Establishment and Characterization of a Cell Line (KU-8) from Squamous Cell Carcinoma of the Penis

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Abstract

The KU-8 cell line was established from a lymph node metastasis of human squamous cell carcinoma (SCC) of the penis. The cells grew rapidly (doubling time 20 hours) as adherent monolayers, and were tumorigenic in nude mice. The carcinoma cells showed epithelial characteristics by observation with a phase contrast microscope. The cells retained the ultrastructural characteristics of squamous epithelium including tonofilaments and desmosomes. In addition to the morphological characteristics of SCC, this cell line preserved specific molecular markers of epithelium, such as desmoplakin, cytokeratin, and involucrin, all of which were demonstrated by immunofluorescent studies. Furthermore, SCC-related antigen (SCC-RA), a tumor marker for SCC, was produced in KU-8. Moreover, immunofluorescent study showed that KU-8 cell line expressed the specific receptor of epidermal growth factor (EGF), which turned out to increase the cell growth of KU-8. These results indicate that this new cell line could provide an excellent model for the basic research and development of new therapeutic modalities of penile carcinoma.

Key words: penile carcinoma, squamous cell carcinoma, cultured cell line

Introduction

Although the penile skin comprises only a small proportion of the volume of the penis, it is the site of almost all penile neoplasms. The majority of penile carcinomas
arising from the foreskin are histologically squamous cell carcinoma (SCC). As for the etiology of penile SCC, recent interest has focused on viral infections. Additionally, SCC of the penis, when compared with other SCCs originating from various organs, is assumed to differ in its characteristics because the foreskin is androgen dependent. These unique properties of penile carcinomas demand the need for model systems for the basic study of this disease. While the number of available SCC cell lines is expanding, as for penile SCC, only two cell lines have been reported in the literature.

In this paper establishment of a new cell line derived from penile SCC and designated as KU-8 is described. To evaluate the state of cellular differentiation, specific markers for squamous epithelium were immunohistochemically identified. Moreover, the effect of epidermal growth factor (EGF) on cellular proliferation was investigated.

**Materials and Methods**

**Cell culture**

Surgical specimens were obtained from an inguinal lymph node metastasis of SCC of the penis on February 15, 1988. The specimens were rinsed in Eagle's minimum essential medium (Eagle's MEM, Chiba Serum Institute) containing 100 U/ml of penicillin and 100 µg/ml of streptomycin, and then minced by scalpel into small-size pieces of about 1 mm³. The pieces were placed in 25 cm² tissue-culture flasks and maintained in Eagle's MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, Flow Lab. Co.) and 100 U/ml of penicillin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The culture medium was renewed every 3 days. The monolayer cells were harvested by enzymatic disaggregation using 0.02% EDTA in 0.25% trypsin (Chiba Serum Institute). The viable cells excluding trypan blue were counted in a hemocytometer chamber and replated in the culture flasks.

**Cytogenetic analysis**

The exponentially growing cells were treated with 0.2 µg/ml of Colcemid for 2 hours. The cells were then harvested by trypsinization and swollen in a hypotonic solution of 0.075 M KCl at 37°C for 10 minutes. The cells were then fixed with glacial acetic acid: methanol (1:3 by volume), and dropped onto slides. The spreads were air dried and stained with 5% Giemsa.

**Tumorigenicity of cultured cells in nude mice**

Male athymic nude (nu/nu) mice (Sankyo Lab. Co.) were given subcutaneous injections of 5×10⁵ cells suspended in the culture medium. The mice were maintained under pathogen-free conditions. When the tumors grew to about 2 cm in diameter the mice were killed, and the tumors were excised and sectioned in paraffin for histochernical examination.
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Immunoradiometric assay of SCC-RA

The SCC-RA level was determined with immunoradiometric assay using on SCC RIAHEAD kit (Dinabot Ltd.). The primary antibody reagent is a monoclonal antibody reacting to human SCC-RA, which is extracted and purified from the liver metastasis of cervical carcinoma. Using this assay, SCC-RA level of 1 to 150 ng/ml can be determined.

Immunoperoxidase staining of SCC-RA

A highly specific polyclonal antibody to SCC-RA (Dinabot Ltd.) was applied as a primary antibody in an indirect immunoperoxidase method. For staining of original tumors and tumors transplanted to nude mice, formalin-fixed, paraffin-embedded specimens were deparafinized with xylene and rehydrated with graded alcohol. For staining of the cultured cells, the cells grown in Lab-Tec chambers (Miles Lab. Inc.) were fixed in -20°C methanol. The specimens were incubated with the primary antibody, and then horseradish peroxidase-conjugated antibody (Cappel Lab. Co.) was applied. Peroxidase activity was detected with 3,3'-diaminobenzidine tetrahydrochloride (DAB). The specimens were counterstained with methyl green. Specific staining was confirmed by replacing the primary antibody with normal preimmune rabbit serum.

Immunofluorescent staining

The expression of the molecular markers of epithelial differentiation and receptor of EGF were investigated by indirect immunofluorescent technique using following primary antibodies: 1) rabbit polyclonal anticytokeratin (DAKO Co.), which stained the entire epidermal layers; 2) rabbit polyclonal anti-human involucrin (Biochemical Technique Co.); 3) monoclonal antibody to desmoplakin 1 & 2 (Progen Biotechnik GBBH); and 4) monoclonal anti-EGF receptor antibody (Oncogene Science, Inc.).

For immunohistochemical staining, the cells grown in Lab-Tec chambers were fixed in -20°C methanol, and then the primary antibody was applied to the cells. After washing the cells, an appropriate second fluorescein isothiocyanate (FITC)-labeled antibody was applied. The specimens were investigated under fluoroscopy. Non-specific stainings of each specimen were assessed in parallel studies where normal serum was substituted for the primary antibody.

Results

Establishment of the cell line KU-8

Within 7 days, epithelial cells predominantly migrated from the tissue explants with some contamination of fibroblastic cells. On culture day 46 when the epithelial cells reached confluency, they were harvested by trypsinization and subcultured. A small number of fibroblasts were contaminated even at the second subculturing. The KU-8
Fig. 1 A phase contrast micrograph of the KU-8 cells showing typical epithelial cobblestone patterns (×100).

Fig. 2 Growth curve of the KU-8 cells.

Cells are seeded at $5 \times 10^4$ cells/2 cm$^2$ multiwell plate. At 24-hour intervals, the cells were harvested and counted in a hemocytometer chamber. Each point represents the average cell counts of 3 separate cultures.
cells were passaged weekly at a split ratio of 1:10, and have been serially passaged over 50 times for 12 months.

The KU-8 cells grew in a monolayer, producing colonies with epithelioid morphology, and were trigonal to polygonal in shape, with a distinct cell border when observed under a phase contrast microscope (Fig. 1).

Figure 2 indicates the cell growth curve of the KU-8 cells when they were seeded at $5 \times 10^4$ cells/2.0 cm$^2$ multiwell plate. Their growth, after an initial lag phase, was exponential up to confluency, with an approximate population doubling time of 20 hours. After reaching confluency, the carcinoma cells piled up and gradually increased their population to more than $9 \times 10^5$ cells per well. The morphology of KU-8 was further characterized by means of light and electron microscopic examinations. A colony of KU-8 stained with hematoxylin and eosin was composed of pleomorphic cells showing cytologically anaplastic changes including large and variable nuclei with conspicuous and numerous nucleoli (Fig. 3). In the electron microscopic examination desmosomes were observed between the cells, indicating the epithelial origin of this cell line (Fig. 4).

The chromosomal analysis at the 8th passage was completely aneuploid human karyotype with a range of 65 to 76 and a modal number of 70, 71 (Fig. 5).

Nude mice given injections of the KU-8 cells developed tumors at the injection

![Fig. 3](image.png)

*Fig. 3* Light micrograph of a KR-8 colony. The cells contain large nuclei with prominent, multiple nucleoli. Mitotic figures are frequently observed (H & E ×100).
Fig. 4  Electron micrograph of the KU-8 cell line shows occasional microvilli on the cell surface. Desmosomes (arrow) are frequently observed between the cells (×2,000): inset. higher magnification of the desmosome (×30,000).

site. The morphology of the resulting tumors sectioned in paraffin and stained with hematoxylin and eosin is shown in Fig. 6. There were diffuse sheets of neoplastic cells which themselves showed great variation in size and shape, darkly stained nuclei with prominent nucleoli, and atypical mitotic figures. The tumors contained areas of maturation where parakeratotic horny pearls were seen. In the electron micrographs, the nude mouse tumor cells were seen to be rich in tonofilaments and to have occasional endoplasmic reticulum profiles. The mitochondria were sparse, and Golgi apparatus poorly developed. The cells were attached to neighboring cells by the desmosome (Fig. 7).

Localization of involucrin, cytokeratin, and desmosome

Monolayer cells reacted with desmoplakin antibodies indicated that definite staining could be seen along the cell borders in a linear pattern. There seemed to be little difference in the staining intensity. In addition to the dense staining of cell borders, weak ones were seen within the cytoplasm (Fig. 8a).
All of the KU-8 cells presented immunoreactivity with the anticytokeratin antibodies. Immunofluorescent staining demonstrated the fine filamentous network in the cytoplasm with some difference in staining intensity (Fig. 8b).

The majority of the cultured cells were unstained by involucrin, although a few cells indicated homogeneous intracellular staining (Fig. 8c).

Production of SCC-RA in KU-8

The cells seeded at $5 \times 10^4$ cells/2.0 cm$^2$ multiwell plate were harvested and counted every 24 hours. The SCC-RA level of the culture medium was also determined at the same interval. In the semiconfluent phase on day 5 when the cells counted $4 \times 10^5$ cells/well, they started secreting the detectable range ($>1.0$ ng/ml) of SCC-RA. During confluency the cells kept producing an increasing amount of SCC-RA up to 26.9 ng/ml on day 10 (Fig. 9).

The immunohistochemical study for localization of SCC-RA in the cultured cells revealed that staining was diffuse in appearance and always confined to the cytoplasm.
Fig. 6 Representative histological appearance of nude mouse tumor showing pearl formation (H & E ×50).

Fig. 7 Electron micrograph of nude mouse tumor. The cells show numerous tonofilaments (arrow) within the cytoplasm. Desmosome-tonofilament complexes (arrowhead) are frequently observed (×5,000).
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a) Staining for desmoplakin demonstrates a linear pattern along the cell borders and weakly stained cytoplasm (×100).

b) Immunofluorescent staining for cytokeratin demonstrates the fine filamentous network in the cytoplasm (×100).

c) Homogeneous cytoplasmic staining for involucrin is occasionally observed (×200).

Fig. 8 Localization of differentiating antigens in the KU-8 cells by indirect immunofluorescent method.
The serum level of SCC-RA in tumor-bearing nude mice was significantly higher (130 ng/ml; n=3) than the control (2.5 ng/ml; n=2). The immunoreactivity for SCC-RA antibodies was definitely seen in the tumor cells (Fig. 10b).

The serum level of SCC-RA of the patient from whom KU-8 was derived also elevated from 3.8 ng/ml to 13.8 ng/ml, as the disease progressed. The immunohistochemical study with the SCC-RA antibodies also showed positive in the original tumors (Fig. 10c).

Effect of EGF on cell growth

To investigate the effect of EGF on cellular proliferation, the carcinoma cells plated at $5 \times 10^4$ cells/2.0 cm$^2$ multiwell plate were incubated with or without 10 ng/ml of EGF extracted from mouse submaxillary gland (Collaborative Research, Inc.). The cell were harvested and counted in a hemocytometer chamber at 24-hour intervals.

The addition of EGF to the medium resulted in enhancement of the proliferation of KU-8. When incubated with 10 ng/ml of EGF the cells reached the confluent phase approximately 2 days earlier than did those with the control medium (Fig. 11). The immunohistochemical study with the monoclonal antibody to EGF receptor revealed its localization at the cell surface of KU-8 (Fig. 12), whereas the cell lines derived from renal cell carcinoma (KU-215), bladder carcinoma (KU-716), and prostatic carcinoma (PC-317) showed negative (data not shown).
Fig. 10. Indirect immunoperoxidase staining with anti-SCC-RA antibody. Reactive products are distinctly observed in cultured cells and tumor cells.

a) Cultured cells (x100).
b) Nude mouse tumor (x50).
c) Original tumor (x50).
Fig. 11  The effect of EGF on KU-8 growth.
The cells seeded at $5 \times 10^4/2 \text{ cm}^2$ well were incubated in the presence (-----) or absence (----) of 10 ng/ml of EGF. Values are the means of three determinations.

Fig. 12  Immunofluorescent micrograph of the KU-8 cells stained for EGF receptor.
The KU-8 cells grown in Lab-Tec chambers were reacted with monoclonal antibodies against EGF receptors. The EGF receptors are labeled along the cell boundaries ($\times$200).
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Discussion

Adequate surgical treatments for localized penile carcinomas can produce a 5-year survival rate of 70–80%,\(^1,18\) advanced counterparts, however, are refractory to currently available therapeutic modalities such as chemotherapy and radiation therapy,\(^1,18\) indicating the need for a new approach in treatments. It is well known that progress toward a better understanding and successful management of malignant disease depends significantly on laboratory experiments with appropriate model systems utilizing malignant cell lines. As for penile carcinoma, however, only 2 cell lines have been reported,\(^6,7\) reflecting the low incidence of penile carcinomas\(^18\) and difficulty in obtaining sterile surgical specimens because of concomitant infection commonly observed with penile carcinomas.\(^7\)

In the present study a cell line derived from a metastatic node of human penile SCC was established and designated as KU-8. The evidence that the KU-8 cell line originated from human penile carcinoma tissue was provided by karyotype analysis, morphological observation, and identification of differentiating molecular markers of the epithelium.\(^19\) In the previous two reports, recognition of the origin of the cell lines was simply based on findings of their morphological characteristics as observed under light and electron microscopy.\(^6,7\) Morphological characterization of cultured cell lines by light and electron microscopy is well established means to identify their origin, however, has certain shortcoming.\(^10,19\) Recent progress in cell and molecular biology has disclosed the presence of several molecular markers specific for a certain tissue,\(^8-10\) and studies on such markers should provide a valuable non-morphological criterion for identifying the origin of cell lines.\(^8-10,19\) In the present study expressions of three markers were investigated in KU-8. Cytokeratin, a part of the cytoplasmic cytoskeleton, is consistently expressed in all epithelial cells.\(^8\) Desmoplakin is a biochemical composition of desmosome, which is a very characteristic structure of epithelial cells.\(^9\) Both are thought to be reliable markers for identifying the epithelial nature.\(^8,9\) Studies of cell culture have shown that the synthesis of these two markers is influenced by the growth state and that these markers are not necessarily coupled in their synthesis.\(^9,20\) Therefore, Moll et al. recommend the use of both markers to confirm the epithelial nature of a given cell line.\(^9\) Involucrin is known to be expressed during terminal differentiation of squamous epithelium and is therefore thought to be a specific marker for squamous epithelium.\(^10\) The evidence that these three markers could be positively identified in KU-8 indicated that this cell line originated in SCC.

Late in the terminal differentiation of the squamous epithelium, an insoluble protein envelope, the cornified envelope, is synthesized immediately beneath the cellular plasma membrane.\(^10,21\) Involucrin is a major structural subunit of this envelope,\(^10,21\) and is immunohistochemically first recognized in the cytoplasm, then becomes localized in
the periphery of cells as keratinization of the cell progresses. Recent studies have shown that the expression of involucrin is altered or markedly reduced in neoplastic conditions, indicating that this molecular marker reflects the cellular state of squamous differentiation. In this study the presence of involucrin in KU-8 proved the ability of this cell line to undergo squamous differentiation. Because of the homogeneous staining of involucrin within the cytosol, however, the KU-8 cells were thought to be in the process of terminal differentiation.

SCC-RA, a glycoprotein extracted and purified from a metastatic lesion in the liver from cervical carcinoma, is a useful marker for this disease. Since it is also raised in SCC of the lung, esophagus, and head and neck, this marker has attracted considerable attention as a useful tumor marker of SCC. In view of the fact that there is currently no clinically useful tumor markers available for penile carcinoma, the pursuit of such markers is important. The serum level of SCC-RA in the patient from whom this cell line was derived increased as his disease progressed and reached as high as 13.8 ng/ml. The serum level of SCC-RA of tumor-bearing nude mice also increased approximately 70-fold over those without tumors. In addition, this marker was also detected in the culture medium after the cells reached confluence. Immunohistochemically SCC-RA could be positively identified in the cytosol of KU-8. However, the expression of SCC-RA could not be detected in the cell line derived from the bladder carcinoma (KU-7), the renal carcinoma (KU-2), or the prostatic carcinoma (PC-3) (data not shown). These findings clearly indicate that SCC-RA was produced in KU-8. Therefore, usefulness of SCC-RA as a reliable tumor marker for penile carcinoma is strongly supported.

EGF stimulates the proliferation of a variety of cell types, especially epithelial cells by binding to a specific cell surface receptor. In the present study KU-8 was proven to have EGF receptors on its cell surface as determined by immunohistochemical method, and the proliferation of the cell was shown to be stimulated by EGF, suggesting that EGF could be involved in regulation of the proliferation of KU-8. In recent years, there have many reports dealing with immunotherapy against carcinomas, using monoclonal antibodies. Masui et al. have reported that monoclonal antibodies against the EGF receptor can block the access of EGF to their receptors on tumor cell surface membranes and prevent the growth of some cultured tumor cells bearing these receptors. As for advanced penile carcinomas, the lack of currently effective treatments necessitates development of new approach in the treatments. The immunotherapy with the anti-EGF receptor monoclonal antibodies, even though preliminary, is thus attractive as a potential alternative approach to this disease.

The foreskin and prostate are known to be androgen-dependent. With prostatic carcinoma, antiandrogen treatment is well established as an effective treatment. However, no such progress as made in the treatment of prostatic carcinoma has not been
made against penile carcinoma, and future study is needed in this regard. The presently established cell line can thus serve as an ideal *in vitro* and *in vivo* model for basic studies of entirely new treatment modalities such as immunotherapy and antiandrogen therapy for this disease.

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