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Immunohistochemical reaction patterns of keratins in MNNG-induced shrew oesophageal carcinomas

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Abstract The distribution of keratins in *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine-induced oesophageal carcinomas in shrews was tested immunohistochemically, using a panel of seven different monoclonal antibodies. The studies were done on methacarn-fixed paraffin-embedded tissue, using the labelled streptavidin biotin method, and the relationship between morphological characteristics and keratin reaction patterns in carcinomas was analysed and compared with that in adjacent "normal" oesophageal epithelium. In the normal oesophageal epithelia, KL1, AE1, AE3, CK8.12 and CK4.62 stained suprabasal cells, 312C8-1 reacted to basal cells, and KS-1A3 labelled all epithelial cells. In squamous cell carcinomas, almost all the cancer cells were labelled strongly by 312C8-1 and weakly by KS-1A3, while a few cells in the centres of the keratinized foci were stained by KL1, AE1, AE3, CK8.12, and CK4.62. Like human oesophageal carcinomas, shrew oesophageal carcinomas maintain expression of human keratin 14, as determined by 312C8-1. The expression of human keratin 13, as determined by KS-1A3, was down-regulated.

Key words Keratin · Immunohistochemistry · MNNG · Oesophageal carcinoma · Shrew

Introduction

The mortality rate for oesophageal cancer varies greatly worldwide, geographic differences suggesting that environmental factors play a role in its aetiology. One such factor, the presence of nitroso compounds in the diet, is probably the major risk factor (Moses 1991; Wu et al. 1993). Indeed, oesophageal tumours have been produced experimentally with nitroso derivatives in various species, including rodents (mice, rats, and hamsters; Herrold 1966; Sander and Schweinsberg 1973; Iizuka et al. 1980), carnivores (dogs; Takubo et al. 1981) and primates (Adamson and Sieber 1983); we have recently succeeded in inducing such tumours in insectivora (shrews; Tsubura et al. 1993b). Phylogenetically, insectivora are considered to be the most primitive class of primates (Romer and Parson 1978). One member of the class insectivora, the house musk shrew (*Suncus murinus*; family Soracidae), is small and has a short lifespan, and has been bred under laboratory conditions (Matsuzaki et al. 1992). As in humans, the oesophagus of these animals is lined with stratified non-cornified squamous epithelium, and they have no forestomach. They are therefore suitable for experimental use in investigating oesophageal carcinogenesis, and the data obtained may easily be extrapolated to humans. In this respect, shrews represent a suitable animal model for studying the histology of oesophageal carcinoma.

Keratins have a number of distinct advantages as marker proteins. Keratin polypeptides are the products of various genes and are expressed in different cells and in cells at different stages of differentiation (Cooper et al. 1985). Keratins are epithelial-specific intermediate-sized filament proteins that have been characterized and classified on the basis of molecular weight and charge; they are identified numerically as keratins 1–19 (Moll et al. 1982). Carcinoma cells retain the capacity to produce the keratins of their normal progenitor cells and they may also develop new types of keratin filaments. Keratins are, therefore, suitable and specific markers both for studies of the classification of epithelial cell types and for the identification of epithelial neoplasms. Biochemical analysis by two-dimensional gel electrophoresis has been the standard method for determining the keratin composition of various tissues and tumours (Moll et al. 1982). However, biochemical analysis cannot discern any differences among individual cells in a population. Immunohistochemistry, in contrast, can relate keratin expression to specified cell populations or to the state of differentiation of the cells. Subdivisions of

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epithelial tumours in accordance with antibody specific to a keratin polypeptide have also been suggested. Several anti-keratin antibodies have been produced that can label human oesophageal epithelium as well as oesophageal carcinomas (Grace et al. 1985; Yang and Lipkin 1990). Since no immunohistochemical examinations of shrew oesophageal carcinomas have yet been performed, we carried out this study to examine the relationship between keratin immunohistochemical reaction patterns and the morphological features of oesophageal carcinomas in these animals. We used seven well-characterized monoclonal antibodies which cross-react to shrew epithelial cells (Tsubura et al. 1991), and we compared the results in oesophageal carcinomas with those in adjacent "normal" epithelium.

Materials and methods

Twenty-five female 4-week-old Japanese house musk shrews, Jic: SUN strain, were purchased from Clea Japan (Osaka) and housed in plastic cages. Room temperature was $22 \pm 2°$ C and relative humidity was $60 \pm 10\%$. They were fed a special pellet diet for shrews (CIEA-305, Clea Japan) and had free access to water. From the age of 6 weeks, 20 animals received *N*-metyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG at a dose of 50 μg/ml) in the drinking water for 30 weeks, followed by tap water until the end of the experiment. They were than killed when they became moribund, and the experiment was terminated when the animals were 54 weeks of age. Fourteen shrews survived until the end of MNNG administration, mean survival age being 48 weeks. Six shrews that died before the end of MNNG administration were excluded from the experiment. The 5 control animals were killed at the termination of the experiment.

The oesophagus was fixed in methacarn (60% methanol, 30% chloroform, and 10% acetic acid) fixative and embedded in paraffin. One to three representative blocks were made from each specimen, and serially cut 4-μm sections were stained with haematoxylin and eosin and used for immunohistochemistry.

We used seven anti-keratin antibodies, some plurispecific and some monospecific, which recognized different keratin polypeptides. Their specificity and sources are shown in Table 1. Their production and characterization are documented elsewhere (Huszar et al. 1986; Levy et al. 1988; Tsubura et al. 1991).

For immunohistochemistry, after deparaffinization and rehydration, the sections were incubated in 0.3% hydrogen peroxide in ethanol for 20 min, followed by incubation in 0.5% bovine serum albumin for 20 min, and subsequent incubation with optimized diluted primary antibodies. Immunostaining was then performed by the labelled streptavidin biotin (LSAB) method (DAKO, Carpinteria, Calif.), carried out according to the manufacturer's instructions. The colour was developed with diaminobenzidine (Wako Pure Chemical, Tokyo) and counterstained with Gill's haematoxylin. These antibodies worked consistently with the LSAB system in methacarn-fixed paraffin-embedded sections when the tissue was treated with actinase, if necessary, prior to incubation with each of the anti-keratin antibodies (Table 1). In brief, sections were placed in 0.01% actinase (Kaken Pharmaceutical, Tokyo) in phosphate-buffered saline (pH 7.2) for 15 min at 37° C. Proteolytic treatment significantly enhanced the immunoreactivity. For negative controls, serial sections were incubated with non-immune mouse serum instead of the primary antibodies.

Results

All 14 shrews that survived until the end of carcinogen treatment developed multiple tumours that protruded or were ulcerative or superficial and restricted to the oesophagus. Microscopically, all animals had invasive squamous cell carcinoma, which had often invaded the adventitia, or deeper into adjacent organs. However, no remote metastasis was seen. The carcinomas were generally well differentiated, characterized by keratin pearl formation and easily detectable intercellular bridges. Squamous cell carcinoma developed selectively and no adenocarcinoma of the oesophagus was seen. Anatomically, there are no mucous glands in the oesophagus of shrews. Papillomatous areas were also identified. None of the 5 untreated shrews had developed tumours at the termination of the experiment (54 weeks of age).

Table 2 shows the main features of keratin expression in normal oesophageal epithelium and in tumours, obtained using the seven monoclonal anti-keratin antibodies. All these antibodies cross-reacted specifically with squamous cells of the oesophagus in all shrews examined. In normal oesophageal squamous epithelia, KL1, AE1, AE3, CK8.12 (Fig. 1a), and CK4.62 showed prominent suprabasal staining, 312C8-1 reaction was restricted to the basal cells (Fig. 1b), while KS-1A3 reacted with all cell layers (Fig. 1c); all produced strong cytoplasmic staining. In papillomas, there was no essential difference from keratin expression in normal epithelia. In carcinomas, the most striking features were that almost all cancer cells were homogeneously labelled by 312C8-1 (Fig. 2). In addition to the labelling of cancer cells, the "normal" epithelium adjacent to the cancer cells showed an abnormal staining pattern, with some positive cells appearing in the suprabasal layer, or positive staining being shown in the entire epithelium in addition to the basal cell staining. Normal epidermis distant from the tumour showed a normal basal staining

Table 1 Specificity and sources of keratin antibodies used (*K* keratin)

Antibody	Specificity (Moll's number)	Source	Proteolytic digestion
KL1	K1,2,5-8,10,11,17	Immunotech, Marseilles, France	−
AE1	K10,14-16,19	ICN Immunobiologicals, Lisle, Ill., USA	+
AE3	K1-8	ICN Immunobiologicals	+
CK8.12	K13,16	BioMakor, Rehovot, Israel	−
CK4.62	K19	BioMakor	−
312C8-1	K14	Supplied by Dr. S.H. Dairkee[a]	+
KS-1A3	K13	BioMakor	+

[a] Lawrence Berkeley Laboratories, Berkeley, Calif., USA

Table 2 Positive keratin reactions in normal epithelium, papilloma, and squamous cell carcinoma in the shrew oesophagus

Antibody	Normal	Papilloma	Carcinoma
KL1	Suprabasal cells	Same as normal	Keratinized cells
AE1	Suprabasal cells	Same as normal	Keratinized cells
AE3	Suprabasal cells	Same as normal	Keratinized cells
CK8.12	Suprabasal cells	Same as normal	Keratinized cells
CK4.62	Suprabasal cells	Same as normal	Keratinized cells
312C8-1	Basal cells	Same as normal	All cancer cells
KA-1A3	All cells	Same as normal	All cancer cells

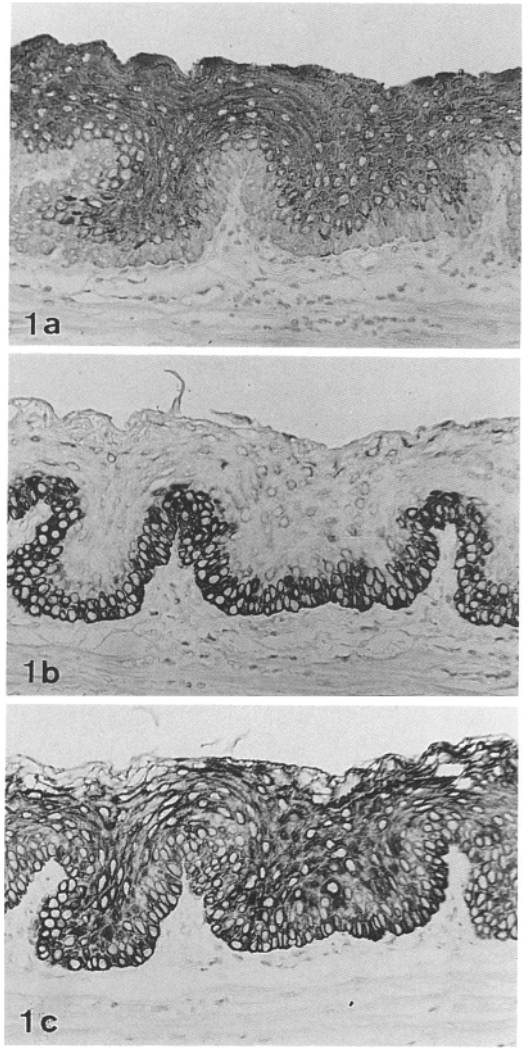

Fig. 1a–c Normal shrew oesophagus. **a** Suprabasal staining by CK8.12. **b** Basal cells are selectively labelled by 312C8-1. **c** Entire epithelium reacts with KS-1A3. × 200

pattern of 312C8-1 localization. A marked decrease in KS-1A3 expression was seen in cancer cells, compared with adjacent "normal" epithelial cells, but this antibody labelled almost all cancer cells (Fig. 3). In cancer cells, KS-1A3-positive material was mainly located in the keratinized nests, being weak at the periphery of the nests (Fig. 4). In contrast, cells positive for KL1, AE1, AE3, CK8.12, and CK4.62 (Fig. 5) were limited predominantly to the keratinized nests. Cells in one to several layers of the periphery of the nests were negative.

Discussion

The characteristic keratin pattern in human oesophageal epithelium (non-cornified squamous epithelium) is the presence of keratins 4–6, 13–17, and 19, shown by two-dimensional gel electrophoresis and immunoblotting (Moll et al. 1983; Grace et al. 1985). Keratins 4, 5, and 13 are seen in large proportions. In squamous cell carcinoma arising from these non-cornified stratified epithelia, keratin 13, a major component, is usually reduced (Moll et al. 1983), while in contrast, levels of keratins 14, 15, and 17 increase in the carcinoma (Grace et al. 1985). As the demonstration of keratins at the cellular level is not possible with biochemical techniques, immunohistochemical methods are used to classify cell phenotypes in terms of keratin distribution. Monoclonal antibodies are of great value in providing information on complex antigens such as keratins, by permitting the analysis of one antigenic determinant at a time.

Keratin expression differs in basal and suprabasal cells during the course of differentiation. In the human oesophagus, KL1 stains only the upper cell layers (Viac et al. 1983), while the basal layers remain negative. AE1 labels basal cells, while AE3 reacts with all epithelial cells from the basal layer to the top of the spinous cells (Yang and Lipkin 1990). The keratin composition of oesophageal epithelia shows species differences (Banks-Schlegel and Harris 1983). There are also possibilities that different keratin polypeptides may be recognized in the different species by each monoclonal antibody. In our present study, we observed similarities and differences in keratin immunoreactivity in the shrew oesophagus with these broad reacting antibodies. A single or small subset of keratins may provide more detailed information in regard to fine typing of cells and tumours. 312C8-1, which is specific for human keratin 14, was found to stain basal cells in normal shrew oesophageal epithelia. Also, in concordance with biochemical analysis in human oesophageal epithelia, almost all the cells in squamous cell carcinoma strongly expressed 312C8-1. In humans, keratin 13, which is normally present in non-keratinizing oesophageal epithelia, is considerably decreased or absent in carcinomatous cells (Malecha and Miettinen 1991). In the shrew carcinoma, although all cancer cells were KS-1A3 (specific for human keratin 13)-positive, this expression was weak immunohistochemically. In squamous cell carcinomas in shrews, in agreement with findings in humans, keratin 14 is maintained and keratin 13 is down-regulated. Compared with KS-1A3 immunoreactivity, which labelled all layers of normal oesophageal cells, CK8.12, which is specific for human keratins 13 and 16 (Huszar et al. 1986), did not label basal cells. This suggests that

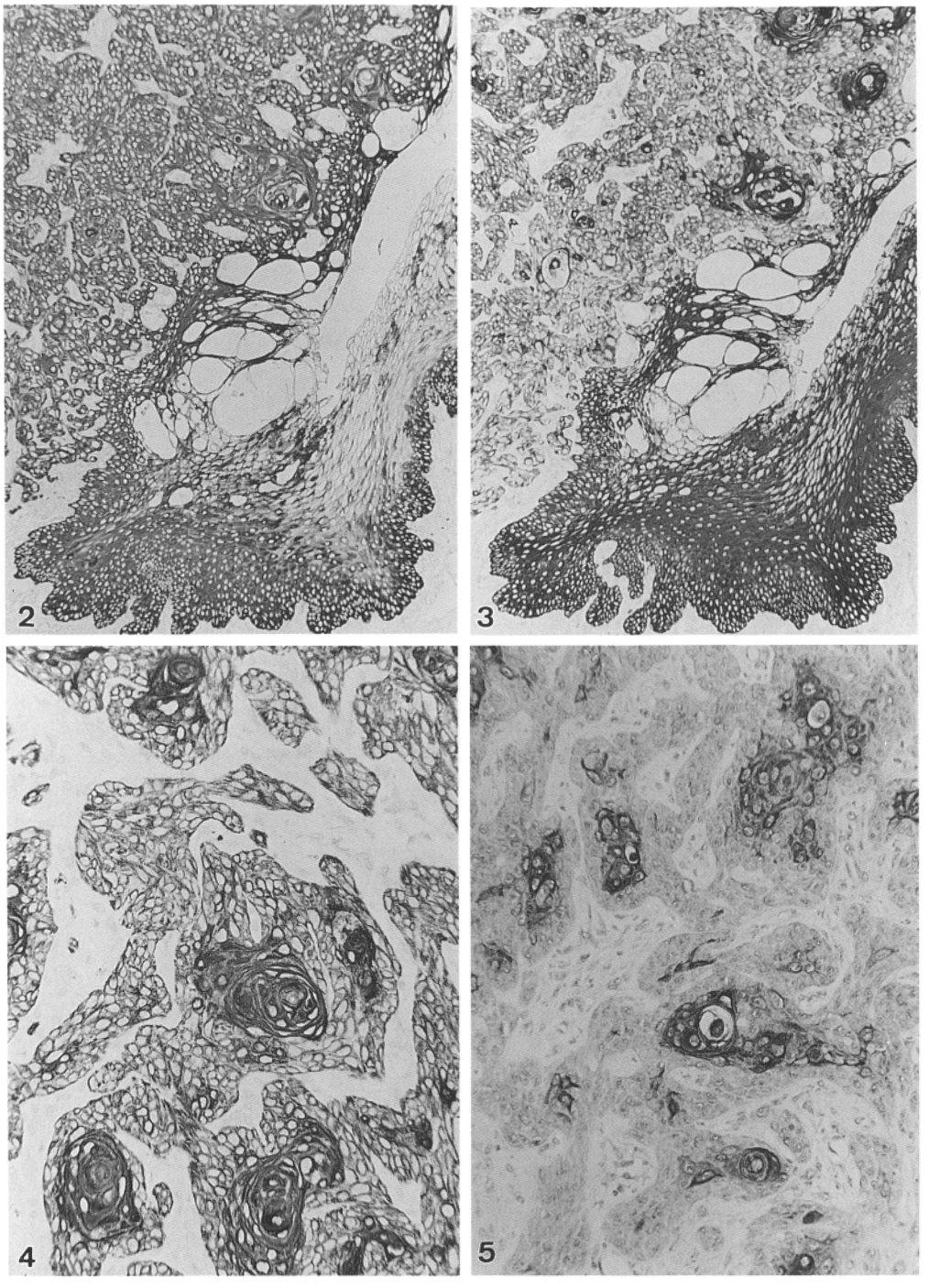

Fig. 2 Protruded squamous cell carcinoma. Strong keratin 14 staining by 312C8-1, is seen in all carcinoma cells. Note positive staining in the entire "normal" epithelium adjacent to the carcinoma. × 100

Fig. 3 Protruded squamous cell carcinoma. Weak keratin 13 staining by KS-1A3, compared to adjacent "normal" epithelium, is seen in carcinoma cells. × 100

Fig. 4 Squamous cell carcinoma. Keratinized nests show cytoplasmic staining by KS-1A3; weak staining is seen in peripheral cells. × 200

Fig. 5 Squamous cell carcinoma. Keratin 19-positive cells, as determined by CK4.62, are localized around the keratinization centre, while the periphery of the nests is negative. × 200

CK8.12 may represent human keratin 16, but that the keratin 13 epitope may be masked in shrews. In the squamous cell carcinomas of shrews, the expression of CK8.12 and CK4.62 (human keratin 19) was limited to keratinizing centres.

In the study of carcinogenesis, it is of particular importance to recognize and define the characteristics of premalignant lesions. Here, we found that many cancers arose abruptly from the normal oesophageal epithelium, without passing through the papilloma stage. However, the presence of papillomatous areas in association with frank carcinoma supports the suggestion that these lesions are precursors of malignancies. As they are in rodents (Napalkov and Pozharisski 1969), papillomas may be an essential step in the development of oesophageal carcinomas in shrews. Of the anti-keratin antibodies tested, we found 312C8-1 to be the most sensitive for examining the premalignant stage. In "normal"-appearing epithelium adjacent to oesophageal cancer, it is conceivable that a suprabasal staining pattern, in addition to basal staining, may be related to a hyperproliferative state, suggesting an increased rate of

cell turnover (Tsubura et al. 1993a). Thus, 312C8-1 may serve as a biomarker, identifying early abnormalities in oesophageal epithelial cells that have an increased predisposition to malignancy. However, papillomas showed similar 312C8-1 immunoreactivity to that seen in normal epithelia. In conclusion, this immunohistochemical study of MNNG-induced oesophageal carcinoma is shrews may serve as a good model for analysing the morphogenesis of the comparable human carcinoma.

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