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Establishment and Characterization of a Human Cell Line Derived from a Squamous Cell Carcinoma of the Tongue

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SUMMARY

A cell line designated as OSC-19 was established from the metastatic tumor which was found in a cervical lymph node of a male patient suffering squamous cell carcinoma (SCC) of tongue. OSC-19 cells were polygonal in shape and grew in a cobblestone pattern. Desmosomes and microvilli were observed by electron microscopic examination, but tonofilament bundles were scarce. However, immunofluorescence studies showed cytokeratins in the cytoplasms of OSC-19 cells. When inoculated into nude mice, OSC-19 cells had many distinctive desmosomes and plenty of tonofilament bundles. These observations suggested strongly that OSC-19 cell line was SCC in origin. OSC-19 cells had receptors of epidermal growth factor (EGF), but the growth of the cells in dish culture was inhibited by 1 to 100 ng/m*l* EGF in dose-dependent manners. OSC-19 cells could not grow in soft agar, suggesting that they were strongly anchorage dependent. However, in contrast to dish culture, EGF stimulated colony formation of OSC-19 cells in soft agar. These results suggested that EGF has complex effects on the cell growth.

Key words: Squamous cell carcinoma, Cell line, Epidermal growth factor

INTRODUCTION

Squamous cell carcinoma (SCC) is the most common of human cancers of the oral cavity, representing over 90% of all oral cancers(3). The establishment of cell lines from human cancers is important for investigating the biology of cancer

* Abbreviations: SCC, squamous cell carcinoma; EGF, epidermal growth factor; HBSS, Ca^{++}, Mg^{++}-free Hanks' balanced salt solution; DME, Dulbecco's modified Eagle's medium; F12, Ham's F12; BrdU, bromodeoxyuridine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; NP-40, Nonidet P-40.

cells at the cellular level. However, SCCs including the oral origin are generally difficult to propagate in culture, though the reasons for this are obscure, and relatively few cell lines have been established from the head and neck SCCs(4, 9, 10, 22, 28, 29, 31, 32). These cell lines have been used for in vitro studies which include the secretion of proteinases(1), the resistance to anti-tumor agents(16), and the effects of retinoid(23).

EGF is a polypeptide originally isolated from submaxillary glands by Cohen(7), and it stimulates the growth of the wide variety of cultured cells(6, 15). However, evidence has been accumulating that the growth of various cell lines is often inhibited by EGF(2, 12, 13, 18, 20, 21). Among these cell lines, A-431 cell line derived from human vulva has been widely used, because it possessed a high amount of EGF receptor. The present paper describes the establishment of a new cell line from a tongue SCC, and the effect of EGF on this cell line. A preliminary report of the results has been presented(34).

MATERIALS AND METHODS

Clinical course

A 61-year-old man was admitted to the hospital with complaint of a painful mass on the right lateral margin of his tongue. The histopathological examination of the biopsy specimen revealed a well differentiated SCC. The patient was treated with the chemotherapy followed by the total resection of the tumor. Six months after the operation, he readmitted because of the appearance of a large lump on the right side of the neck. A right radical neck dissection was performed after chemotherapy. A diagnosis of metastases of a poorly differentiated SCC was made. A portion of the tumors was used for culture. The patient died 3 months after the second operation in spite of radiation therapy.

Establishment of a cell line

Surgical specimen was minced with a surgical blade and the fragments with a size of 1-3 mm were prepared. These fragments were rinsed several times with Ca^{++}, Mg^{++}-free Hanks' balanced salt solution (HBSS) containing 40 U/m*l* penicillin G and 40 μg/m*l* streptomycin sulfate and placed in 60-mm dishes. They were then dipped in 1 m*l*/dish of the culture medium, which consisted of a 1 : 1 mixture of Dulbecco's modified Eagle's medium (DME) and Ham's F12(F12) supplemented with 10% heat-inactivated fetal bovine serum and the antibiotics mentioned above, and cultured at 37℃ in a humidified atmosphere of 5% CO_2 in air. Cell outgrowth occurred from the tumor fragments within 48 hr of explanting. Spindle-shaped cells were often observed in the periphery of the epithelial-like cell sheets. These cells were removed with a rubber policeman. At day 20 of primary

culture, the epithelial cell sheets having a diameter of over 1 cm were subcultured using 0.01% trypsin (2914 units/mg ; GIBCO) and 0.01% EDTA in HBSS. At the third passage, the cells were cloned by the ring cylinder technique of Marcus *et al.* (24) with some modifications. Thus, one clone was established and designated as OSC-19.

Growth characteristics

For determination of population doubling time, the OSC-19 cells were seeded in 60-mm dishes at 1×10^5 cells/dish. The number of viable cells/dish in a logarithmic growth phase was counted in triplicate.

Plating efficiency was determined in the cultures with a seeding density of 1×10^3 cells/60-mm dish. The cells cultured for 2 weeks were stained with Giemsa and the number of colonies/dish was counted macroscopically.

To examine anchorage independent growth, the cells were suspended in 0.3% agarose (Difco Labs., Detroit, USA) in a culture medium at 1×10^4, 5×10^4 and 1×10^5 cells/m*l*. The basal layer was formed in a 35-mm dish with 1.5 m*l* of 0.5% agarose in the culture medium. The cell suspensions were placed on the basal layer at 1 m*l*/dish. To investigate the effect of EGF on the soft agar growth of OSC-19 cells, the cells (5×10^4 cells/35-mm dish) were cultivated in soft agar which contained EGF at 100ng/ml. Thereafter, the cells were cultured for 30 days and the number of colonies/dish was counted with an inverted phase contrast microscope.

Electron microscopy

The OSC-19 cells and the tumor tissues grown in nude mice were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and were postfixed with 2.0% osmium tetroxide in the cacodylate buffer. They were then dehydrated in a graded series of ethanols and then hydroxypropyl methacrylate for the cultured cells or propylene oxide for the tumor tissues, followed by embedding in Epon 812. The thin sections were stained with uranyl acetate and lead citrate.

Immunofluorescence for cytokeratins

The cells grown on glass coverslips were fixed in methanol for 5 min at −20℃. The fixed cells were incubated with the mouse monoclonal antibody to cytokeratins-PKK1 (Labsystems Oy, Helsinki, Finland) for 1 hr at 37℃ and then incubated with a biotinylated anti-mouse IgG (Vector Labs., Burlingame, USA) for 30 min at 37℃. Subsequently, they were incubated with FITC-conjugated avidin (Vector Labs., Burlingame, USA) for 30 min at 37℃. Each step was followed by several washings with PBS.

Tumorigenicity in nude mice

To examine the tumorigenicity, the cells were injected subcutaneously into the back of two Balb/c nude mice (CLEA Japan Co., Shizuoka, Japan) at 1×10^7 cells/mouse.

Chromosome analysis

Colchicine (Katayama Chemical Industries Co., Japan) at 10^{-6} M was added to the dishes in which the cells were in an exponential growth phase. After incubation for 2 hr, the cells were harvested by trypsinization and treated with 0.075 M KCl for 20 min at 37℃. They were then fixed with Carnoy's fixative and stained with Giemsa for the determination of chromosome number.

Effect of EGF on cell growth

The cells were plated in 35-mm dishes at 5×10^4 cells/dish and at 24 hr in culture, they were rinsed two times with HBSS and maintained in a defined serum-free medium with 0 to 100 ng/m*l* EGF (Collaborative Research Inc., Waltham, USA). This medium consisted of DME and F12 (1 : 1) supplemented with insulin (5 μg/m*l*), transferrin (5 μg/m*l*), selenium (5 ng/m*l*) and the antibiotics. The number of viable cells were counted at 1 or 2-day intervals, and the growth rates were determined.

The effect of EGF on the labeling index of nuclei in S-phase was examined with the use of bromodeoxyuridine (BrdU). The cells maintained for 3 days with or without EGF were exposed to BrdU at 10^{-6} M for 30 min and fixed in 70% ethanol for 30 min. The fixed cells were treated with 2 N HCl and stained by the avidin-biotin-peroxidase complex method using an anti-BrdU monoclonal antibody (Becton Dickinson, California, USA). The labeling index was expressed by the percentage of the positive cells.

Immunoprecipitation of EGF receptor

OSC-19 cells were radioiodinated by adding 100μl of 2 mg/m*l* lactoperoxidase (Sigma Chemical Co., St. Louis, USA) and 1 mCi of Na ^{125}I to 1×10^7 cells in PBS. Thirty microliters of 0.03% hydrogen peroxide were added 3 times at 5 min intervals. After iodination, the cells were washed with PBS and solubilized for 1 hr at 4℃ by adding the lysis buffer (0.05 M Tris HCl buffer, pH 7.4, containing 0.14 M NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1μg/m*l* pepstatin, 0.05% sodium azide, 0.2 TIU/m*l* aprotinin, and 5 mM EDTA). Aliquots of the labeled materials were incubated with a mouse monoclonal antibody to EGF receptor (Amersham, Buckinghamshire, UK) and an anti-mouse IgG coupled to Affi Gel 10

beads (Bio-Rad Labs., Richmond, USA) at 4℃ for 12 hr. The beads were then washed with the washing buffer (0.05 M Tris HCl, pH 7.4, 0.14 M NaCl, 0.1% NP-40) and extracted with the SDS-PAGE sample buffer (10% SDS, 10% glycerol, 0.01% bromophenol blue, and 62.5 mM Tris-HCl, pH 7.0). The SDS-PAGE was carried out under reducing conditions, where the samples were boiled for 5 min in the SDS-PAGE sample buffer with 50 mM dithiothreitol. After electrophoresis, the gel slab was dehydrated by a gel slab dryer. The radioactivity in the slab gel was visualized by autoradiography with a Cronex intensifying screen (Dupont Instruments, Wilmington, USA).

RESULTS

Morphological and Immunocytochemical properties

On phase contrast observation, OSC-19 cells were polygonal in shape and grew in typical cobblestone patterns. Nuclei were large and contained several nucleoli. A piling-up of the cells were rarely seen (Fig. 1).

Ultrastructurally, nuclei of the cells were mainly oval in shape and had distinct nucleoli. A few of organelles such as mitochondria and rough endoplasmic reticulum were observed. A large number of polysomes were seen throughout the cytoplasms. Microvilli were frequently found on the cell surface (Fig. 2A). Desmosomes were infrequently seen between adjacent cells (Fig. 2B) and, in some of the cells, desmosomes were also observed in their cytoplasms (Fig. 2C). Tonofilaments were scarcely observed in the cultured cells.

OSC-19 cells were examined for cytokeratins using the immunofluorescence technique. As shown in Fig. 3A, many cells contained a faintly stained cytoplasmic network of cytokeratins. Some cells showed strongly stained thick filaments in their cytoplasms (Fig. 3B). They did not contain vimentin or desmin (data not shown).

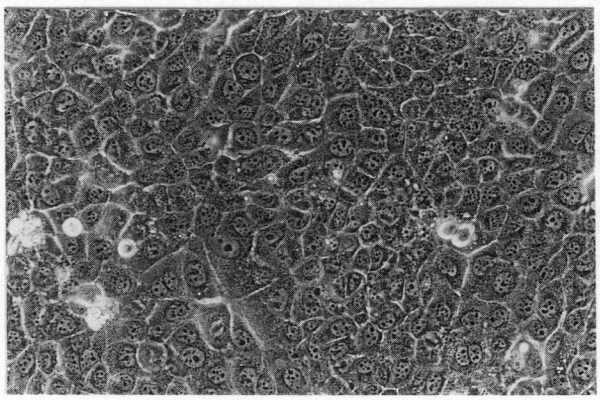

Fig. 1 A phase contrast microphotograph of OSC-19 cells in the primary culture, ×100.

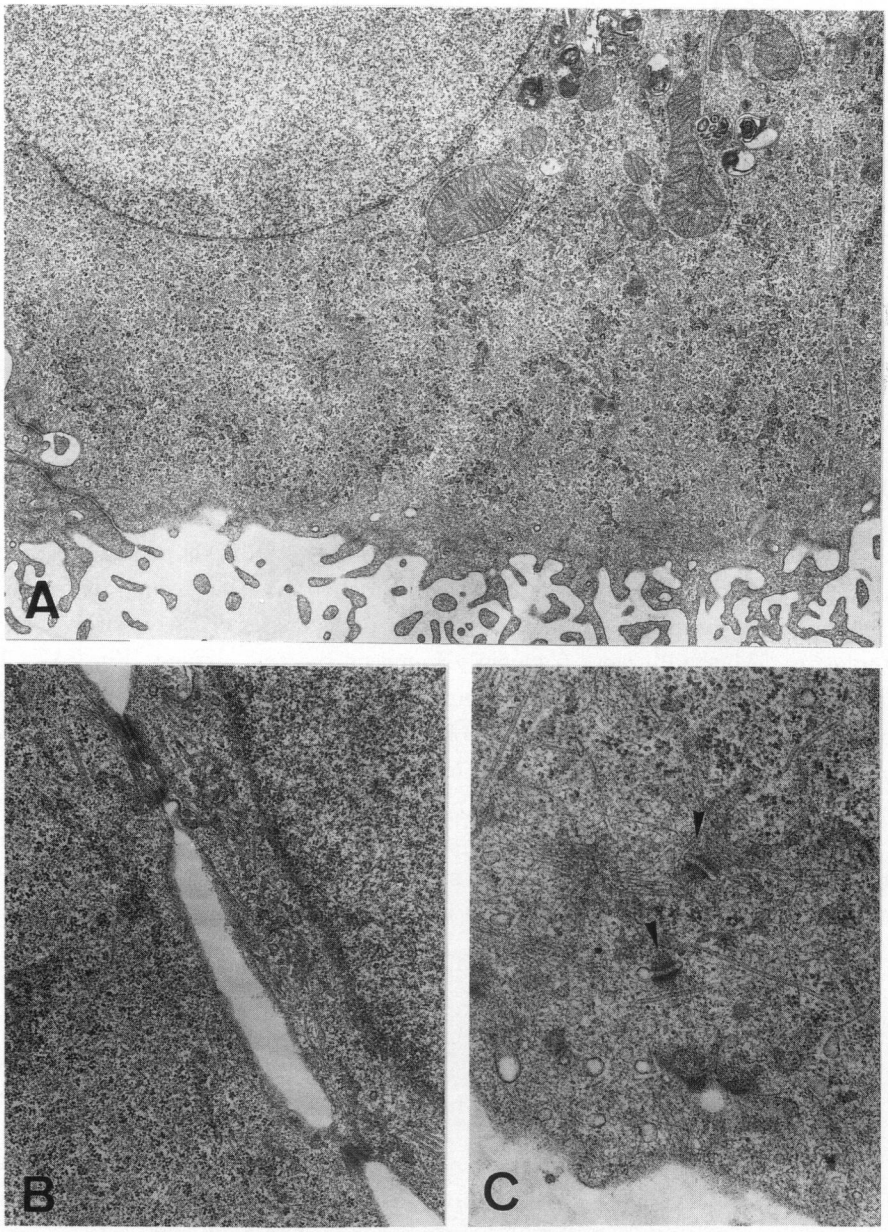

Fig. 2 Electron microphotographs of OSC-19 cells A. ×8,000. B. Desmosomes, ×15,000. C. Intracytoplasmic desmosomes (*arrow heads*), ×30,000.

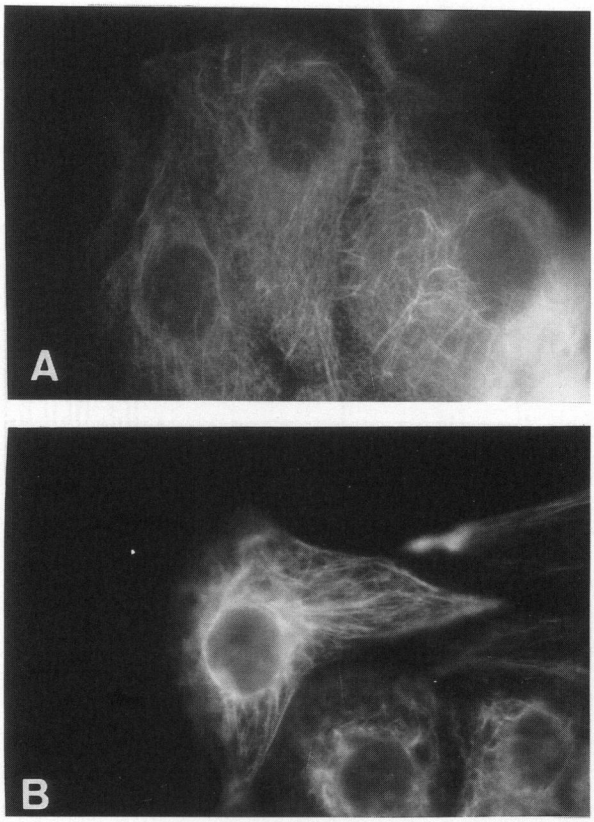

Fig. 3 Immunofluorescent microphotographs of OSC-19 cells stained for cytokeratins. A. Fine filaments, ×240. B. Thick filaments, ×240.

Growth properties

The population doubling time of OSC-19 cells at the 12 th, 28 th and 55 th passages was 33.4 hr, 25.1 hr and 16.7 hr, respectively. Thus the population doubling time became shorter as the passage number advanced. The plating efficiency was 0.47% at the 32 nd passage, and the colony forming efficiency in semi-solid agar was less than 0.001% at the 25 th passage.

Chromosome number

Chromosome of OSC-19 cells at the 50 th passage were aneuploid, showing 48 to 146 chromosomes (Fig. 4). Forty-five percent of the cells had 110 to 120 chromosomes.

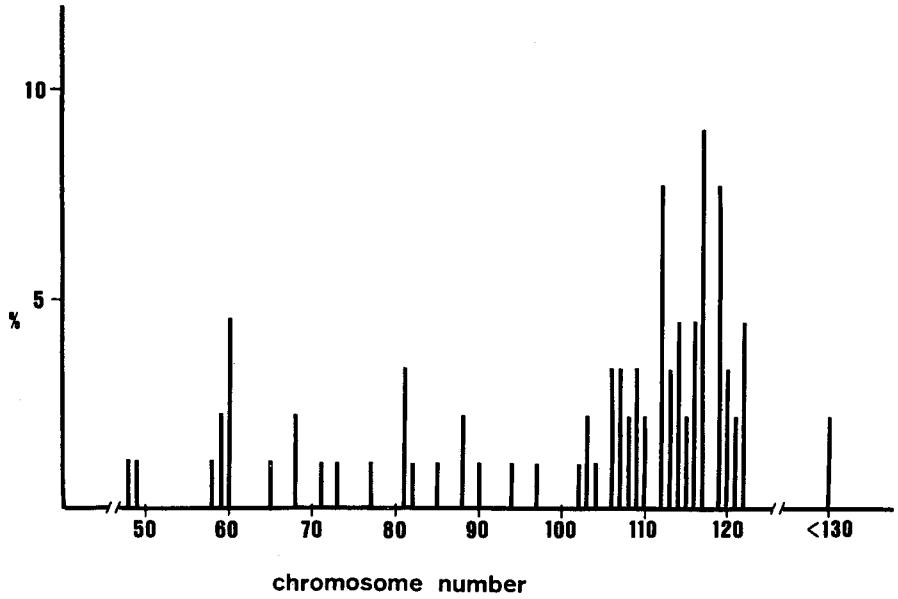

Fig. 4 Chromosome distribution in OSC-19 cells at the 50 th passage. *Ordinate*, the percentage of cells having each number of chromosomes. *Abscissa*, the number of chromosome per cell.

Tumor formation in nude mice

OSC-19 cells formed tumors in both the two nude mice. Tumors developed into 7 and 15 mm in diameter in 20 days after injection. The tumors did not show invasive growth beyond the dermis. No metastases were observed. Histologically, they did not show cancer pearls, but a few cells had abundant eosinophilic cytoplasms which mimicked individual keratosis (Fig. 5A). The tumors possessed a histological appearance similar to that of the original tumor excised from the lymph node of the patient (Fig. 5B).

On electron microscopic observation, cytoplasmic organelles of the tumor cells were developed not so well, but desmosomal junctions were frequently seen between the cells. Abundant tonofilament bundles were observed in the cytoplasms, and some of them connected with desmosomes (Fig. 6A, 6B).

Effect of EGF

As shown in Fig. 7A, EGF inhibited the growth of OSC-19 cells at 10 ng/m*l*. The inhibitory effect was dose-dependent from 1 to 100ng/m*l* and there was no significant effect of EGF at the concentration of 0.01 and 0.1 ng/m*l* (Fig. 7B). The nuclear labeling indices of the cells cultured with or without EGF at 10 ng/m*l* were 10.9% and 25.2%, respectively. Whereas EGF stimulated the colony forma-

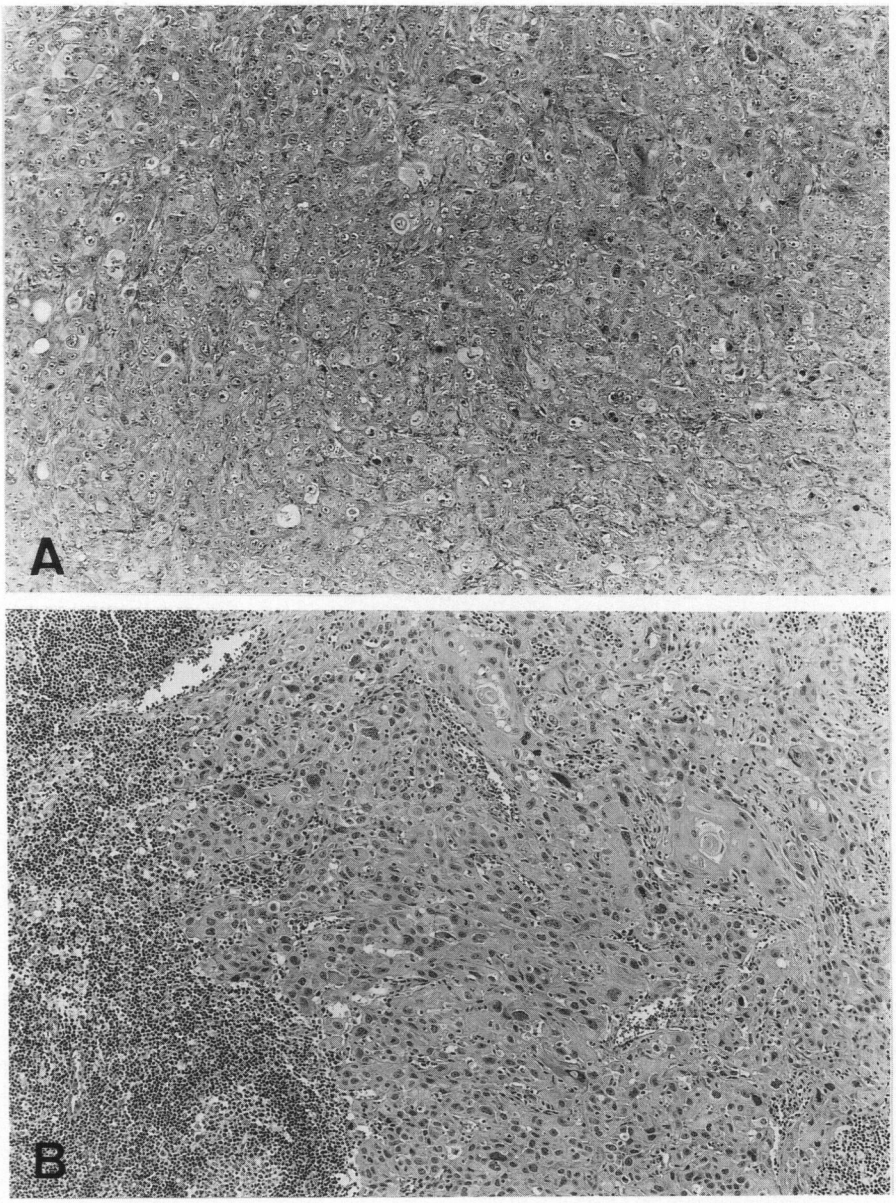

Fig. 5 Histological microphotographs of the tumors, H.-E. staining.
A. The tumor developed in nude mice, ×85, B. The original tumor from which OSC-19 was derived, ×85.

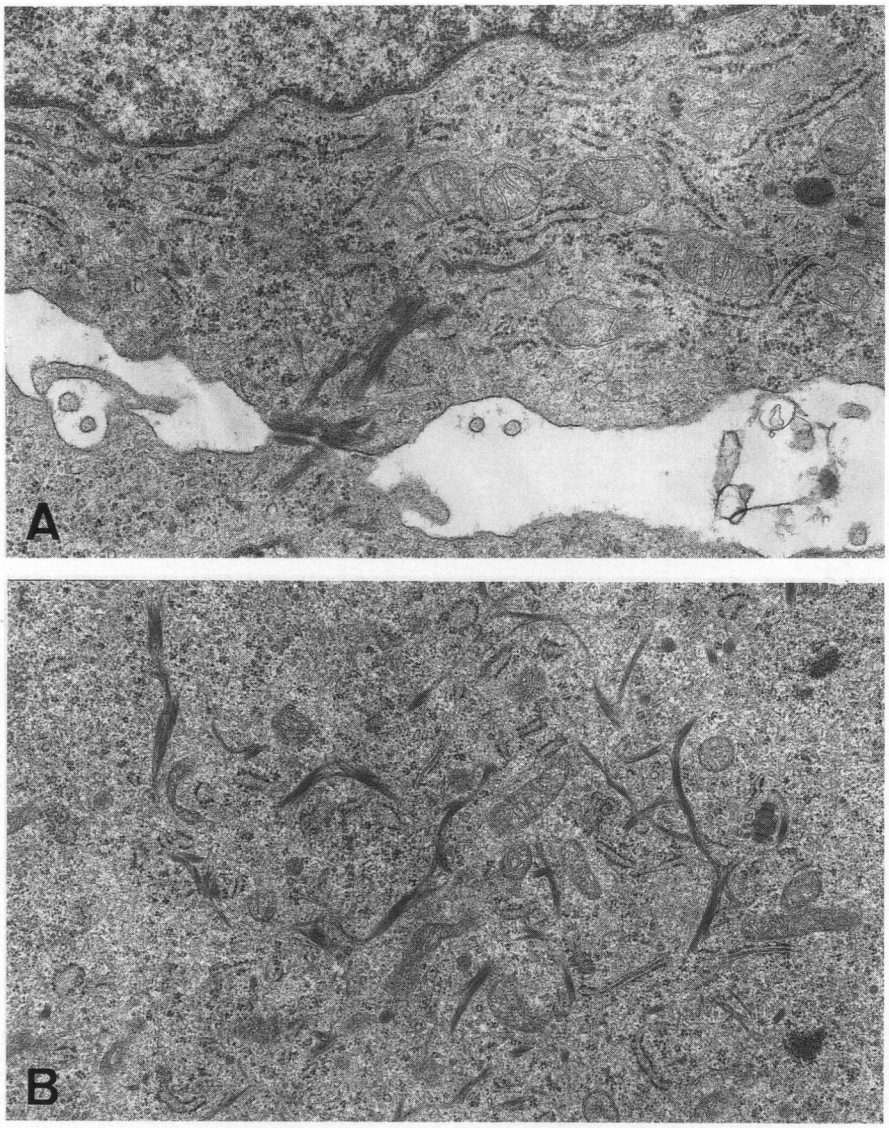

Fig. 6 Electron microphotographs of the tumor cells developed in nude mice. A. Desmosomes connected with tonofilament bundles, ×22,500. B, ×15,000.

tion in soft agar media. OSC-19 cells formed colonies in soft agar which contained EGF at 100 ng/m*l* (Fig. 8), but did not form colonies without EGF.

Since EGF acts on the cells through the receptors on plasma membranes, we examined whether OSC-19 cells really had EGF receptors. In the immunoprecipitation assay, a strong band with M_r. 170,000 protein corresponding to the EGF

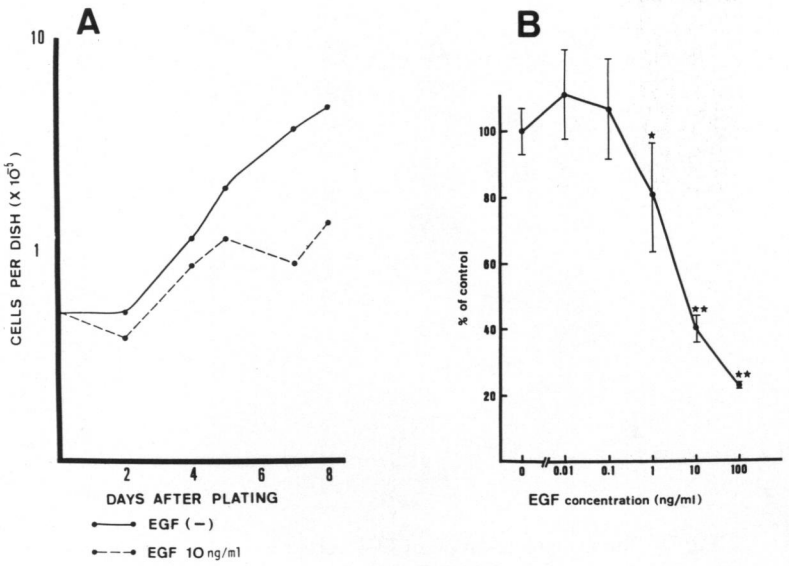

Fig. 7 Effect of EGF on the growth of OSC-19 cells.
A. OSC-19 cells were cultured in the serum-free medium with or without EGF. Numbers of viable cells/dish were counted at days 2 to 8. Each point represents the mean value of triplicate dishes.
B. OSC-19 cells were cultured for 6 days in the serum-free medium containing EGF at concentrations of 0-100 ng/m*l*. The number of viable cells/dish of the EGF-treated cells was expressed by the percentage of that of the control without EGF. Points and bars are averages and S. D., respectively, for 3 dishes. *Asterisks* represent significant differences from the control ; *, $p<0.025$; **, $p<0.001$.

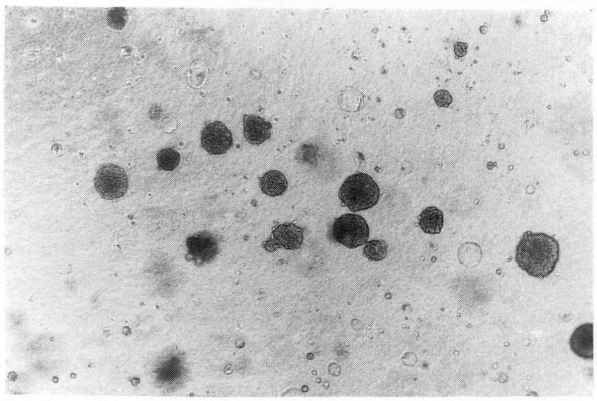

Fig. 8 The colonies of OSC-19 cells in the soft agar which contains EGF at 100 ng/m*l*, ×20.

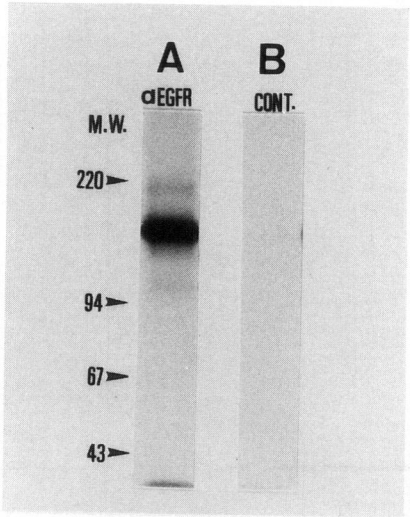

Fig. 9 Immunoprecipitation of EGF receptor.
1×10^7 of OSC-19 cells was labeled with Na ^{125}I and solubilized by the method described in MATERIALS AND METHODS. Extracts were immunoprecipitated with mouse anti-human EGF receptor and anti-mouse IgG coupled to Affi Gel beads (*lane A*). *Lane B* represents the control without the anti EGF-receptor.

receptor was detected (Fig. 9).

DISCUSSION

The establishment of human SCC cell lines is considered to be difficult, although the reasons for this are not clear, and only relatively few SCC cell lines of the head and neck have been reported since the early 1950s (4, 9, 10, 22, 28, 29, 31, 32). For example, Krause *et al.* (22) have established three SCC cell lines from 194 patients, and more successfully Rheinwald and Backett(31) have obtained 6 SCC cell lines from 22 patients using 3T3 fibroblast feeder layers. We have made an effort to establish cell lines from human oral SCCs. Among 20 different oral SCCs, we succeeded in establishing only one line(OSC-19). Krause *et al.* (22) suggested three major factors in a failure of establishment of cell line ; inadequate nutrients in the culture medium, fungal or bacterial contamination, and overgrowth of fibroblasts. Indeed, our failure was mainly due to fungal contamination and overgrowth of fibroblasts. OSC-19 cell line was established from the lymph node metastases which seemed to be less contaminated. It was our impression that metastatic tumors are more favorable to establish cell lines.

Surgical specimens contain several kinds of cells. It is necessary to study

several parameters in order to identify OSC-19 cells as SCC origin. OSC-19 cells were polygonal in shape and grew in cobblestone patterns on the plastic dish. Ultrastructurally, they had desmosomes and microvilli. Although OSC-19 cells did not show distinctive tonofilament bundles, immunocytochemically, they contained cytokeratins which are characteristic of epithelial cells. Furthermore, the tumor cells grown in nude mice contained numerous tonofilament bundles, which were typically observed in squamous epithelia(14), and many desmosomes associated with tonofilaments. These characteristics of this cell line were identical with those of the SCC lines reported by Carey(5). Thus, OSC-19 cells have retained certain phenotypic traits of squamous cells. It is also suggested that the conditions in nude mice are more favorable for OSC-19 cells to differentiate than those in culture.

EGF, which is a polypeptide originally isolated from submaxillary glands of male mice by Cohen(7), plays an important role in regulating the proliferation of cells. It is widely accepted that EGF stimulates the growth of a variety of cultured cells of both epithelial and mesenchymal origin(6, 15). However, there are sporadic reports that EGF inhibits the growth of cell lines derived from human carcinomas(2, 12, 13, 18, 20, 21). Among these cell lines, A-431 cell line derived from human vulva has been studied for this model(2, 13). Because this cell line has a large amount of EGF receptor(11, 17), EGF-mediated growth inhibition has been suggested to be due to high numbers of EGF receptors. Using other human carcinoma cell lines, Kamata *et al.* (19) also found that EGF inhibited the growth of all 14 lines of human SCC cells examined, but not inhibited the growth of other tumor cells, such as adenocarcinomas. Also they found that the sensitivity to the inhibitory effect of EGF correlated well with the elevated level of EGF receptors. Although we did not investigate the number of EGF receptors, OSC-19 cells seemed to possess a sufficient amount of EGF receptor, because their growth was clearly inhibited by EGF. The inhibitory effect was dose dependent from 1 to 100 ng/m*l*. It is interesting that, though the statistical significance was not obtained, the lower doses of EGF seemed to stimulate the growth of OSC-19 cells. Kawamoto *et al.* (20) also found that the growth of A-431 cells was stimulated at low concentrations of EGF, in the range of 3-100 pM. Furthermore, they reported that, in the presence of anti-EGF receptor monoclonal antibody, EGF stimulated the proliferation of A-431 cells at concentration up to 3 nM. They suggested that EGF receptors might be heterogeneous and a minor population of high affinity EGF receptors might involved in stimulation of A-431 cell proliferation.

It is widely accepted that there is a correlation between tumorigenicity and anchorage independence(8, 33). However, Marshall *et al.* (25) reported that the correlation was not always found in cases of epithelial cell lines. In fact, the SCC cell lines, SCL-1 developed by Boukamp *et al.* (4) and HSC-3 established by

Momose *et al.* (27) were unable to form colonies in semi-solid agar despite of their tumorigenicity. OSC-19 cells also could not form colonies in soft agar when seeded at 1×10^5 cells/dish. Thus, OSC-19 is suggested to be highly anchorage dependent, such as these two SCC lines.

It is surprising to observe that the growth of OSC-19 cells in soft agar was stimulated by a high concentration of EGF at which their growth in dish culture was prominently inhibited. This result suggests that OSC-19 acquire anchorage independence in the presence of EGF. EGF receptors may decrease in number in soft agar, especially the low-affinity EGF receptors, or EGF may act in a different manner in soft agar. In this connection, it is noteworthy that the growth of A-431 cells in nude mice was stimulated by EGF(30) and inhibited by anti-EGF-receptor antibodies(26). It is the subject for a future study to clarify the mechanism(s) not only leading to inhibitory or stimulatory effect of EGF on cell growth, but also acquiring the anchorage independency in the presence of EGF.

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