Cutaneous Epithelial Lesions Induced by *N*-Methyl-*N*-nitrosourea in Male Sprague-Dawley Rats: A Possible Animal Model for Human Keratoacanthoma

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Abstract. *A single intraperitoneal injection of 50 or 75 mg/kg N-methyl-N-nitrosourea in male Sprague-Dawley rats at 4 weeks of age, dose-dependently resulted in cutaneous epithelial cysts and tumors of pilosebaceous origin. Cysts were composed of epidermal cysts or mixed epidermal and inner root sheath hybrid cysts. The majority of induced tumors were keratoacanthomas. A few tumors were trichofolliculomas, trichoblastomas, pilomatricomas, or sebaceous adenomas. All tumors were benign pilosebaceous tumors. Keratoacanthomas were crater-shaped tumors with thick infoldings of epithelium containing keratohyalin granules (epidermal lip) that abruptly changed to epithelium containing trichohyalin granules. The morphological similarity and resemblance of keratin 1, 10, and 14 profiles, and p63 and β-catenin expression between mixed epidermal and inner root sheath hybrid cysts and keratoacanthomas suggests that hybrid cysts progressed to keratoacanthomas, and the cells from infundibular cells to inner root sheath cells of the pilar segment seem to be the origin of rat keratoacanthomas. Immunohistochemical localization of keratins 1, 10 and 14, p63, and β-catenin in trichofolliculoma, trichoblastoma, and pilomatricoma, as well as keratoacanthoma, may indicate tumor histogenesis.*

Cutaneous adnexal tumors are relatively uncommon but often difficult to diagnose. As such tumors exhibit a broad repertoire of morphologies, determination of their exact histogenesis is controversial.

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Studies of animal models add to the understanding of human disease. The rat is unique among laboratory animals in that a variety of tumors develop spontaneously (1-4) and can be artificially induced by using carcinogens, such as aromatic hydrocarbons (5) and nitroso compounds (6). One nitroso compound, *N*-methyl-*N*-nitrosourea (MNU), produces a wide variety of tumors in different organs and tissues in rats (7). Topical cutaneous application of MNU induces cutaneous tumors at the site of application but systemic administration of MNU can induce tumors at remote sites. Although hair follicles accompanied by sebaceous glands are located throughout the rat body, sweat glands are located only in the skin of foot pads. Therefore, we targeted the pilosebaceous apparatus and evaluated whether systemic administration of MNU induces formation of pilosebaceous tumors and whether MNU-induced tumors exhibit morphological similarities to human tumors (8).

Keratins are a highly diverse family of cytoskeletal proteins and important markers of epithelial cell differentiation. Keratins are differentiated into 20 sub-types according to their molecular weight and isoelectric pH, and different keratin sub-types can be used to label different components of pilosebaceous cells (9). Among keratins, keratin 1 (K1) and K10 are found in the suprabasal layer of the infundibular epithelium, as well as the interfollicular epithelium, while K14 is seen in basal cells of the epidermis in normal human skin. K14 is also found in outer root sheath cells, sebaceous cells, and sebaceous ducts. The protein p63 plays distinctive roles in the physiology of skin, and is expressed in the nuclei of outer root sheath cells (but not inner root sheath cells), hair matrix cells, and peripheral sebaceous cells in normal human skin (10). β-Catenin controls hair follicle differentiation and regeneration. In addition to being expressed in epidermal cells and sebaceous cells, β-catenin is expressed in inner and outer root sheath cells and in hair matrix cells in anagen phase in normal human skin (11,12). An evaluation of the expression of

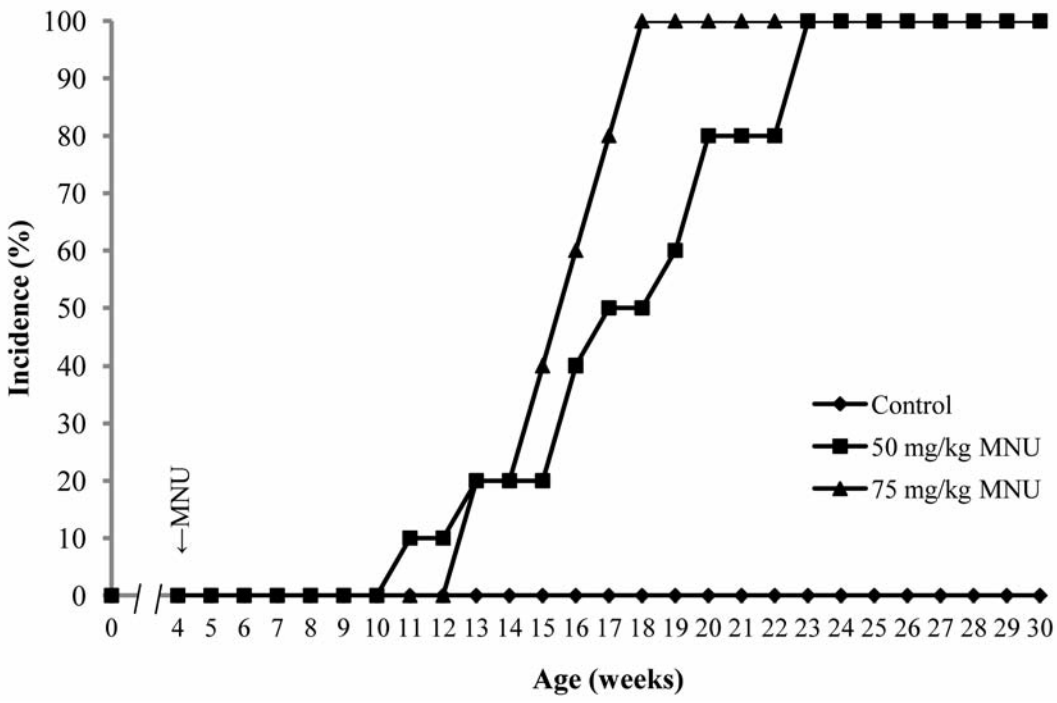

Figure 1. *N-methyl-N-nitrosourea (MNU) dose and the occurrence of palpable skin lesions in male Sprague-Dawley rats.*

keratins, p63, and β-catenin in pilosebaceous lesions will enable our understanding over the histogenetic origin of pilosebaceous tumors.

Therefore, immunohistochemistry was applied to determine if these markers are useful in understanding the histogenesis of MNU-induced skin tumors in rats. As there was a high incidence of keratoacanthoma, the histogenesis of keratoacanthoma was discussed.

Materials and Methods

Carcinogen. MNU in a powder form obtained from Sigma (St. Louis, MO, USA) was stored at 4˚C in the dark. Immediately before use, MNU was dissolved in physiological saline containing 0.1% acetic acid.

Animals and experimental procedures. The study protocol and animal procedures were approved by the Animal Care and Use Committee of Kansai Medical University (permit no. 14-032). In brief, 4-week-old male Sprague-Dawley rats (Crl:CD, SD) were purchased from Charles River Japan (Hino, Japan). They were housed in groups of four or five in plastic cages with paper bedding (Paper Clean, SLC, Hamamatsu, Japan) in a specific pathogen-free environment maintained at 22±2˚C and 60±10% relative humidity with a 12-h light/dark cycle (lights on at 8:00 AM and lights off at 8:00 PM). The rats were randomly divided into three groups, one treated with 50 mg/kg MNU (n=10), another with 75 mg/kg MNU group (n=10), and a control group without MNU (n=10). A single dose of either 50 mg/kg or 75 mg/kg body weight was administered intraperitoneally (*i.p.*) at 4 weeks of age. Animals were maintained on a commercial pellet diet (CMF, 30 kGy; Oriental Yeast, Chiba, Japan). Diet and water were available freely. During the experiment,

Table I. *Expression of keratin 1 (K1), K10, K14, p63 and β-catenin in normal pilosebaceous units and pilosebaceous lesions.*

	K1	K10	K14	p63	β-Catenin
Normal					
Epidermis	+/−	+/−	−/+	−/+	+/+
Infundibulum	+/−	+/−	−/+	−/+	+/+
Sebocyte	−	−	+	−	+
Seboblast	−	−	+	+	+
Inner root sheath	−	−	−	−	+
Outer root sheath	−	−	+	+	+
Hair matrix	−	−	−	+	+
Lesions					
Cysts					
Epidermal	+/−	+/−	−/+	−/+	+/+
Hybrid	+/−.−	+/−.−	−/+.−	−/+.+	+.+
Tumors					
Keratoacanthoma	+/−.−	+/−.−	−/+.−	−/+.+	+.+
Trichofolliculoma	+/−	+/−	−/+	−/+	+
Trichoblastoma	−	−	+	+	+
Pilomatricoma	−	−	−	−/+	+
Sebaceous adenoma	−	−	+	−/+	+

+/−: Positive or negative in suprabasal/parabasal-basal cells. Expression patterns of respective proteins; front of dot is keratohyalin granule area and behind of dot is trichohyalin granule area.

Table II. *N-Methyl-N-nitrosourea (MNU)-induced pilosebaceous lesions in male Sprague-Dawley rats.*

	MNU dose					
	50 mg/kg (n=10)			75 mg/kg (n=5)		
Lesion	No. of lesions	Multiplicity	Ratio (%)[a]	No. of lesions	Multiplicity	Ratio (%)[a]
Cysts						
Epidermal/inner root sheath	66	6.6±1.1	49	77	15.4±2.9	49
Tumors						
Keratoacanthoma	62	6.2±1.3	46	74	14.8±3.2	47
Trichofolliculoma	2	0.2±0.1	1.4	1	0.2±0.2	0.7
Trichoblastoma	2	0.2±0.1	1.4	2	0.4±0.2	1.1
Pilomatricoma	2	0.2±0.1	1.4	2	0.4±0.2	1.1
Sebaceous adenoma	1	0.1±0.1	0.8	2	0.4±0.4	1.1

[a](No. of respective lesions/Total no. of lesions) ×100.

moribund animals were sacrificed and excluded from the study, and the experiment was terminated when animals reached 30 weeks of age. At necropsy, all organs were macroscopically examined, and macroscopically abnormal organs, as well as all cutaneous lesions, were histologically examined. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (HE), and a few were used for immunohistochemistry. Throughout the experiments, animals were cared for in accordance with the Guidelines for Animal Experimentation of Kansai Medical University.

Immunohistochemistry. Serially-cut paraffin-embedded tissues were deparaffinized in xylene and dehydrated in ethanol. After the blocking of endogenous peroxidase, pressure cooking antigen retrieval was performed. Slides were tested for the expression of K1 (clone 34βB4; Leica Biosystems, Nussloch, Germany), K10 (clone LHP1; Leica Biosystems), K14 (clone LL002; Leica Biosystems), p63 (clone 4A4 ThermoFisher Scientific, Waltham, MA, USA), and β-catenin (14/Beta-Catenin; BD Biosciences, San Diego, CA, USA) using the respective antibodies. Immunohistochemistry was performed with a labeled streptavidin biotin (LSAB) staining kit (Dako, Glostrup, Denmark). 3,3'-Diaminobenzidine tetrahydrochloride was then used as the chromogen to assess positive reactions with hematoxylin as counterstain.

Results

MNU at a dose of 75 mg/kg at 4 weeks of age was toxic in five rats that died at 5 weeks of age (one week after MNU). The remaining five rats in the 75 mg/kg group and all rats from the other two groups (control and 50 mg/kg MNU) survived until the end of the experiment (at 30 weeks of age) and were included in the study.

Cutaneous lesions became palpable when they reached 2-3 mm in diameter. In MNU-treated rats, the 75 mg/kg-treated group tended to have accelerated development of palpable cutaneous lesions compared to the 50-mg/kg group. The 75 mg/kg and 50 mg/kg MNU doses caused 100% incidence by 18 and 23 weeks of age, respectively, while the untreated control group had developed no cutaneous lesions at 30 weeks of age (Figure 1). The cutaneous lesions had an average diameter of 5 mm at the end of the study. Histologically, in the control group, the skin was composed of approximately four layers of epidermis with scattered pilosebaceous apparatus and a lack of sweat glands, and no cutaneous lesions were detected. Immunohistochemically, K1 and K10 were expressed in the suprabasal cells (but not in the basal cells) of interfollicular and infundibular epithelium, but the pilosebaceous apparatus below the infundibulum was totally negative. In contrast, K14 was expressed in the basal cells of interfollicular and infundibular epithelium, sebaceous cells and sebaceous ducts, and outer root sheath cells. However, inner root sheath cells and hair matrix cells remained negative. p63 was expressed in the nuclei of outer root sheath cells (but not in inner root sheath cells) and hair matrix cells and in peripherally located sebaceous cells as well as epidermal cells. β-Catenin was expressed in all layers of epidermal cells and sebaceous cells, inner and outer root sheath cells, and hair matrix cells. Fibroblasts of dermal papilla were also reactive. Representative immunohistochemical expression of each marker in hair follicles is shown in Figure 2, and the expression of K1, K10, K14, p63, and β-catenin in normal pilosebaceous units is summarized in Table I.

At the end of the study, it was possible to differentiate histologically detectable lesions into cysts and tumors (Table II). In MNU-treated rats, the 75 mg/kg group tended to develop a higher incidence of cutaneous lesions compared to the group treated with 50 mg/kg as evaluated by multiplicity, but the ratio of induced lesions (*i.e.* percentage of each lesion induced by 50 or 75 mg/kg MNU, respectively) was not influenced by the dose of MNU. MNU-induced cysts were distinguished into two types, epidermal cysts and hybrid cysts. In epidermal cysts, the entire cyst wall was composed of a

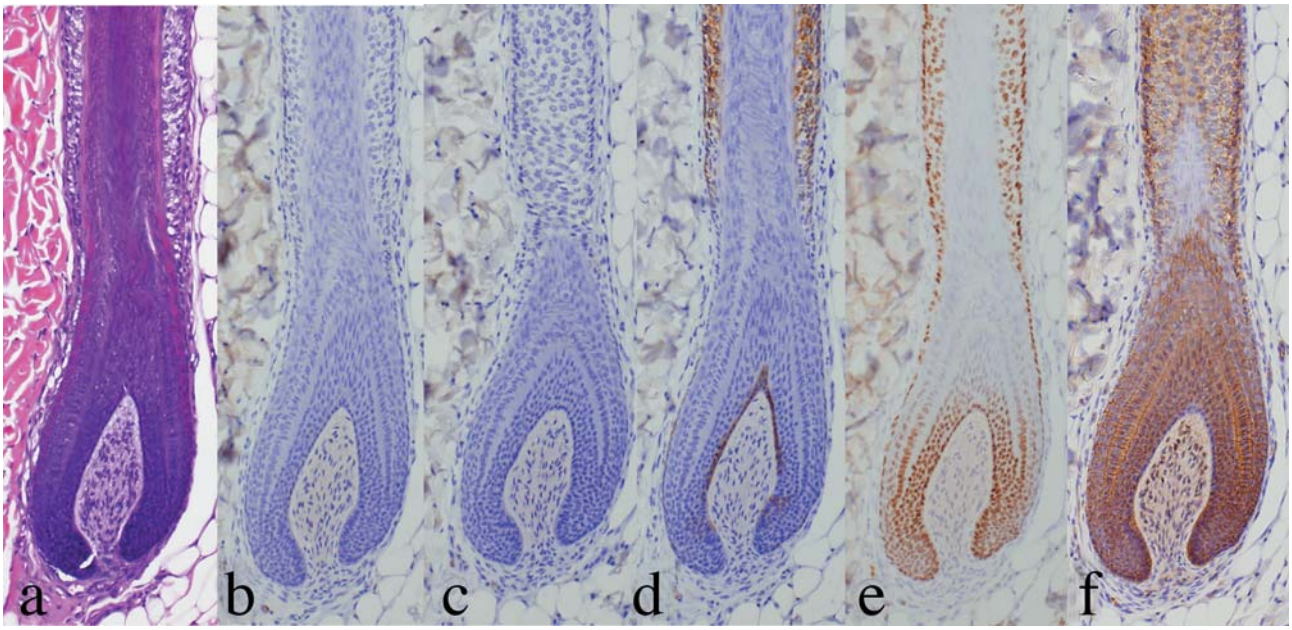

Figure 2. *a: Hematoxylin and eosin staining in normal hair follicles. Representative immunoprofiles of keratins 1 (b), 10 (c) and 14 (d), p63 (e), and β-catenin (f) in normal hair follicles (all ×100). Keratin 14 expression is apparent in the outer root sheath cells; p63 expression is visible in the outer root sheath cells; and β-catenin expression can be seen in the inner and outer root sheath cells and in hair matrix cells. Fibroblasts of dermal papilla are also reactive.*

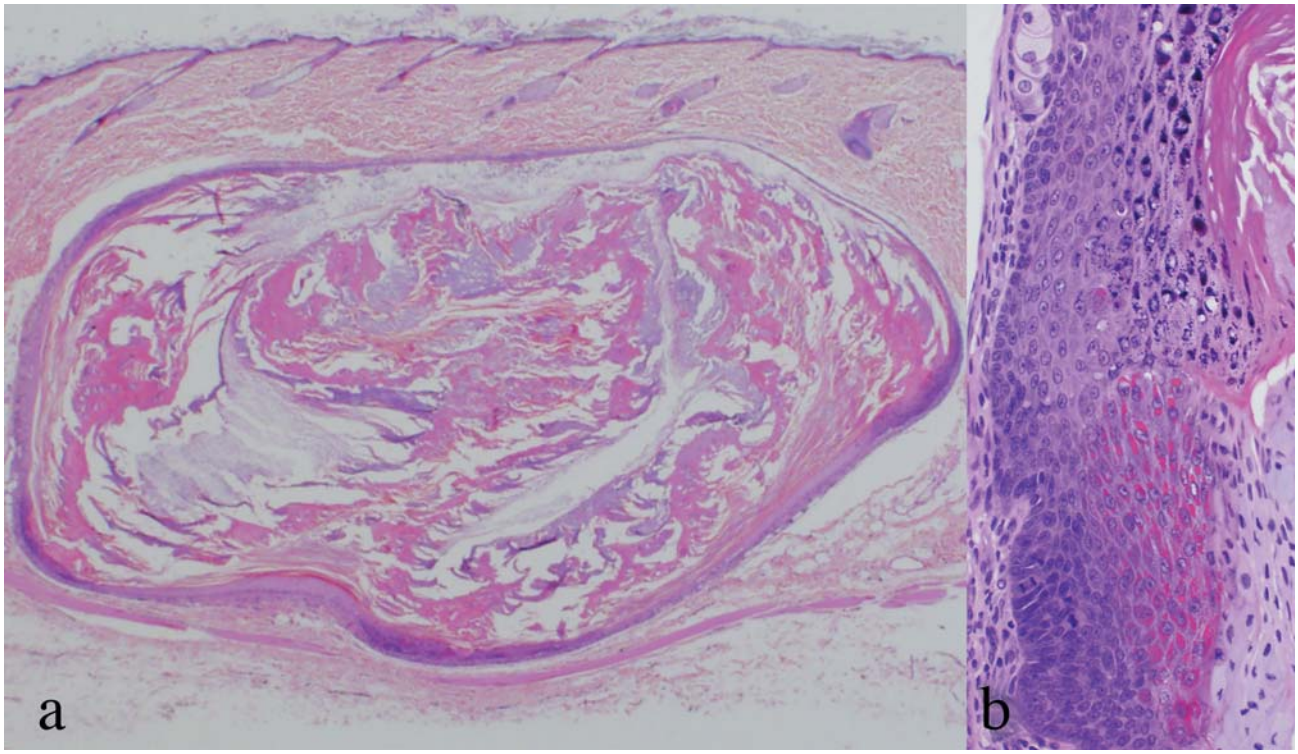

Figure 3. *Mixed epidermal and inner root sheath hybrid cyst by hematoxylin and eosin staining. a: Low-power view (×10.25) ; b: high-power view (×100). Keratohyalin granules are a component of epidermal cysts (upper portion), and trichohyalin granules are a component of inner root sheath cysts (lower portion).*

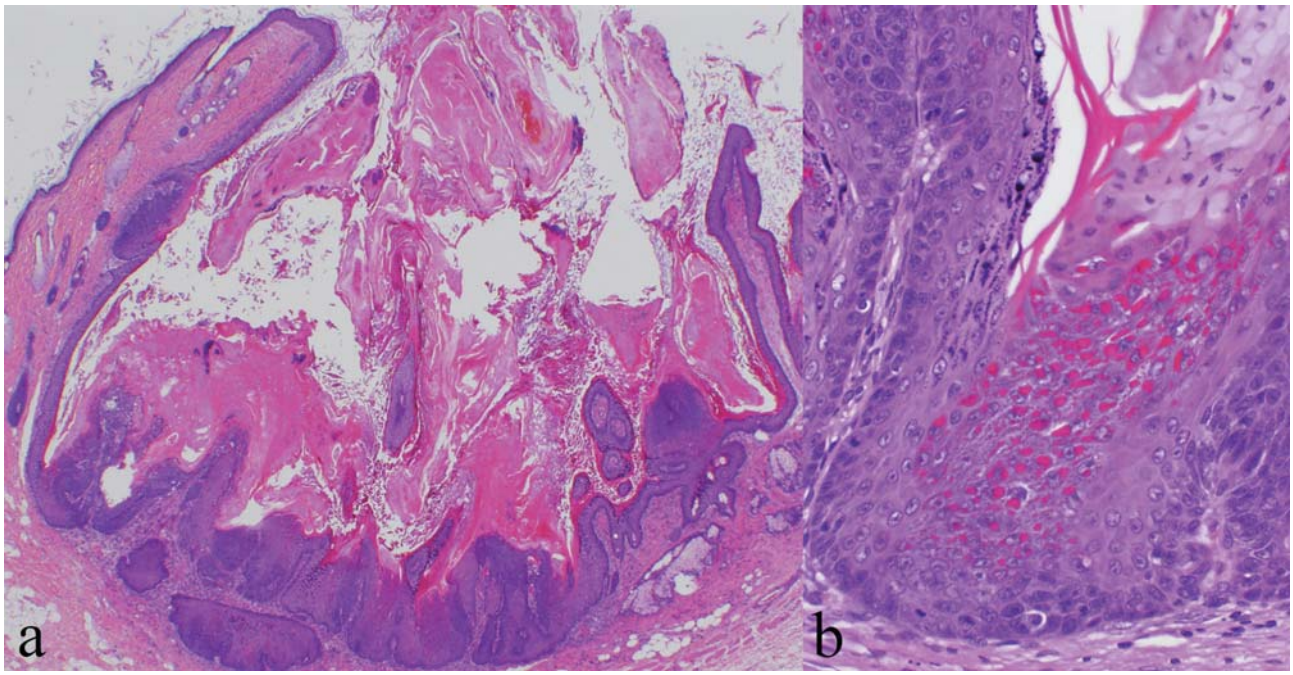

Figure 4. *Keratoacanthoma by hematoxylin and eosin staining. a: Low-power view shows dome-shaped tumor with central keratinization (×20). Epidermal lip suggestive of infundibular epithelium can be seen in the upper right corner. b: High-power view (×100). Both epithelial layers contain keratohyalin granules indicative of infundibular cells and trichohyalin granules indicative of inner root sheath cells.*

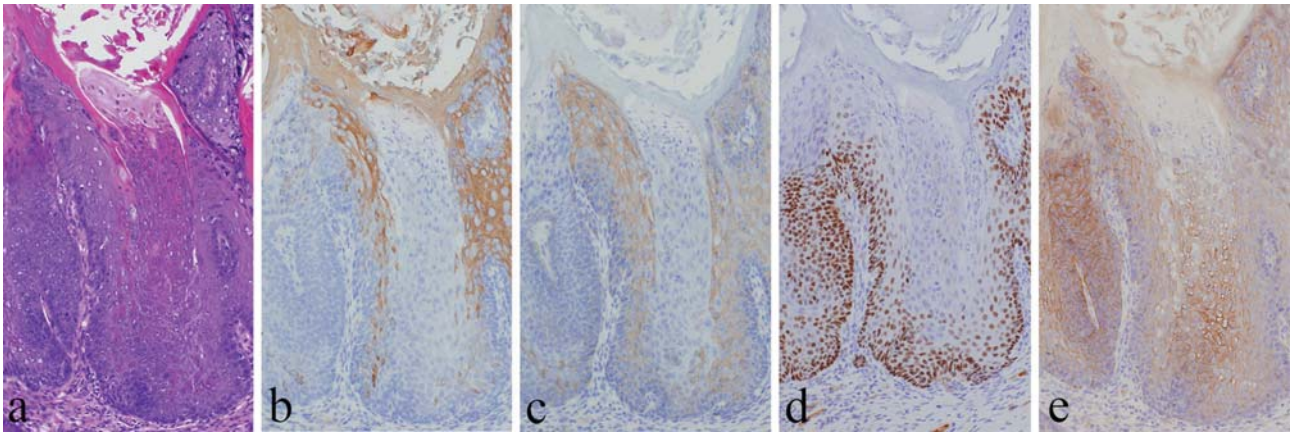

Figure 5. *Staining profiles of keratoacanthoma (all ×100). a: Hematoxylin and eosin staining. Immunostaining for keratin 1 (b), keratin 14 (c), p63 (d), and β-catenin (e). Staining patterns of trichohyalin granule-containing layer indicate inner root sheath keratinization.*

granular layer with keratohyalin granules, which produced horny material arranged in a laminated layer. In hybrid cysts, the cyst wall was composed of keratohyalin granules that abruptly changed into a cyst wall with trichohyalin granules (Figure 3). Immunohistochemically, the epidermal cyst wall was composed of K14-positive and p63-positive cells in the basal to parabasal layer and K1- and K10-positive cells in the suprabasal layer, while the trichohyalin-containing portion of the hybrid cyst lacked K1and K10 in the suprabasal layer; β-catenin labeling was seen in all parts of the epithelial layer.

The majority of MNU-induced skin tumors were keratoacanthomas with crater-shaped tumors with central keratinization incidentally accompanied by groups of sebaceous structures irregular in size and shape embedded at

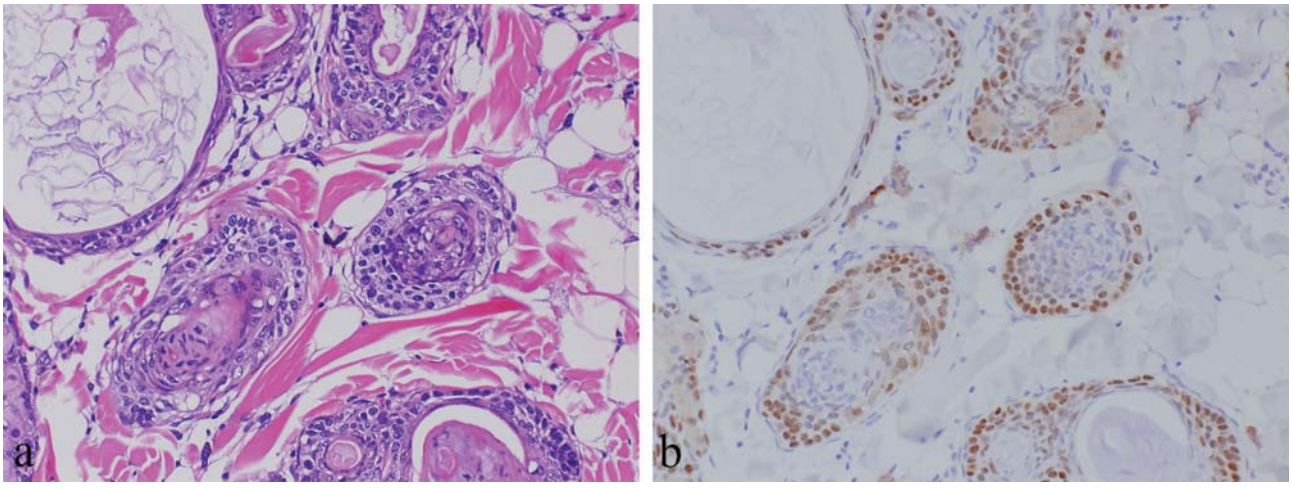

Figure 6. *Trichofolliculoma (all ×200). a: Hematoxylin and eosin staining; b: p63 immunostaining. p63 expression is restricted to germinative cells.*

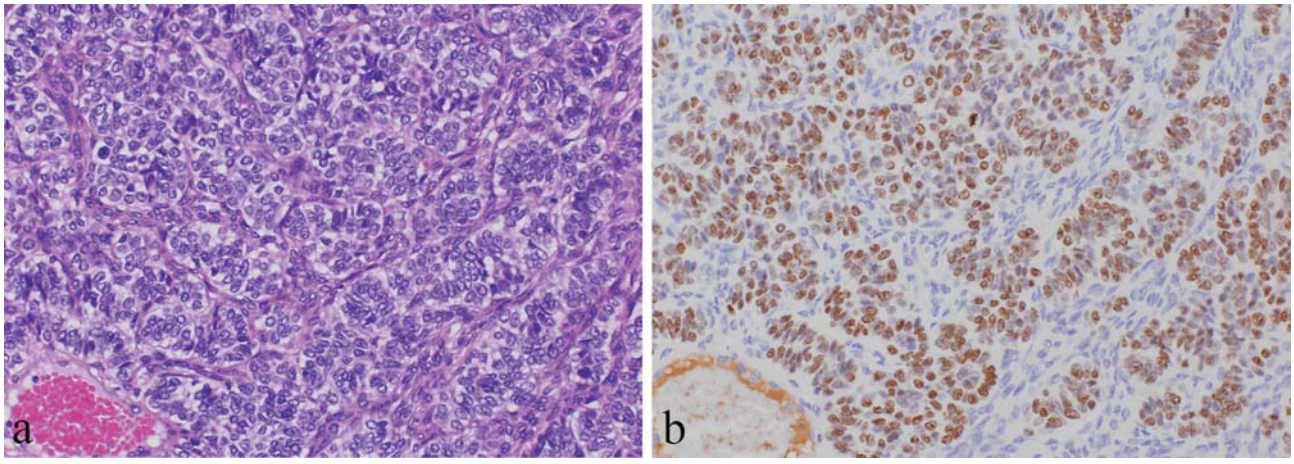

Figure 7. *Trichoblastoma (all ×200). a: Hematoxylin and eosin staining; b: p63 immunostaining. Consistent p63 expression was seen in basophilic cells.*

the periphery of the tumor cell nest (Figure 4a). Invagination of the proliferating epithelial cells comprised of two distinct morphologies. Some areas of the epithelial cell layer contained keratohyalin granules and produced a laminated keratin mass suggestive of infundibular cells (epidermal lip), while the other area contained trichohyalin granules suggestive of inner root sheath cells that converted to blue-gray corneocytes (Figure 4b). Immunohistochemically, the epithelial cell layer with keratohyalin granules was K1- and K10-positive in the suprabasal layer, and K14- and p63-positive in the basal and parabasal layer, while the cell layer with trichohyaline granules lacked K1and K10 expression, and K14 and p63 expression was restricted to the basal to parabasal portions of the epithelial layer, leaving the upper part negative (Figure 5). However, the unstained upper part was labeled with β-catenin, and the staining was similar to that of hybrid cysts.

Although low in frequency, trichofolliculoma, trichoblastoma, pilomatricoma, and sebaceous adenoma also occurred after MNU treatment. Trichofolliculoma is characterized by a dilated keratin-filled primary follicular cyst associated with a secondary follicular structure consisting of germinative cells or mature hairs (Figure 6a). Germinative cells at the periphery of the cell nest were K14 and p63 positive (Figure 6b), while β-catenin expression was not restricted to germinative cells but was also found in hair surrounding more mature cells. Trichoblastoma is characterized by a monotonous basophilic epithelial cell proliferation in cribriform, nodular, retiform, and strand form (Figure 7a). Basophilic cells

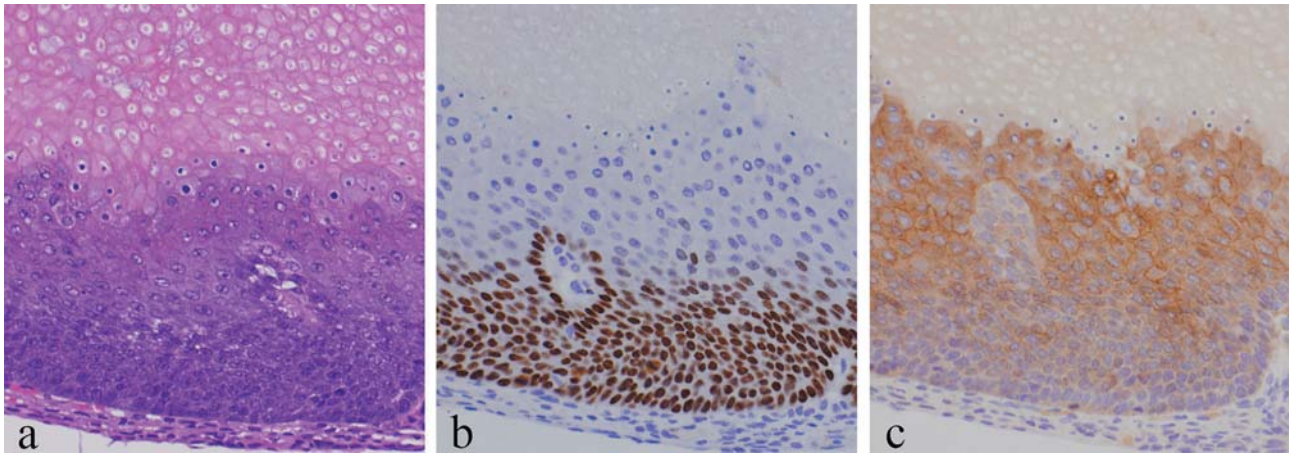

Figure 8. *Pilomatricoma (all ×200). a: Hematoxylin and eosin staining. Immunostaining for p63 (b) and β-catenin (c). Basaloid, transitional, and shadow cells are clearly distinguished.*

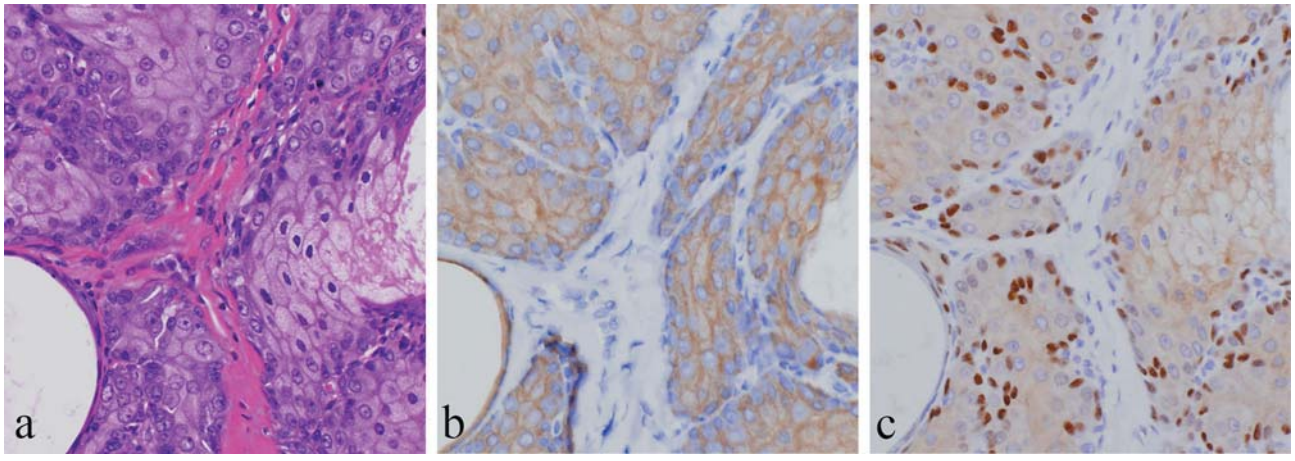

Figure 9. *Sebaceous adenoma (all ×200). a: Hematoxylin and eosin staining; b: keratin 14 and c:p63 immunostaining (each, ×200). Both sebocytes and seboblasts are labeled by keratin 14 antibody, while p63 antibody labels seboblasts only.*

germinative in nature were K14-, p63-(Figure 7b), and β-catenin-positive. Pilomatricoma differentiates towards the matrix and inner root sheath of a normal hair follicle as well as hair matrix, which consists of basaloid cells, transitional cells, and shadow cells (Figure 8a). Antibodies to the tested keratins failed to label pilomatricoma cells, whereas p63 labeled basaloid cells (Figure 8b) and β-catenin labeled basaloid cells and transitional cells (Figure 8c).

Immunohistochemistry suggested that trichofolliculoma and trichoblastoma originated from germinative cells with outer root sheath differentiation and that pilomatricoma was of hair matrix origin. Finally, sebaceous adenoma was composed of incompletely differentiated sebaceous lobules (Figure 9a). Sebaceous lobules were consistently labeled with K14 (Figure 9b) and β-catenin, and seboblasts located at the periphery were labeled with p63 (Figure 9c). The expression of K1, K10, K14, p63, and β-catenin in pilosebaceous lesions is summarized in Table I.

Discussion

MNU dose-dependently caused the formation of various cysts and tumors in male Sprague-Dawley rats. The lethal dose 50% for both oral and intravenous MNU administration in rats is 110 mg/kg (7). Although 75 mg/kg MNU administered to 4-week-old male Sprague-Dawley rats was

toxic and caused death in half of the animals, all surviving rats, including the 50-mg/kg group, had developed multiple cutaneous lesions when examined at 30 weeks of age. The carcinogenic potential of 75 mg/kg MNU was more potent compared to 50 mg/kg MNU. A 100% tumor incidence occurred in the 75 mg/kg MNU group at 18 weeks of age and in the 50 mg/kg MNU group at 23 weeks of age. We observed the rats until 30 weeks of age, and no malignant transition occurred; all induced tumors were benign pilosebaceous tumors. The keratin profile can differentiate between different epithelial cells and predict the histogenetic origins of tumors, including human pilosebaceous tumors (13). However, it is possible that there are species differences in keratin profiles. The K1, K10, and K14 labeling pattern in rat pilosebaceous units was similar to that in humans. K1 and K10 labeled the suprabasal cells of infundibular and interfollicular areas, while K14 labeled the basal and parabasal cells. K14 also labeled sebaceous cells and outer root sheath cells. Characteristically, hair papilla was sometimes covered by a single layer of K14-positive outer root sheath cells (14). Moreover, p63 and β-catenin labeling was similar to that in humans (10, 15). p63 labeled peripheral cells of sebaceous glands, outer root sheath cells, and hair matrix cells, leaving inner root sheath cells negative. β-Catenin labeled all components of cells composed of pilosebaceous units, as well as epidermal cells. Taken together, K1, K10, K14, p63 and β-catenin labeling can clearly differentiate cell components of pilosebaceous units in rats as well as humans.

In the present study, various cysts were found in the MNU-treated rats. Cutaneous cysts spontaneously occur in Sprague-Dawley rats (1). Epidermal cysts are most commonly observed, and some of the epidermal cysts have epidermal openings suggestive of a follicular infundibular origin (16). Hybrid cysts, in which the cyst wall is composed of more than one component of the pilosebaceous unit, occur in humans (17) and rodents (18). Hybrid cysts contain various combinations of superficial infundibular (epidermal) cyst, trichilemmal cyst, sebaceous cyst, inner root sheath cyst, and pilomatrical cyst (19-21). In humans, the most frequent combination is epidermal and trichilemmal cyst (17). The existence of keratohyalin granules is a hallmark of infundibular as well as interfollicular epithelium. In contrast, trichohyalin is a major structural protein of the inner root sheath cells, and the existence of trichohyalin granules indicates inner root sheath keratinization/cornification (19, 22). In the present study, in addition to epidermal cysts, hybrid cysts were commonly seen, in which the cyst wall containing keratohyalin granules abruptly changed to cyst wall with trichohyalin granules, suggestive of a combination of epidermal cyst and inner root sheath hybrid cyst. Taken together, the cysts seen in MNU-treated rats were derived from different segments of the pilosebaceous apparatus.

The majority of human keratoacanthomas occur in men (23). In Sprague-Dawley rats and Wistar rats, keratoacanthoma is the most frequent spontaneous epithelial skin neoplasm in males (1, 2, 4). As male rats were used in the present study, sex differences in MNU-induced keratoacanthoma should be further investigated. Moreover, although the role of chemical carcinogens in human keratoacanthoma is speculative, MNU is a likely carcinogen in humans (7). In the present study, almost all of the MNU-induced skin tumors in male Sprague-Dawley rats were keratoacanthomas. Keratoacanthoma induction by a single *i.p.* injection of MNU was simple, and multiple tumors developed throughout the body. Keratoacanthoma can be differentiated from cutaneous cysts by the proliferation and thickening of the epithelium lining the tumor (epidermal lip). In agreement with previous reports on human keratoacanthoma (24), K1 and K10 were expressed in the epidermal lip. Abrupt changes from a keratohyalin-containing proliferating epithelial cell layer to a trichohyalin-containing proliferating epithelial cell layer closely resemble hybrid cysts with epidermal keratinization and inner root sheath keratinization. β-Catenin-positive but K14- and p63-negative trichohyalin-containing epithelia suggest inner root sheath cells (11). Human keratoacanthoma is considered to originate from infundibulum to isthmus cells of hair follicles (24), the upper portion of outer root sheath cells (25-26), or the outer root sheath cells beneath the opening of the sebaceous duct (27). Our animal study showed a greater range of cells involved in the development of rat keratoacanthoma.

Other MNU-induced tumors, although low in frequency, were restricted to the pilosebaceous apparatus origin, and the interfollicular epidermis was free from MNU damage. In the same was as keratoacanthomas develop from cutaneous cysts, trichilemmal cysts and pilomatrical cysts are early lesions of proliferating trichilemmal tumors (28) and pilomatricoma (20-21, 29-30), respectively. Pilomatricoma begins as an infundibular matrix cyst (29). As trichilemmal cysts were hardly detectable, this may explain the lack of proliferating trichilemmal tumors in the present study. In contrast, the present pilomatricoma cases showed morphological transition from pilomatrical cysts to pilomatricomas. However, all cases were diagnosed as pilomatricoma due to their proliferative potential. In agreement with human cases (9), basaloid cells, transitional cells, and shadow cells of pilomatricoma were K1, K10, and K14 negative. However, basaloid cells were labeled by p63, and basaloid cells and transitional cells were labeled by β-catenin (31). The immunohistochemical profile of pilomatricoma indicates that the lesion is derived from hair matrix. Trichofolliculoma and trichoblastoma are formed by the outpouching of hair follicle derived from germinative cells that was differentiated toward outer root sheath cells (13, 32-35). K14, p63, and β-catenin positivity and K1 and

K10 negativity may suggest outer root sheath differentiation. Finally, sebaceous adenoma revealed a keratin profile similar to that of normal sebaceous cells.

In conclusion, the systemic administration of MNU to male Sprague-Dawley rats led to a high yield of keratoacanthoma and a few other tumors that were all of pilosebaceous origin.

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