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Ji, Zheng-Wen
Oku, Naohisa
Komori, Takahide

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ESTABLISHMENT OF A NOVEL HUMAN SQUAMOUS CELL CARCINOMA CELL LINE FROM ORAL PRIMARY TUMOR BY GENETICIN TREATMENT

Zheng-Wen JI, Naohisa OKU, and Takahide KOMORI

Department of Oral and Maxillofacial Surgery,

Kobe University School of Medicine

KEY WORDS

human SCC cell line; head and neck cancers; epithelial cells; xenograft tumors

ABSTRACT

A novel cultured cell line NOS-1, derived from a human oral primary squamous cell carcinoma (SCC) of the lower gingiva, was established without xenografting the tumor into nude mice by means of "Geneticin" treatment, which allowed for elimination of contaminated fibroblasts and produced enriched tumor cells at an early stage of the culture. NOS-1 cells showed numerous desmosome structures and some intermediate filaments. To determine tumorigenicity and to establish an orthotopic tissue invasion model for oral carcinoma, the NOS-1 cells were injected into the back and the tongue of male athymic nude mice. The back tumors showed an expansive growth pattern without significant invasion of surrounding tissues, while the tongue-implanted tumors exhibited invasive growth. The establishment of a novel oral primary tumor cell line and a new orthotopic tissue invasion model is expected to be useful for the study of biological characterization and for the identification of the invasive mechanism of human oral cancers.

INTRODUCTION

Establishment of cancer cell lines has been the subject of intensive efforts over the past 30 years since cell lines are extremely important for basic molecular research as well as for diagnostic and therapeutic development. However, the establishment of human oral-carcinoma cell lines has been mostly very difficult and few successful attempts have been reported.[1-3] Moreover, most cancer cell lines have been established from the metastatic foci of lymph nodes, the pleural cavity and tumor cells transplanted into the subcutaneous tissue of the back of nude mice.[2, 4-8] Thus, there are not enough cell lines for research on oral primary tumors. Moreover, cell lines derived from recurrences and metastases appear to be less differentiated, less well organized in culture, and demonstrate a

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morphological divergence displaying fewer desmosomes and tonofilaments than cells in primary tumor lines.[9] Therefore, the establishment of many cell lines from a primary tumor is regarded as desirable for obtaining a cell line which retains the characteristics of the primary tumor. However, one of the major problems with a successful culture is the overgrowth of host fibroblasts.[10] We have applied "Geneticin treatment" for the selective elimination of fibroblasts to establish a new cell line from oral primary tumors.

This report describes the biological and molecular characterization of a cell line established from an oral primary tumor without xenografting into nude mice.

MATERIALS AND METHODS

Cell culture and geneticin treatment

A new tumor cell line designated as NOS-1 was established by using a squamous cell carcinoma (SCC) of the right lower gingiva from a 47-year-old Japanese male patient who underwent surgery at Kobe University Hospital. Fresh primary tumor tissues were obtained at operation of the patient with untreated primary oral SCC (Stage IV). After the surrounding fat, necrotic tissue, connective tissues and hemorrhagic regions were carefully removed, the tumor tissue was rinsed three times with phosphate buffered saline (PBS) to minimize microbial contamination, and then rinsed three times in serum-free GIT medium (Nihon Seiyaku, Tokyo, Japan).[11] which contained penicillin G (100 U/ml: Meiji Seika, Tokyo, Japan), streptomycin (100 mg/ml: Meiji Seika), and fungizone (5μg/ml: GIBCO, NY, USA). The tumor tissue was minced with scalpels into small cubes, 1-3 mm in size. Neither enzymatic nor mechanical dissociation of the tumor cells was performed. The dispersed tumor cells were then suspended in medium on collagen-coated 60-mm plastic dishes (Corning, Michigan, USA), which were placed inside 90-mm dishes to avoid bacterial contamination in the primary culture. The medium for the primary culture comprised GIT medium containing antibiotics, as well as antimycotics for 2 to 5 passages. For cell characterization, the cell line was maintained in GIT medium containing antibiotics. The cells were then kept at 37°C in a humidified atmosphere of 5% CO_2 in air, and the medium was replaced every 3 days. Fibroblast overgrowth was controlled by cell scraping and selective chemical elimination with the antibiotic agent, Geneticin (G418 sulfate, GIBCO, NY, USA).[12, 13] The Geneticin treatment (100μg/ml) was repeated until all fibroblasts had been eliminated. When the epithelial cells in the dishes had reached 75 to 100% confluency, they were sub-cultured in GIT medium with an initial split ratio of not more than 1:2. This operation was repeated for 2 to 7 passages. The cultured cells were passaged twice a week by means of trypsinization for one year after the initial cultivation. This line was considered "established" (capable of being routinely passaged) after about one year in culture.[6]

Light- and electron-microscopic examination

Unstained cultured cells were photographed with a phase contrast microscope. Cells cultured directly on Lab-Tek chamber slides (NUNC, Roskilde, Denmark) were fixed with 2% glutaraldehyde, post-fixed in 1% osmium tetroxide (OsO_4), and dehydrated through a series of graded alcohols. Tissue samples were embedded in Epon 812 (Nissin, Tokyo, Japan) with propylene oxide, cut into thin sections and examined with an electron microscope H-600A (Hitachi, Hitachinaka, Japan).

Growth curve and colony formation in soft agar

Cells were plated in duplicate at 1 x 10^5 cells in a 35-mm Petri dish and counted on days 0, 1, 3, 5, 7 and 9; doubling times were calculated from the exponential growth phase.

Anchorage-independent growth was assayed by suspending known numbers of log phase cells in 1.0 ml of 0.3% agar (bact agar; Difco, Detroit, USA) over a 0.5% agar base. The agar was supplemented with GIT medium and the number of cells per dish was varied from 5 x 10^3 to 1 x 10^4. Colonies were grown for 14 to 21 days and the dishes stained with vital stain trypan blue before counting. A colony was defined as a cluster at least 60 μm in diameter.

Immunohistochemical analysis

An LSAB kit (DAKO, Kyoto, Japan) was utilized to examine paraffin-embedded samples from orthotopic tumors in nude mice for keratin and vimentin immunolocalization. Diaminobenzidine was used as the chromogen for peroxidase reaction, and the IgG fraction of normal rabbit serum was used as negative control. The antibodies employed were polyclonal anti-cytokeratin for human keratin and V9 for vimentin (DAKO).

Tumorigenicity and establishment of a new orthotopic tissue invasion mode

To determine tumorigenicity and to establish an in vivo local invasion model for oral carcinoma, 10^7 viable cells /0.025 ml in culture medium per mouse were injected into the tongue and the back of male athymic nude mice (6-week old Balb/c nu/nu mice) kept under laminar-flow hoods and under pathogen-free conditions. When the tumors had reached a size of between 0.5 and 1 cm in diameter, they were removed and examined histologically.

Chromosome analysis

Two hours after treatment with colcemid (0.1 μg/ml), mitotic shake-off yielded highly condensed chromosomes. The cells were treated with 0.075 M KCl, 10% sodium citrate and glacial acetic acid.[14] Several slides were stained with Giemsa for chromosome counting. The modal numbers of minimum of 50 metaphase spreads were counted and at least three G-banded karyotypes were generated for this cell line.

RESULTS

Establishment of a cell line by means of Geneticin treatment

We succeeded in directly establishing the cell line designated as NOS-1 from the oral primary tumor without employing transplantation into the back of nude mice. Approximately 5 days after the primary culture, contaminating fibroblasts had started to increase. And 5 days after that, the subculture was performed. In the third passage, epithelial cells became dominant, but because contaminating fibroblasts were observed in the 6th passage, we performed selective elimination by using three consecutive Geneticin treatments (100μg/ml with 2 days exposure; Fig. 1).[13] Epithelial cells with a cobblestone pattern were growing in a dominant manner. At present, the number of passages of NOS-1 has reached 150.

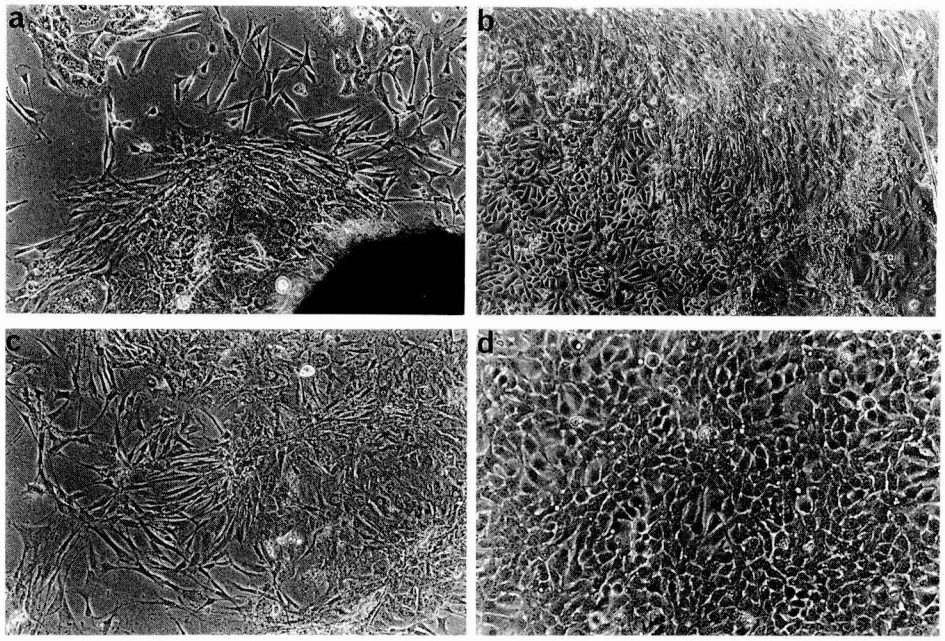

Fig. 1. Effect of Geneticin exposure on contaminating fibroblasts.
(a) First passage (b) Third passage: sudden overgrowth of fibroblasts (c) Sixth passage: immediately after Geneticin exposure (100 μg/ml for 2 days) (d) Eighth passage: after selective elimination by Geneticin treatment. Original magnification: x 100.

Morphological and growth characteristics of the cell lines

Fresh primary tumor tissues were obtained at operation from a patient with untreated primary oral squamous cell carcinoma (SCC) (Fig. 2a). This line started to grow in the form of adherent monolayers with characteristic cobblestone epithelial morphologic features (Fig. 2b). Electron microscopic examination showed many desmosomes, atypical nuclei and some intermediate filaments (Fig. 2c, 2d). Growth characteristics are summarized in Table I. The NOS-1 cells grew loosely, and adhered to the surface of the plastic culture dishes (Fig. 2b). The doubling times of the 50th passage was 15.7 and, that of the 100th passage was 16.1 hours, while the respective colony-forming efficiencies in soft agar on plastic were 0.83 and 0.99 percent.

Immunohistochemical analysis

Immunohistochemical studies confirmed the epithelial origin of the cells, which stained positive for keratin and negative for vimentin.

Tumorigenicity and establishment of a new orthotopic tissue invasion model

This line was tumorigenic both in the heterotopic transplantation in the back and the orthotopic transplantation to the tongue of athymic nude mice. The back and the tongue tumors grew slowly and reached

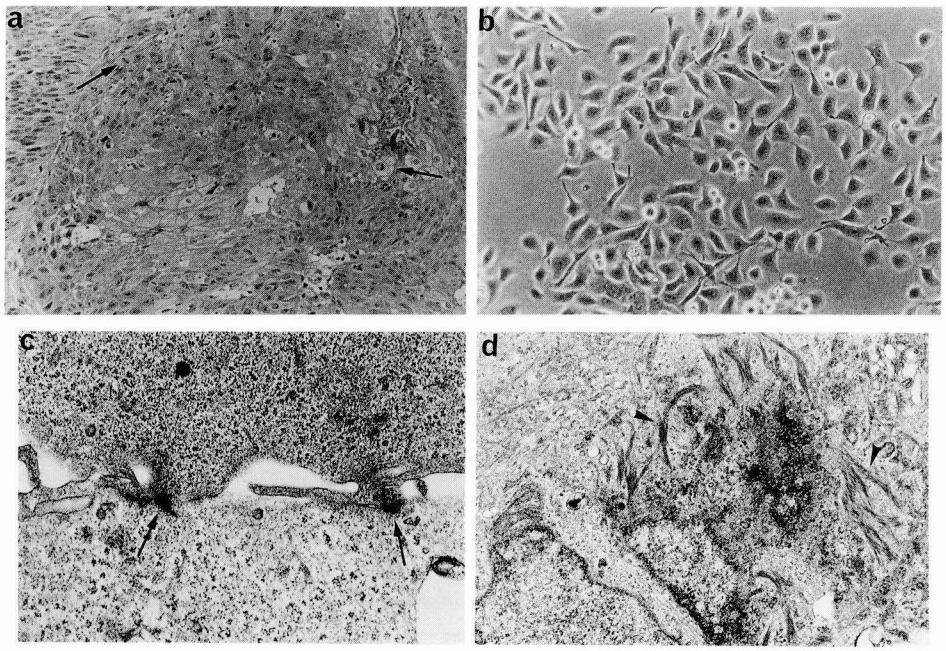

Fig. 2. Histopathologic findings of established culture cell NOS-1.

(a) Histopathologic micrographs taken from oral biopsy specimens. Arrows show mitotic figures.

(b) Cell grown in GIT medium after 150 passages. Cells attached with bipolar and tripolar processes.

(c) Arrows indicate desmosomes.

(d) Electron micrograph of culture cells after 50 passages. Some intermediate filaments (arrowheads) are seen in the cytoplasm.

Original magnification: (a) × 100; (b) × 100; (c) ×10,000; (d) × 8,000.

Table I. Characteristics of established cell line.

Cell line	Passage*	Doubling time (hr)**	CFE (%) 1×10^4	Tumorigenicity 1×10^7 S.C.	Tongue
NOS-1	50	15.7	0.83	4/6	3/6
	100	16.1	0.99	4/6	4/6

CFE: colony-forming efficiency
S.C.: subcutaneous implantation
*Assessed for cells during the exponential phase of growth.
**Number of passages in December 1999.

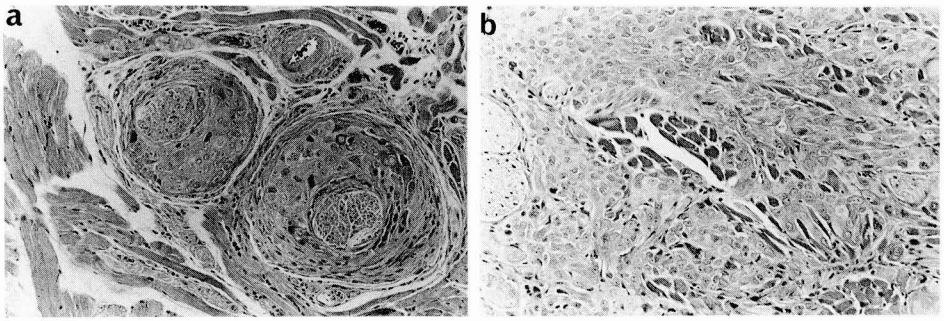

Fig. 3. Histopathologic findings for a xenograft of NOS-1.

(a) The subcutaneous tumors were encapsulated by fibrous tissue without invading neighbouring tissues.

(b) The orthotopically transplanted tumors showed invasive growth with various round tumor nests in one month or less.

Original magnification: (a) × 100; (b) × 100

diameters ranging from 3 to 9 mm after less than 1 month. Histological analysis of the tumors showed moderately differentiated SCC. The back tumors remained encapsulated with fibrous tissue and did not invade neighboring tissues (Fig. 3a), while the orthotopically transplanted tumors showed an invasive growth pattern with various round tumor nests (Fig. 3b). The fibrous stromal reaction was poorly developed, and many infiltrating mononuclear cells were observed around the tumor nests. The mode of invasion of these tumors was similar to that of the original tumor.

Chromosomal analysis

NOS-1 was nearly hypotriploid (range: 55 to 60) with a modal chromosome number of 59 (Fig. 4).

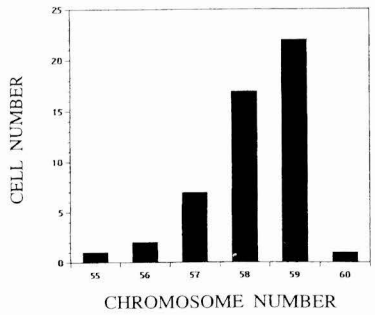

Fig. 4. Distribution of number of chromosomes of NOS- 1 cells. Cells were shown to be heterogenous with chromosome numbers ranging from 55 to 60 with a modal number of 59.

DISCUSSION

The establishment of SCC cell lines is considered to be difficult and low success rates have been reported: at most three cell lines out of 100 attempted have been successfully established.[1-3] Moreover, these cell lines include those established from metastatic foci from lymph node, pleura, and tumor cells transplanted into the back of nude mice.[2,4-8] Cell lines established from recurrences and metastases appear to be less differentiated. With a less organized culture, and with a morphological divergence displaying fewer desmosomes and tonofilaments than cells in the primary tumor line.[9]

We succeeded in establishing the human SCC cell line NOS-1 from oral primary tumor more efficiently than previously reported.[1-3] Our success appears to be the result of precautions taken in the following areas: (a) isolation of pure tumor cells, (b) disinfection of the surgical specimens, (c) protection from bacterial and fungal contamination and (d) control of the overgrowth of fibroblasts in culture dishes. The last precaution in particular constitutes a major difficulty in the establishment of epithelial cell lines. Our group developed a useful method to overcome this difficulty by using selective-growth control of fibroblasts treated with serum-free GIT medium and their selective elimination with antibiotic Geneticin (G418 sulfate), instead of trypsin treatment, assisted by mechanical scraping. The optimal condition for selectively eliminating normal fibroblast cells from malignant cancer cells was attained with 100μg/ml and 2 days exposure. The mechanism of selective elimination probably depends on differences in chemosensitivity.[13] With the aid of this technique, we succeeded in establishing the human SCC cell line NOS-1 from oral primary tumors without xenografting to nude mice. The growth characteristics of NOS-1, including doubling time and colony-forming efficiency in soft agar, were similar to those of other SCC cell lines from Japanese patients.[5,6,15] NOS-1 grew in the form of an adherent monolayer with epitheloid morphologic features. Cells were polygonal and roundish and showed a cobblestone pattern-like arrangement (Fig.2b). Currently, NOS-1 has been kept in serum-free medium for more than 2 years. This serum-free culture with a chemically defined medium constitutes quite a significant advance in the inhibition of fibroblast overgrowth and in the detection of factors that could affect the growth of tumor cells and substances elaborated by the tumor cells themselves.[16]

The importance of orthotopic implantation has recently been reported for other affected areas.[17] However, few reports deal with orthotopic invasion models using human oral SCC cells.[18] It is noteworthy that NOS-1 cells, which showed no invasive growth when implanted subcutaneously and heterotopically, showed invasion into the surrounding tissue, when they were implanted into tongues of nude mice, in a manner similar to that observed in the original tumor. Nakajima et al.[19] also observed differences in the production and secretion of extracellular matrix-degrading enzymes in tumors growing in different organs: human colon carcinoma cells implanted orthotopically expressed metastatic ability and high extracellular matrix-degrading enzyme activities. In the study presented here, orthotopic implantation was also found to be an important factor for reproducing local tissue invasion. This orthotopic tissue invasion model therefore proved itself useful for the study of the mechanisms of tissue invasion in human oral cancer.

Varying numbers of chromosomes are a common finding in human tumors. The number of chromosomes of the NOS-1 cell line varied within a hypotriploid range between 55 and 60 with a modal number of 59.

The establishment of a cell line from an oral primary tumor is expected to provide a useful model for the study of the biological and molecular characterizations and mechanisms of local tissue invasion in human oral cancers.

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