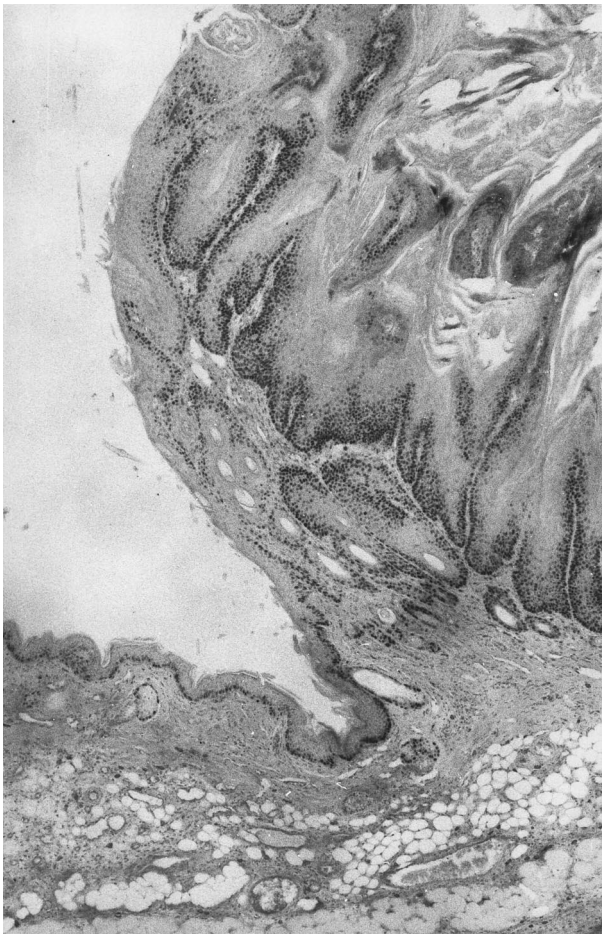

Figure 2. Abundant cell proliferation in keratoacanthoma induced by 7,12-dimethylbenz(a)anthracene (DMBA) in an NMRI mouse; the basal epidermis is also positive. Proliferating cell nuclear antigen (PCNA), original magnification×50.

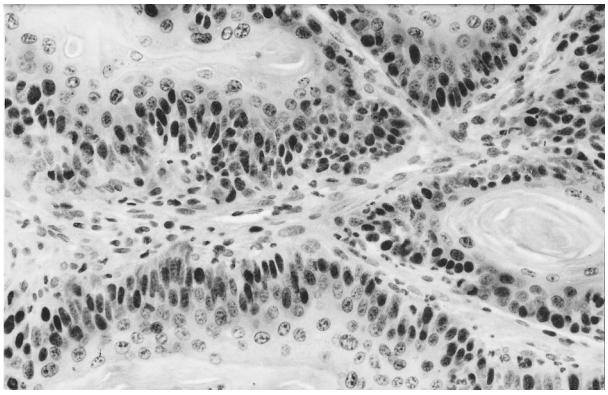

Figure 3. Well-differentiated, 7,12-dimethylbenz(a)anthracene (DMBA)-induced squamous cell carcinoma in an NMRI mouse showing the peripheral arrangement of proliferating cells. Proliferating cell nuclear antigen (PCNA), original magnification×145.

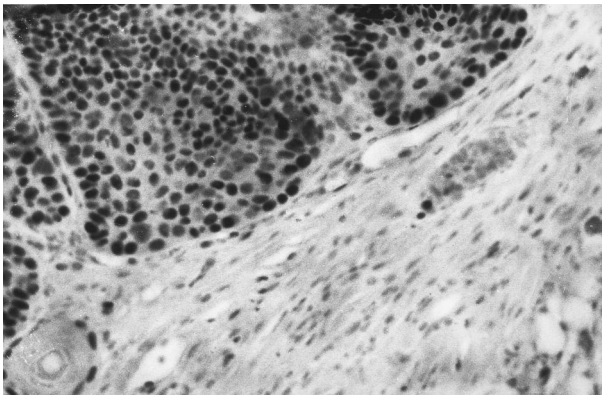

Figure 4. Proliferating cells in a 7,12-dimethylbenz(a)anthracene (DMBA)-induced moderately differentiated squamous cell carcinoma in an NMRI mouse. Proliferating cell nuclear antigen (PCNA); original magnification×155.

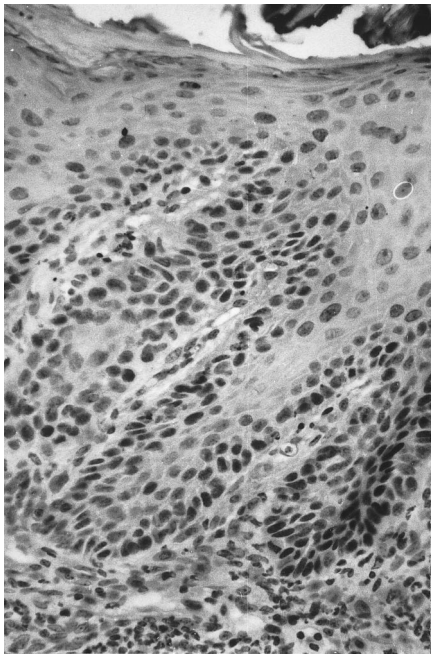

Figure 5. Ultraviolet-exposed C57BL/6 mouse skin with dysplastic cells expressing proliferative cell nuclear antigen (PCNA) staining in basal layers. PCNA; original magnification×155.

moderately pigmented DBA/2 mice (group 7) displayed only a slight hyperkeratosis after extensive UVB exposure; with the exception of two small papillomas no other morphological alterations were found.

The functional properties of the epidermal cells were assessed by their ability to form a basement membrane containing laminin (Table 2). In exposed and hyperplastic epidermis, the basement membrane was continuous and regular. In dysplastic epidermis, laminin expression was slightly irregular in the junctional area, with thickened, slightly discontinuous basement membrane structures; invasion was not observed. In benign papillomas and keratoacanthomas devoid of cellular atypia, the basement membrane was well preserved. Well-differentiated squamous cell carcinomas exhibited a mostly well preserved basement membrane that was absent around cells invading the stroma. In undifferentiated neoplasms, small patches of laminin-positive, basement membrane-like material was found surrounding individual tumour cells.

The localisation of proliferating cells was assessed by staining for PCNA with the antibody PC10. In carcinogen-exposed epidermis, approximately 35–50% of cells in the basal layer were PCNA positive, as were cells in the suprainfundibular region of the hair follicles (Figure 6), while the superficial layer was negative. In UV-exposed animal strains, increased PCNA immunoreactivity was seen in NMRI, C57BL/6 and DBA/2 mice several months after cessation of exposure (Table 2), regardless of the lack of an ensuing neoplasm formation. In hyperplastic epidermis, PCNA staining was observed in 40–50% of cells in the basal layer and was almost totally absent in the superficial keratinising cells. In dysplastic epidermis, PCNA immunoreactivity was observed in multiple layers (Figure 1), extending up to the surface in severe dysplasia, and was present in approximately 30–40% of the epidermal cells. Papillomas exhibited a distinct zonal arrangement of PCNA staining, with 40–50% of the cells being positive in a zone adjacent to the basement membrane, while cells in the superficial zone were PCNA negative. In benign keratoacanthomas, PCNA staining was extensive (Figure 2), affecting several of the more basal layers, but was absent from the superficial layers. In well-differentiated squamous cell carcinomas, PCNA-positive cells exhibited a distinct zonal distribution in well-differentiated squamous cell carcinomas (Figure 3). In cell layers adjacent to the basement membrane, 50–60% of cells exhibited PCNA positivity, while in central keratinising areas only 1–5% of cells were proliferating. In squamous cell carcinomas consisting of epithelial cells devoid of keratin cysts, PCNA staining was diffuse, affecting 20–40% of cells, and was scattered throughout the neoplasm (Figure 4). In anaplastic, undifferentiated

Table 2. Intensity and location of immunoreactivity of antibodies to basement membrane cell proliferation-associated antigens and p53

			PCNA			p53		
Mouse	Morphology	Altered basement membrane	Ba	Su	Lo	Ba	Su	Lo
NMRI	Normal	0	1	0	zonal	1	0	zonal
NMRI	Exposed	0	2	0	zonal	2	0	zonal
NMRI	Hyperplastic	0	2–3	1	zonal	2	1	zonal
NMRI	Dysplastic	0–1 focal, irregular	2	2	diffuse	2	2	irregular
NMRI	Papilloma	0	2	0	zonal	2	0	zonal
NMRI	Keratoacanthomas	0	2	0	zonal	2	0	zonal
NMRI	SCC grade I	0–1 irregular	3	1	zonal	2	1–2	zonal
NMRI	SCC grade II	2 irregular	2–3	2	zonal	2	2	zonal
NMRI	SCC grade III	3 irregular	3	3	irregular	3	3	irregular
NMRI	Spindle cell tumour	3 irregular	1	1	irregular	3	3	diffuse
C57BL/6	Exposed	0	2	0	zonal	2	0	zonal
C57BL/6	Papilloma	0	2	0	zonal	2	0	zonal
C57BL/6	Spindle cell tumour	3 irregular	1	1	irregular	3	3	diffuse
DBA/2	Exposed	0	2	0	zonal	12	0	zonal
DBA/2	Papilloma	0	2	0	zonal	2	0	zonal

Ba, basal cell layer; Su, superficial cell layers; Lo, location; SCC, squamous cell carcinoma; PCNA, proliferating cell nuclear antigen. Extent of alteration, staining intensity: 0, none; 1, 1–25%; 2, 25–50%; 3, > 50%. See text for further details.

neoplasms, 25–35% of the cells were PCNA positive with a diffuse distribution.

Among heavily pigmented C57BL/6 mice (group 6), PCNA immunoreactivity was also observed in UVB-exposed epidermal cells. In tumour-resistant, moderately pigmented DBA/2 mice (group 7), PCNA immunoreactivity was distinct (Figure 5), although they displayed only a slight hyperkeratosis and two small papillomas.

The expression of p53 in non-neoplastic, carcinogen- or UV-exposed skin was seen as CM5 immunoreactivity in 40–50% of cells in a layer adjacent to the basement membrane regardless of sensitivity to carcinogenic exposure, whether sensitive NMRI mice (Figure 7), moderately resistant C57BL/6 or resistant DBA/2 mice.

In the hyperplastic areas, p53 expression was also located in the basal layer, while superficial cells were negative. There were no detectable morphological differences between CM5-positive and -negative cells. In dysplasia, p53-positive cells were observed in several cell layers, although the staining intensity varied, as did the thickness of the positive cell layers (Figure 8). In hyperplastic solar keratosis-like conditions, p53 immunoreactivity was present in all cell layers and affected 40–50% of cells. Neoplastic cells in the basal regions of papillomas were p53 positive, and were distributed in single layers (Figure 9). Regardless of whether the gross appearance of the neoplasms was verrucous, acanthomatous, onion-like, or finger-like, the superficial neoplastic cells were p53 negative.

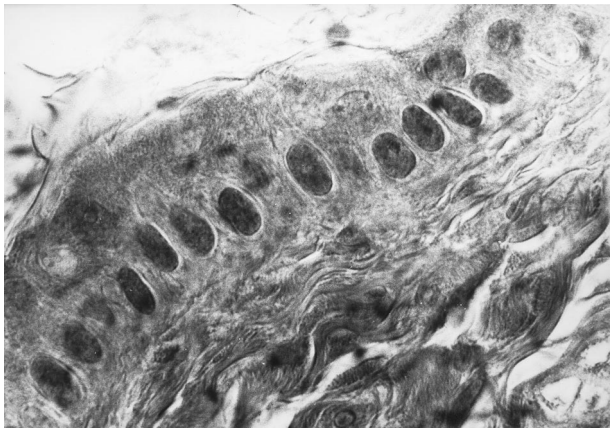

Figure 7. Positive p53 staining in basal layer cells of 7,12-dimethylbenz(a)anthracene (DMBA)-exposed non-neoplastic epidermis in an NMRI mouse. CM5; original magnification×870.

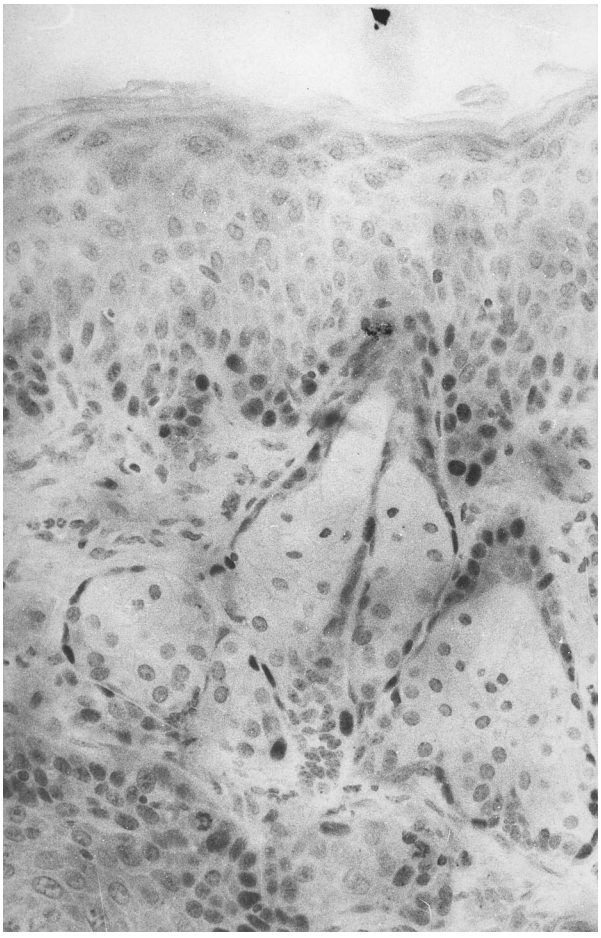

Figure 6. Epidermis in 7,12-dimethylbenz(a)anthracene (DMBA) exposed NMRI mouse skin showing proliferating cell nuclear antigen (PCNA)-positive cells in the basal layer and in the peripheral cells of the hair follicle and sebaceous gland. PCNA; original magnification×240.

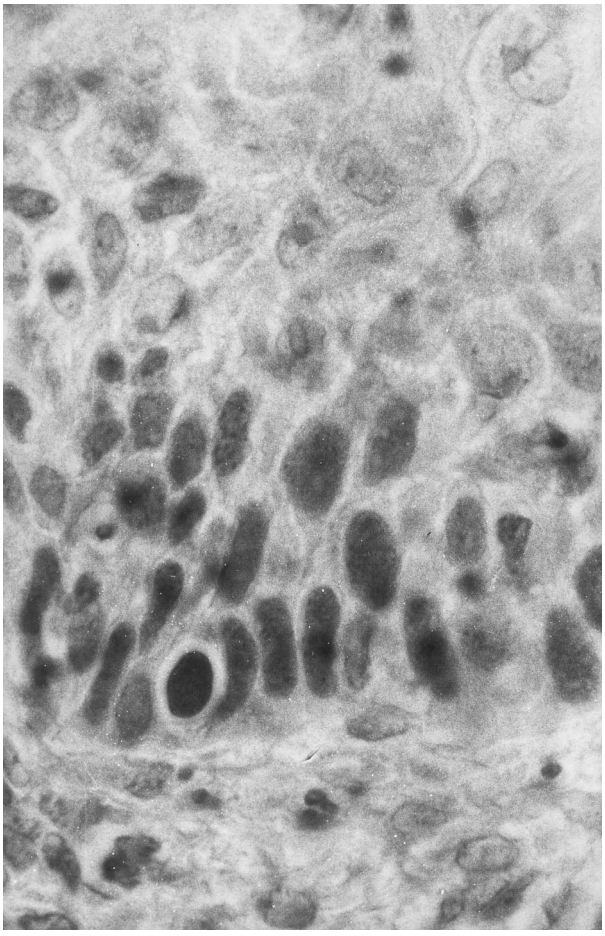

Figure 8. Multiple layers of cells staining with antibody against mutated p53 protein in 7,12-dimethylbenz(a)anthracene (DMBA)-exposed, dysplastic skin in an NMRI mouse. CM5; original magnification×1150.

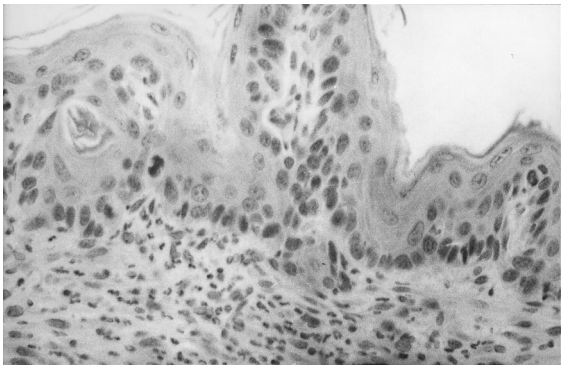

Figure 9. Squamous cells positive for p53 in 7,12-dimethylbenz(a)anthracene (DMBA)-induced papilloma in an NMRI mouse. CM5; original magnification×160.

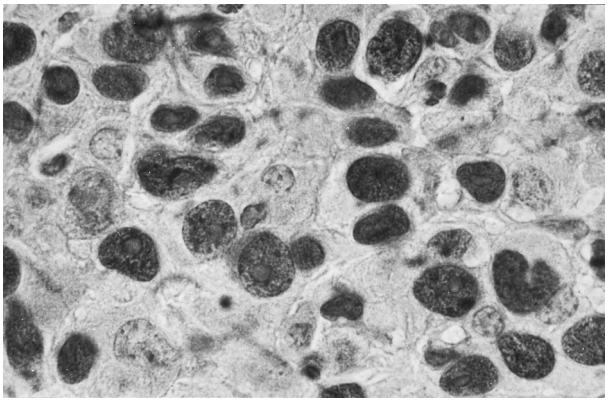

Figure 11. Anaplastic cells positive for p53 with antibody CM5 in an undifferentiated neoplasm induced by 7,12-dimethylbenz(a)anthracene (DMBA) in an NMRI mouse. CM5; original magnification×870.

In keratoacanthomas, p53 staining was observed only in basal cells.

In well-differentiated squamous cell carcinomas, positive nuclear staining for p53 appeared as a rim at the periphery of the keratinised islets, immediately adjacent to the basement membrane (Figure 10). The central region of these keratinised islets, which contained horn cysts, was invariably p53 negative, using the antibody CM5. Neoplasms with acanthosis and irregular rete ridges extending into the dermis, with areas showing signs of invasion and basement membrane disintegration, were also positive for p53. Positive staining for PCNA was also common in these locations. In moderately differentiated squamous cell carcinomas, the p53-positive cells were arranged in irregular sheets and cords, frequently appearing as isolated positively stained cells among morphologically similar atypical, but p53-negative cells. In these neoplasms, the malignant cells were inconsistently positive for p53, while uninvolved epithelium remained negative. In undifferentiated neoplasms, the p53 distribution was diffuse, with cells exhibiting variable p53 immunoreactivity (Figure 11). Epidermal cells displayed p53 immunoreactivity among the other UVB-exposed mouse strains, in heavily pigmented C57BL/6 mice (group 6) and in tumour-resistant, moderately pigmented DBA/2 mice (group 7), as in NMRI mice.

The comparison of p53 immunoreactivity with that of other markers revealed different features. Staining for p53 increased with increased keratin 8 and 18 immunoreactivity and with increased cellular atypia. The expression of p53 was also associated with the invasive behaviour of tumours, but the absence of laminin staining did not correlate with increased p53 immunoreactivity. Individual p53-positive cells were located close to preserved basement membrane structures. However, in invading areas (Figure 8), the number of cells expressing p53 protein increased. The p53 staining pattern mostly corresponded to the distribution of PCNA. However, the zonal distribution in benign neoplasms was not so clear and, in undifferentiated neoplasms, cells expressing p53 protein were more numerous than those expressing PCNA.

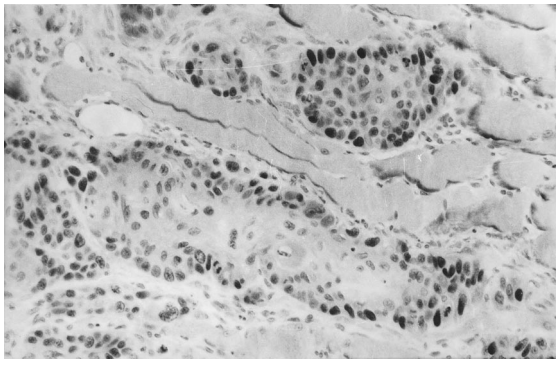

Figure 10. Peripheral arrangement of cells staining positive for p53 in a well-differentiated squamous cell carcinoma in an NMRI mouse. CM5; original magnification×145.

DISCUSSION

In the present study, we observed p53 expression in a high percentage of carcinogen-induced skin tumour cells. In studies of the expression of the *p53* gene in skin tumour cell lines and short-term cultures of skin tumours using Southern and immunoprecipitation procedures [20], the gene appeared to be normal in all papillomas and in early, well-differentiated carcinomas. In contrast, late advanced tumours manifested a 25% incidence of *p53* mutations. A low p53 positivity rate was reported in well-differentiated human skin cancers [41], while high positivity rates were found in moderately, or poorly differentiated carcinomas. Mutated p53-positive compartments may expand more rapidly than p53-negative ones, suggesting a role for p53 in the late stages of neoplasm development.

The distribution of cells expressing p53 protein depended upon the morphology of the skin and the neoplasm, with the staining pattern being focal, zonal or diffuse. A consistently positive and homogeneous staining of morphologically similar malignant cells was, however, not observed. In studies on p53 protein expression in squamous cell carcinomas [42], positive nuclei were seen in the less well-differentiated proliferative zones, with multiple foci of positivity and a clearly defined border. In solar keratosis [15], the distribution was either diffuse or focal along the advancing edge, as also seen here. A similar distribution of p53 expression along the advancing edge of the benign or well-differentiated malignant neoplasms, indicating a role in tumour expansion, has been reported [21]. In the present study, p53 expression was irregular in poorly differentiated carcinomas, with cells varying in both malignant potential and p53 staining.

Positive staining of p53 protein, which is not usually seen in normal cells [22], was observed in non-neoplastic, carcinogen-exposed skin in this study and has also been reported previously [43]. Gusterson and colleagues [42] reported a cut-off point between the tumour and the adjacent normal skin, but multiple foci were observed, possibly due to lateral spread of the neoplasm. In addition to invasive neoplasms, pre-invasive dysplastic lesions in the bronchus are partly positive for *p53* mutations [44]. p53 expression has been reported in cutaneous premalignant conditions, solar keratosis and Bowen's disease [15, 45, 46], as well as in benign keratoacanthoma [45]. False positive p53 immunoreactivity most likely results from the interruption of the normal degradative pathway of p53 [47], while false negative results may arise from fixation [35], or, for example, from a gross deletion which masks all p53 protein production [47]. Barbareschi and associates [48] reported p53 overexpression in normal skin that was not associated with malignant skin lesions. However, UV exposure was not ruled out in the latter study. Immunohistochemical analyses of mutant p53 in solar keratosis [15] revealed a focal and scattered nuclear positivity of cells in the adjacent 'normal' epidermis that was not observed in studies of other tumours. This may represent a potential biological marker of an earlier stage in the evolution of solar keratosis. Skin possesses a p53-dependent 'guardian of the tissue' response to DNA damage that aborts precancerous cell development [23, 49–52]. Thus, p53 immunoreactivity in non-neoplastic epidermal cells may indicate a protective action against UV- and chemical-promoting effects in tumour induction.

In this study, p53 showed a close, although indirect, relationship with PCNA. This is a 36 kDa nuclear protein present in proliferating cells, and essential for cell replication [35, 53]. PC10 is a monoclonal antibody reactive towards a particular epitope of PCNA (polymerase ε accessory protein) and can be used on routine material [53]. PCNA expression increases during the G1 phase, peaks at transition from G1 to S, and decreases through the G2 phase [53–55]. Recent studies have shown that PCNA immunostaining provides information on cell proliferation and has a certain prognostic value in human tumours [33, 34, 53–56] although the idea that cell division is important in cancer causation has been attacked [57]. p53 may be directly involved with cell division [31]. As shown here, cell proliferation occurs very much in the absence of p53 during early carcinogenesis. However, the distribution and intensity of staining of both antigens was similar in different types of malignant neoplasms. Thus, one of the effects of p53 in skin tumour progression may be to alter cell proliferation [58].

Keratin staining and the immunohistochemical analysis of p53 in this study revealed a distinct differentiation pattern related to mutated p53. The differentiation-specific keratins in mouse epidermis are keratins 1,18 and 19 [27, 59]. In hyperplasia and skin papillomas, keratins K1 and K10 are not expressed [59, 60], while keratins 8 and 18 are found in simple and malignant epithelial neoplasms. In this study, mutated p53 was associated with an increase in simple keratins. However, this was not a specific correlation, because many epidermal cells not expressing mutated p53 also exhibited this keratin pattern. Specific alterations in the keratin staining pattern were not observed, as we have shown previously [60].

Invasion is the hallmark of malignant cells and basement membrane disintegration is common in malignant neoplasms of the skin [28, 29]. In squamous cell carcinomas, a decrease in basement membrane deposition was associated with an increased p53 expression. However, mutated p53-positive cells were seen adjacent to a preserved basement membrane in more differentiated neoplasms. Thus, cells expressing p53 protein were also capable of producing basement membrane structures. In this study, neoplasms devoid of basement membrane structures showed distinct staining and a diffuse distribution of mutated p53 that was related to a lack of differentiation itself associated with a high degree of malignancy.

Several exogenous agents, such as polyaromatic hydrocarbons in cigarette smoke, have been implicated in cancer induction. Mutations in the *p53* gene are frequent in cancer patients with a history of heavy smoking [17, 61, 62], e.g. in primary resected non-small cell lung cancers, squamous cell carcinomas of the larynx and squamous cell carcinomas of the head and neck [17, 61]. Most *p53* mutations affect the phylogenetically conserved regions of the gene, exons 5–9, and result in conformational changes that bind interactions between p53 and other molecules [63]. In the study of McGregor and colleagues [45], p53 protein expression in neoplasms associated with UV exposure was similar to that observed in areas not likely to be exposed to UV, suggesting that other carcinogenic agents are involved in skin carcinogenesis. UV light produces distinctive mutations in DNA [49–52] and the presence of these mutations can identify UV as the mutagen. No UV-specific effect was observed in this study, possibly due to the method used. Ongoing studies in our laboratory attempting to detect specific mutations have not yielded conclusive results. p53-positive cells were also observed in non-neoplastic skin of resistant animal strains in this study, indicating a relationship between exposure and p53 protein expression.

The immunohistochemical determination of specific processes is dependent on the application of the method. Many technical aspects, including slight variations in specimen thickness, temperature, duration of the different steps, pH, washes, different batches of antibodies, etc., can all affect the outcome of a manual procedure comprising numerous steps. Visscher and colleagues [64] concluded that a variety of factors may account for discrepancies when immunohistology is used to evaluate p53 status. These include fixation artefacts, different epitope specificities of monoclonal reagents, the presence of immunohistologically 'silent' mutations and, possibly, aberrant overexpression of wild-type protein. Numerous negative and positive controls, different antibodies and other compounds included in the different steps, as well as repeated analyses of the same specimens, were used in this study in order to ensure its reliability. The duration and the method of fixation of the specimens are important. When fixing for PCNA determination [35, 53], formaldehyde preserves a nucleoplasmic and chromatin-associated form of cyclin, and fixation with methanol preserves cyclin bound to DNA replication sites in the chromatin [65]. These observations may partially explain the high variability of the results obtained in studies of the occurrence of mutated p53 in human tumours and why a high proportion of morphologically malignant cells were p53 negative [66]. Different results may also be explained by an interobserver variability in interpreting the staining. The immunoreactivity classification system employed in this study, consisting of computerised videocamera image-based cytometry, is more consistent, and allows the analysis of a much greater number

of cells than conventional visual classification. Previous studies have shown a close correlation between flow and image cytometry, with a high degree of reliability and sensitivity [67].

Accumulation of p53 is essentially due to one of two different mechanisms: as a reaction to DNA damage, or as a result of a mutation of the *p53* gene, in which case p53 is constitutively expressed in the nuclei [68]. These findings were supported by our study involving early accumulation of p53. The p53 mutation-induced accumulation of the corresponding protein is related to the degree of differentiation and is independent of the exposure to the mutagen [68]. These results were also supported by our study showing p53 accumulation in dedifferentiated neoplastic lesions, irrespective of the time of appearance, or the duration of exposure to the inducing agent, DMBA or UV. p53 accumulation induced by UV damage is a dynamic event; the correlation between the *p53* mutation and the accumulation of the corresponding protein is not perfect. For unknown reasons, *p53* mutations do not involve all cells, even when they are exposed to large doses of damaging agents for extended periods of time.

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