Tumor Res. **24**, 57—73 (1989)

Establishment and Characterization of OSC-19 Cell Line in Serum- and Protein-free Culture

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SUMMARY

Morphological properties and keratin profiles of a human squamous cell carcinoma cell line, OSC-19 in chemically defined media were investigated and compared to those under conditions in which 10% fetal bovine serum was supplemented. OSC-19 cells were adapted to continuous culture in serum-free (SF) medium supplemented only with insulin, transferrin and selenium. The cells maintained in SF medium were further adapted to protein-free (PF) medium with no exogenous factors. The cells cultivated in SF medium had a tendency to elongate and grew in scrollwork-like pattern, while the cells under serum containing condition were polygonal in shape and grew in cobblestone pattern. In SF culture condition, the cells formed focal stratifications and an apparent increase in the number of tonofilaments and desmosomes was observed by electronmicroscopic examination. With the morphological changes, keratin profiles were also changed. The cells cultured in SF medium produced two new major keratins (50 and 56 kd) in addition to five major keratins (40, 45, 47, 52 and 58 kd) that were present in cells cultured in serum supplemented medium. The cells maintained in PF medium had the same characteristics as those under SF culture condition. When the cells maintained in SF medium were re-cultured in the serum supplemented medium, the scrollwork-like pattern of growth and two keratin polypeptides (50 and 56 kd) disappeared. These findings suggested that serum contained factor(s) which modulates differentiation of OSC-19 cells.

Key words: Squamous cell carcinoma, Serum-free culture, Protein-free culture, Electronmicroscopy, Keratin

* Abbreviations:
SF, serum-free; PF, protein-free; FITC, fluorescein isothiocyanate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate buffered saline.

INTRODUCTION

In recent years, cell culture technique has been rapidly developed and widely used in many experimental studies. Most cell cultures usually require the addition of serum. However, serum is a complex mixture of many biomolecules and these components are not defined completely. In addition, serum is generally expensive and does not always have a uniform effect among different batches. To conquer these problems, much efforts have been made to cultivate cells in serum-free (SF) medium, and many different kinds of cells have been successfully maintained in SF media(4, 8, 21). In SF culture, exogenous growth factors which support cell growth are generally supplemented, while very limited kinds of cells have continuously grown in protein-free (PF) medium with no exogenous factors(7, 13, 20).

We established a cell line designated as OSC-19 which was derived from an oral squamous cell carcinoma(23). Recently, the subpopulation of this cell line has been successfully adapted to continuous cultivation in SF medium supplemented with insulin, transferrin and selenium. In addition, the cells transferred to SF culture could be adapted to PF medium. When the cells were grown in serum depleted media, morphological and cytoskeletal changes occurred. In this paper, morphological properties and keratin profiles of OSC-19 cells in chemically defined media were described.

MATERIALS AND METHODS

Cell culture

OSC-19 cells were maintained in a medium consisting of equal volumes of Dulbecco's modified Eagle's medium and Ham's F12 medium, supplemented with 2.5 g/*l* sodium bicarbonate, 10 mM HEPES, 40 U/m*l* penicillin G, 40 μg/m*l* streptomycin, and 10% fetal bovine serum (Boehringer Mannheim, Mannheim, W. Germany). Some properties of OSC-19 cells were described previously(23). For serum-free culture, we replaced the serum by 1 μg/m*l* of insulin (Sigma Chemical Co., St. Louis, USA), 5 μg/m*l* of transferrin (Sigma, Chemical, Co., St. Louis, USA) and 5 ng/m*l* of selenium (Merck, Darmstadt, W. Germany) and this medium was designated as SF medium. The cells transferred to the SF condition were then further adapted in the absence of exogenous factors (insulin, transferrin, selenium). This chemically defined medium was defined as PF medium. All these cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37℃. The subculturing was carried out using 0.01% trypsin (2914 units/mg; Gibco, New York) −0.01% EDTA solution and 6-amidino-2-naphtyl p-guanidinobenzoate dimethanesulfonate (Torii Yakuhin Inc. Tokyo) at 10^{-7}M as

a trypsin inhibitor.

Growth examination

The cells were plated at a density of 1×10^5/60-mm dish. At intervals, the number of viable cells was counted by trypan blue exclusion test and the doubling time was calculated.

Morphological observations

The morphology of the cells grown on plastic dishes was examined daily under inverted phase microscopy. Ultrastructural examination of the cells was performed by the previously described method(23). Briefly, the cells were fixed in 2.5% glutaraldehyde, postfixed in 2% osmium tetroxide, dehydrated, and embedded *in toto* in Epon 812. After polymerization, selected areas were bored out with a cork borer, and glued to old Epon blocks with epoxy cement. The horizontal or vertical ultrathin sections were stained with uranyl acetate and lead citrate. For light microscopy, semithin sections (1 μm) were cut perpendicularly to the surface of the plastic dish, mounted on the slide glass, and stained with toluidine blue.

Immunofluorescence for cytokeratins

The polyclonal antiserum against keratins isolated from stratum corneum of human epidermis was obtained from Dakopatts (Carpinteria, USA). The biotinylated anti-rabbit IgG and FITC-conjugated avidin were purchased from Vector Labs. (Burlingame, USA). Immunofluorescence was performed as described previously(23).

Cytoskeletal preparation

Cytoskeletons enriched in intermediate filaments were prepared by a modified method of Franke et al. (5). Briefly, confluent cultures were treated with cold, high-solt detergent buffer [1.5 M KCl, 0.5% Triton X-100, 5 mM EDTA, 10 mM Tris, 0.2 mg/m*l* DNase I (Worthington Biochemical Co., Freehold, USA), 10 mM Mg^{++}, pH 7.6]. Following 30 min incubation in this buffer, the insoluble material was collected by 15 min centrifugation at $8000\times g$ and washed twice in PBS. The insoluble material was suspended in extraction buffer (1% SDS, 0.192M Glycine, 25 mM Tris, 1 mM EDTA, 10 mM Dithiothreitol, pH 8.3) and sonicated. This solution was boiled for 3 min and centrifuged at $10000\times g$ for 30 min. The supernatant was stored at -20℃ until used.

Electrophoresis and immunoblot analysis

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli(12) using a 4% stacking and 10% running gel. Gels were stained with 0.2% Coomassie blue in 45% ethanol/10% acetic acid.

For immunoblot analysis, proteins from unstained polyacrylamide gels were transferred electrophoretically to nitrocellulose paper (Schleicher & Schuell, Dassel, W. Germany). The transfer was checked by staining with 0.1% amido black in 45% methanol/10% acetic acid. Unreacted paper was blocked with Block Ace (Dainihon Seiyaku Inc., Osaka) for 1 hr at room temperature. The nitrocellulose paper was then reacted with mouse monoclonal antibodies, PKK1 (Labsystems Oy, Helsinki, Finland) and MA-903 (Enzo Diagnostics Inc., New York). Subsequently, the paper was incubated with a biotinylated anti-mouse IgG (Vector Labs., Burlingame, USA) and then reacted with avidin-biotin-peroxidase complex (Vector Labs., Burlingame, USA). Primary antibodies were incubated overnight at 4℃, and biotinylated secondary antibody and avidin-biotin-peroxidase complex for 1 hr at room temperature. Each step was followed by several washings with 0.05% Tween 20 in PBS. Proteins were visualized by incubating with 0.02% 3,3′-diaminobenzidine and 0.01% hydrogen peroxide dissolved in 0.05M Tris-HCl (pH 7.6).

RESULTS

Cultivation of OSC-19 cells in chemically defined media

OSC-19 cells maintained in the presence of serum were transferred to SF medium at the 25th passage. In the SF culture, the cells showed unstable growth at early passages. However, following 20 passages, these cells showed a steady growth rate, and the population doubling time was 23.2 hr at the 30th passage. After 20 passages in SF medium, the cells were further adapted to PF medium. It was possible to propagate these cells without falling in senescence. The growth rate had stabilized with a doubling time of 27.4 hr at the 30th passage. On the other hand, the cells maintained in serum supplemented medium had a doubling time of 16.7 hr at the 55th passage.

Morphological properties

When cultured in the presence of serum, OSC-19 cells were polygonal in shape and grew in cobblestone patterns (Fig. 1A and 1B). The cells mostly formed the monolayer or bilayer even after confluence (Figs. 3A and 4A). The cells maintained in SF or PF medium were larger in size and more irregular in shape than those in the serum supplemented medium. Furthermore, many cells

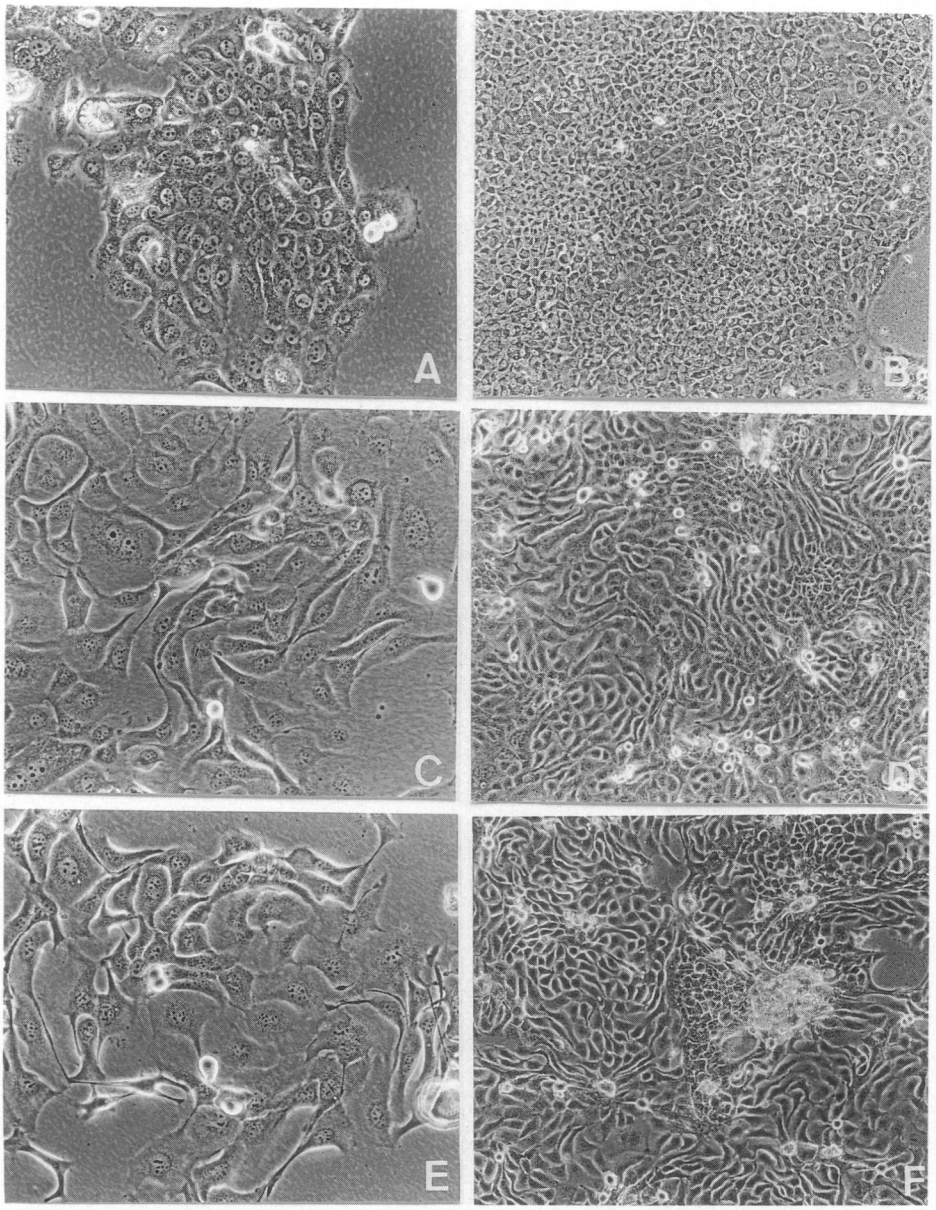


Fig. 1 Phase contrast microphotographs of OSC-19 cells.
A and B: Serum containing culture.
C and D: SF culture.
E and F: PF culture.
A, C and E: Subconfluent culture, ×100.
B, D and F: Confluent culture, ×50.

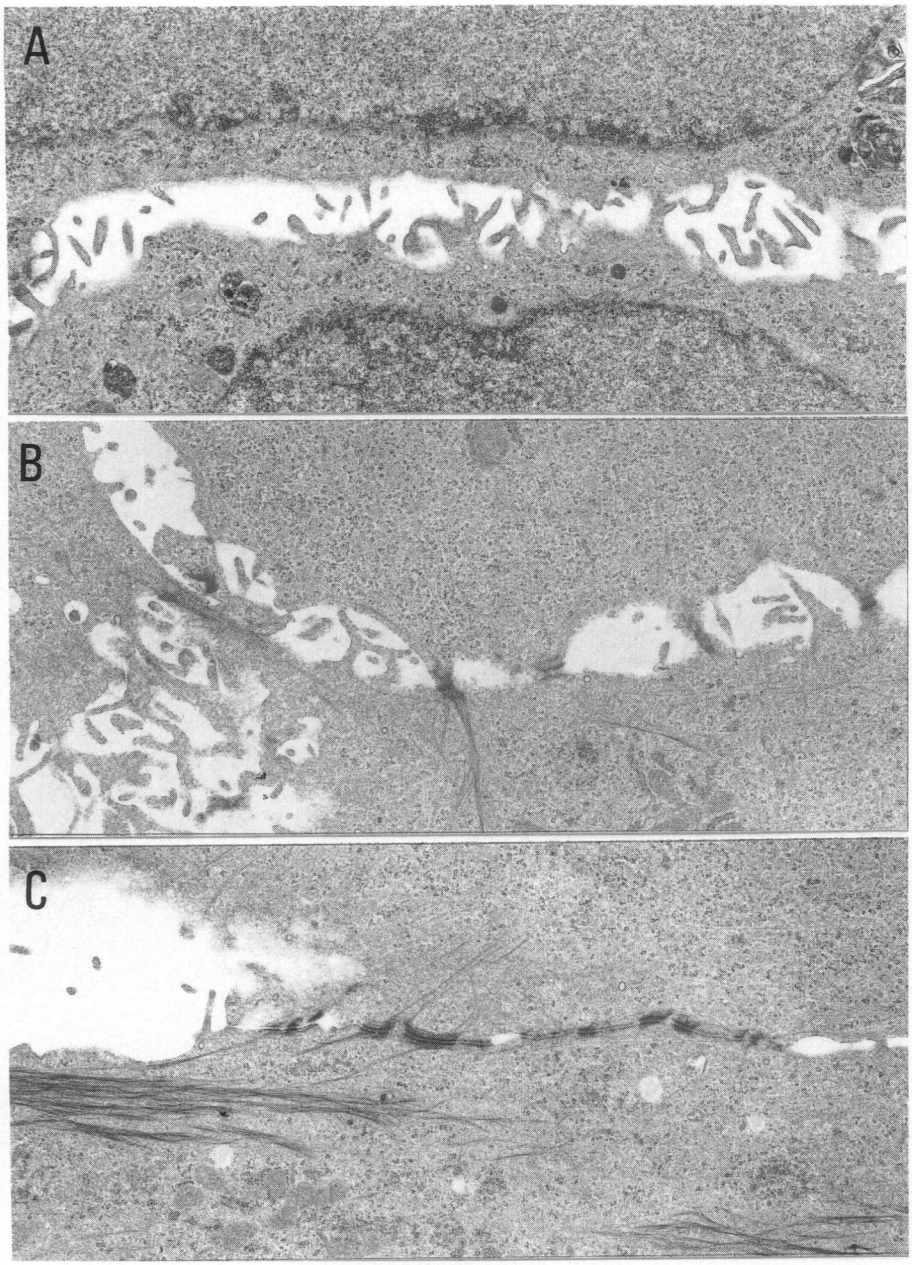


Fig. 2 Electron microphotographs of OSC-19 cells in subconfluent culture.
A: Serum containing culture, ×8000.
B: SF culture, ×8000.
C: PF culture, ×8000.

had a tendency to elongate, resulting in scrollwork-like growth patterns (Fig. 1C, 1D, 1E and 1F). Before the confluence, the cells grown in SF or PF medium had more numerous desmosomes and more abundant tonofilaments than those in the serum supplemented medium (Fig. 2A, 2B and 2C). After the exponential growth phase, focal stratifications were observed from place to place in SF or PF culture. One of such stratifications was shown in Fig. 3D. The stratifications were not observed in the serum supplemented culture. Toluidine stained-Epon sections cut perpendicularly from the stratifications showed several layers of cells (Fig. 3B and 3C). In the lower layers of the stratifications, cells were usually flat, but sometimes showed cuboidal in shape (Fig. 3B). In the upper layers,

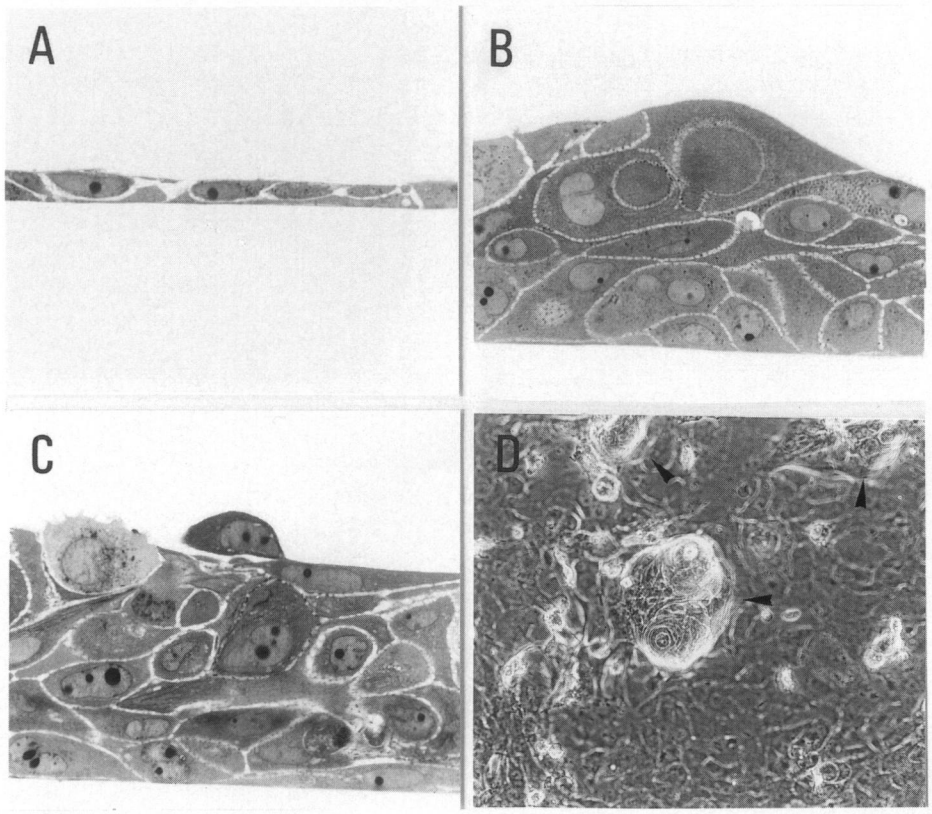


Fig. 3 Toluidine stained-Epon sections cut perpendicularly to the dish surface (A, B and C) and a phase contrast microphotograph (D) of post confluent culture.
A: Serum containing culture, ×500.
B: SF culture, ×500.
C: PF culture, ×500.
D: Stratified area in SF culture (arrow heads), ×100.

cells were generally larger in size than those in the lower layers. Almost all cells in the stratifications had many intercellular bridges (Fig. 3B and 3C). Ultrastructurally, cells in the middle and upper layers of the stratifications had more numerous and longer interdigitating processes, and larger desmosomes than those in the lower layers (Fig. 4B, 4C and 4D). The transition between

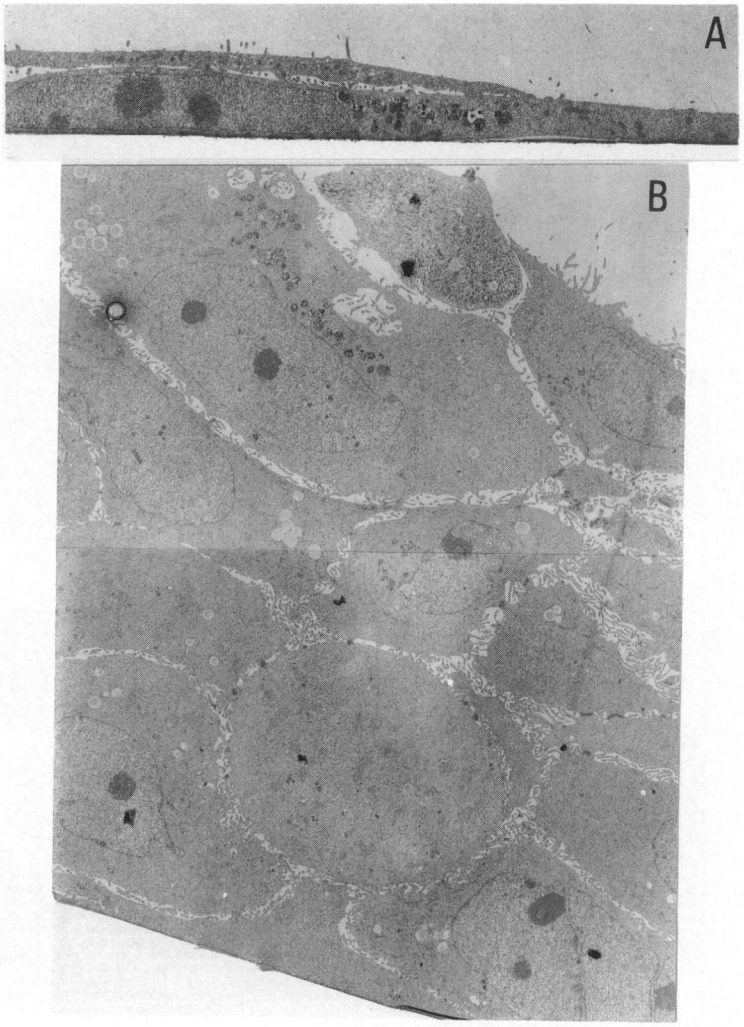
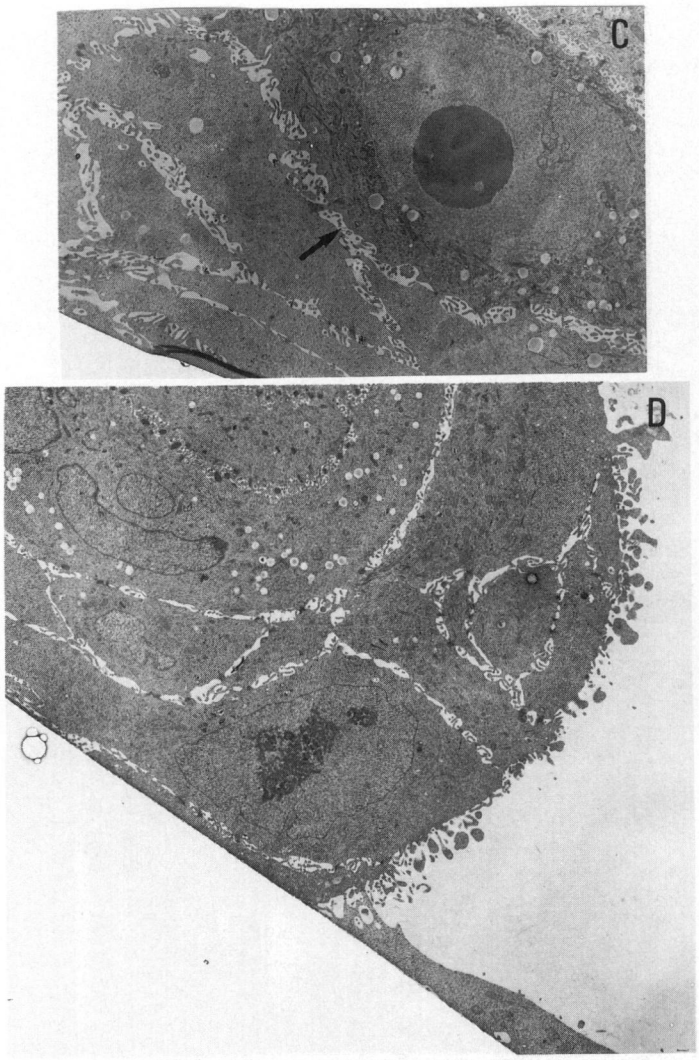


Fig. 4 Electron microphotographs of OSC-19 perpendicularly to the dish surface.
A : Serum containing culture, ×2990.
B : SF culture, ×1990.
C : A tonofilaments enriched cell in SF
D : A transition area between monolayer

monolayer and multilayer was usually gradual, but abrupt transition was occasionally observed (Fig. 4D). Some of the cells which composed multilayered structures contained abundant tonofilaments in their cytoplasm. Such cells were usually observed in the middle and upper layers, and rarely in the basal layer



cells in post confluent culture. Sections were cut

culture (arrow), ×3320.
and multilayer in SF culture, ×2660.

(Fig. 4C). Fig. 5 shows a representative sample of the cells containing a large number of tonofilaments. There were two types of the bundles. One was long and thin bundles (Fig. 5A) and another was short, thick and dense bundles (Fig.

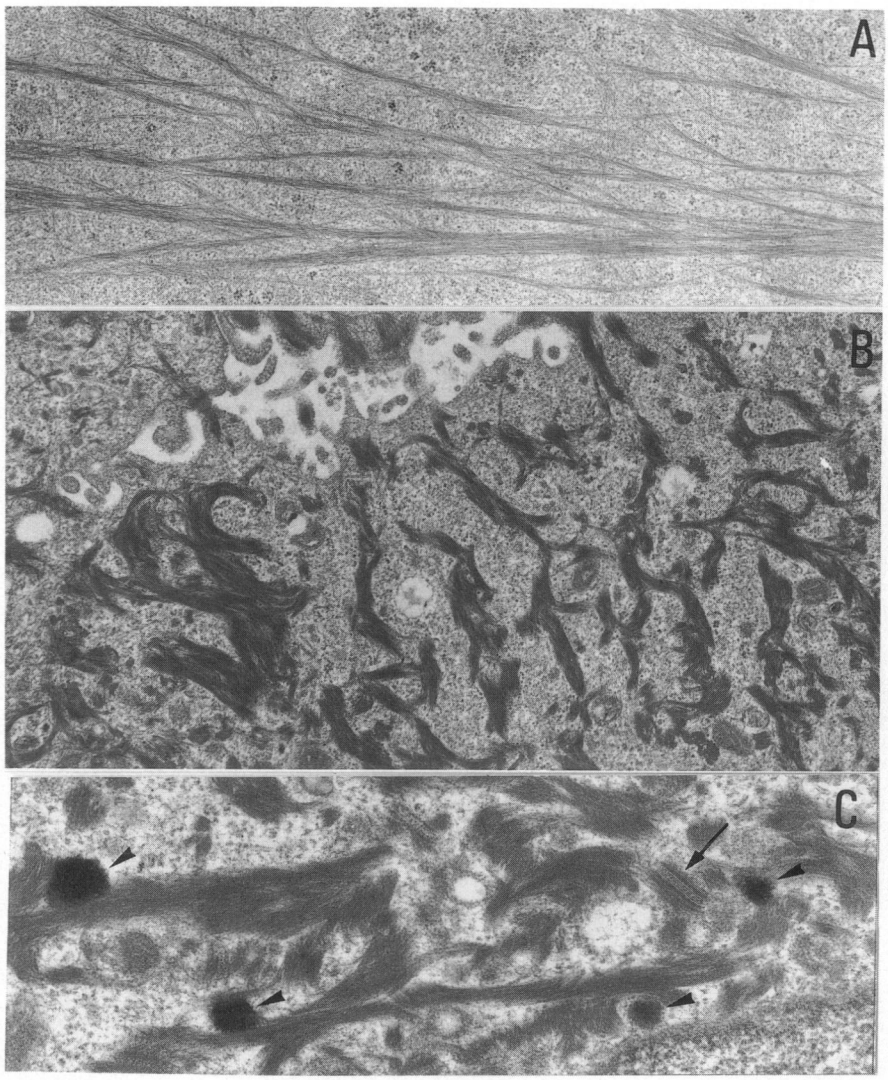


Fig. 5 Tonofilament bundles enriched area in SF post confluent culture.
A: Long and thin bundles, ×16000.
B: Short, thick and dense bundles, ×16000.
C: Keratohyaline granule like osmiophilic substances (arrow heads), Intracytoplasmic desmosomes (arrow) ×32000.

5B and 5C). The latter type of bundles was not observed in the basal layer of the stratifications. Keratohyaline like osmiophilic substances were occasionally found among the latter type of tonofilament bundles (Fig. 5C), but typical keratohyaline granules were not detected. Many desmosomes were observed between adjacent cells in the stratification (Fig. 6). In general, the number and size of desmosomes seemed to be increased in the cells of the middle and upper layers as

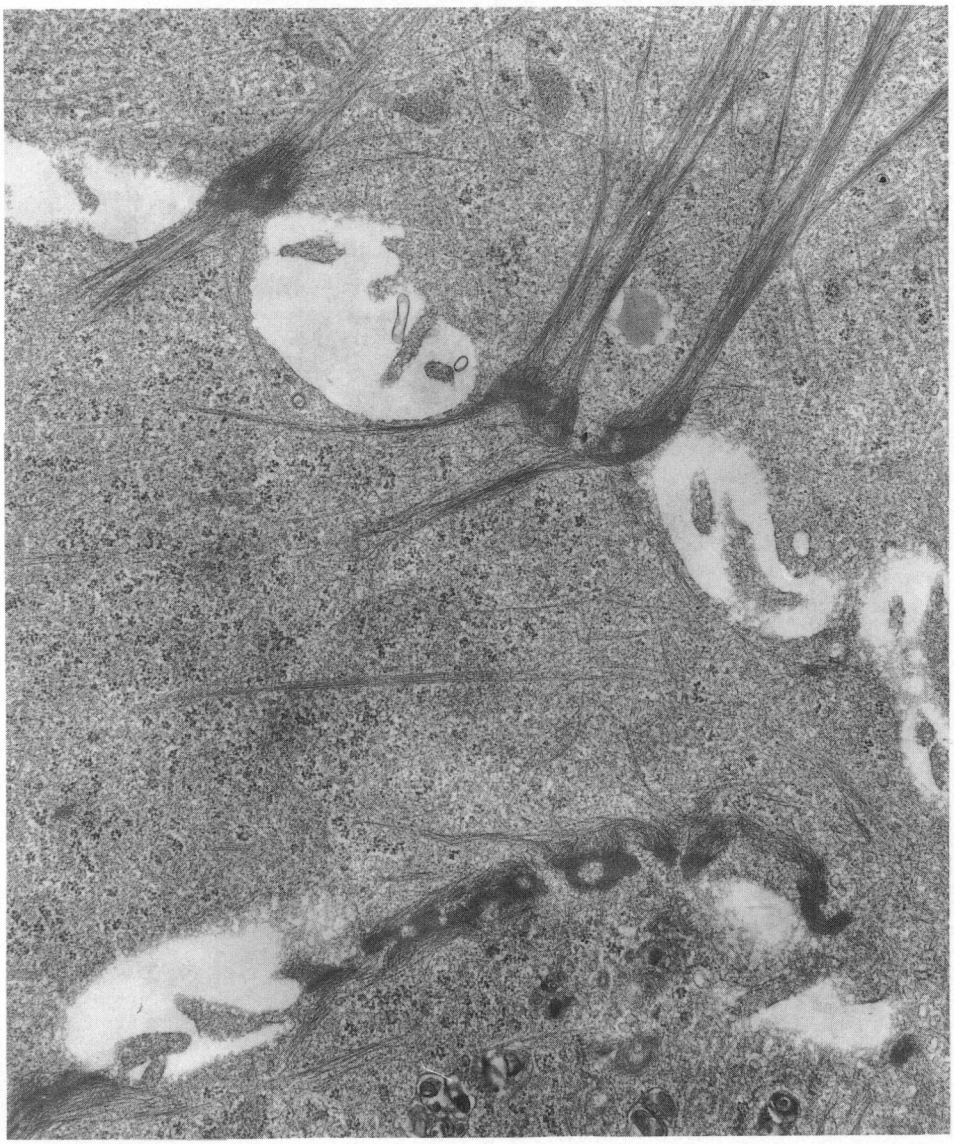


Fig. 6 Well developed desmosomes in SF post confluent culture. ×20000.

compared with those in the basal layers.

Immunofluorescence microscopy

As shown in Fig. 7, the typical fibrils extend throughout the cytoplasm were

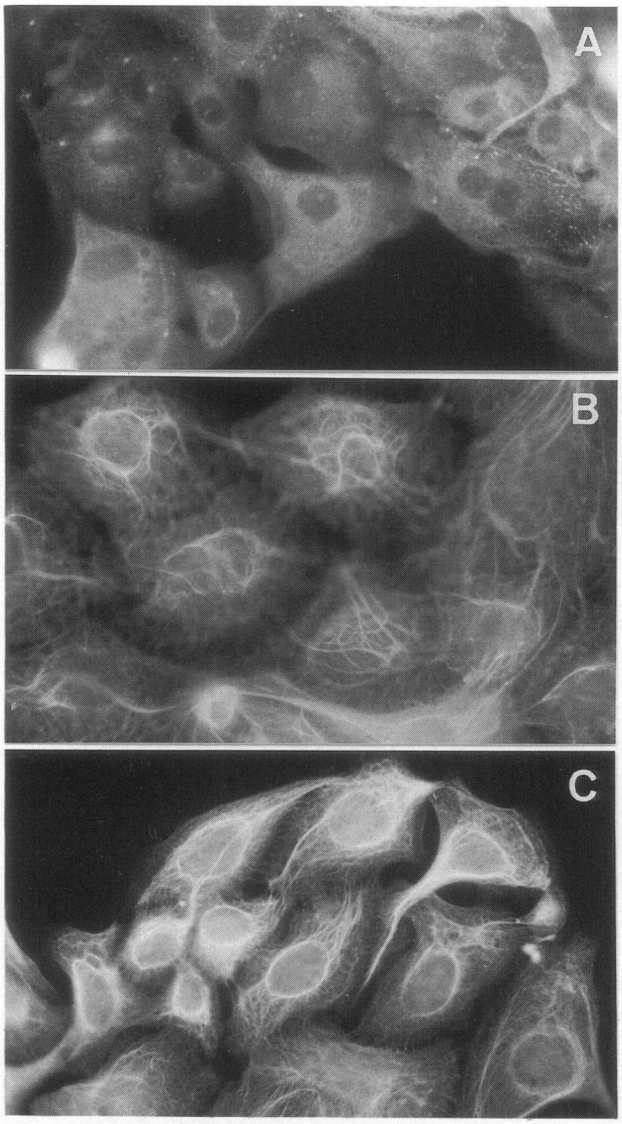


Fig. 7 Immunofluorescent microphotographs of OSC-19 cells.
A: Serum containing culture, ×200.
B: SF culture, ×200.
C: PF culture, ×200.

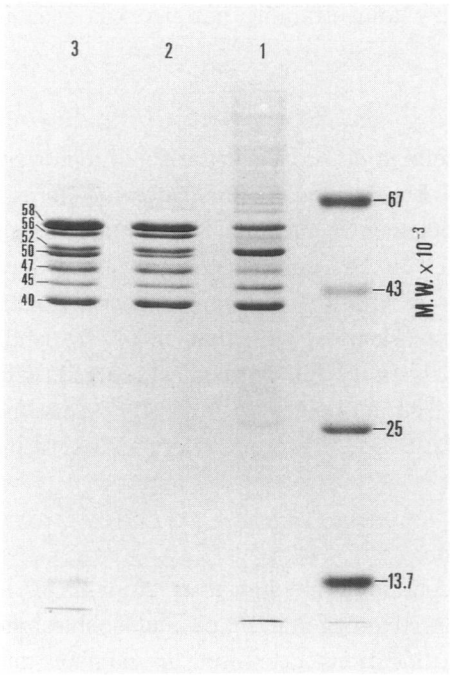


Fig. 8 Comparison of keratin proteins from different culture conditions.
Lane 1: Serum containing culture.
Lane 2: SF culture.
Lane 3: PF culture.
Molecular weight standards on the right side of the gel are; bovine serum albumin (67 kd), ovalbumin (43 kd), chymotrypsinogen A (25 kd), ribonuclease A (13.7 kd).

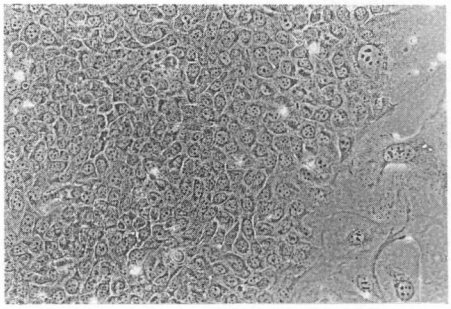


Fig. 10 A phase contrast mcrophotograph of re-cultured cells in serum containing medium. ×100.

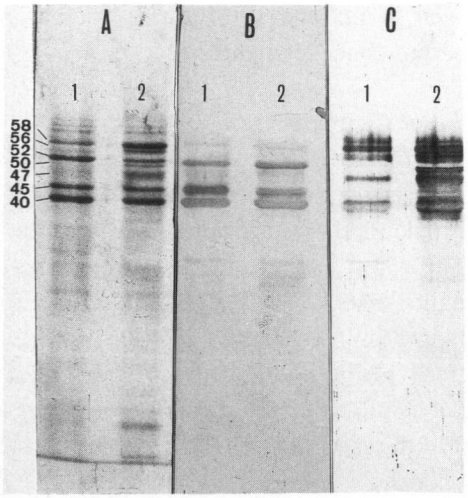


Fig. 9 Immunoblot analysis using PKK1 and MA-903 monoclonal antibodies.
A: Amido black.
B: PKK1.
C: MA-903.
Lane 1: Serum containing culture.
Lane 2: PF culture.

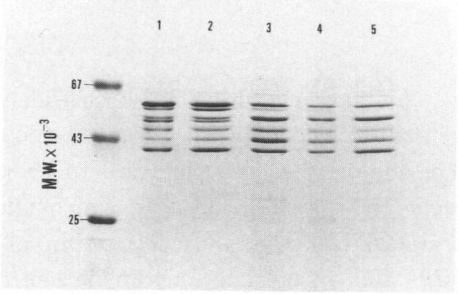


Fig. 11 Keratin profiles of re-cultured cells in serum containing culture. Proteins were extracted at each passages.
Lane 1: PF culture.
Lane 2: The passage 0.
Lane 3: The passage 1.
Lane 4: The passage 2.
Lane 5: Serum containing culture.

seen in SF and PF culture, whereas a very faint staining pattern was seen in serum containing culture.

Keratin profiles

The keratins extracted from OSC-19 cells in different culture conditions were analyzed by one-dimensional SDS-PAGE. Fig. 8 (lane 1) illustrates the keratin profile of the cells maintained in serum supplemented medium. Five major bands (40, 45, 47, 52 and 58 kd) were observed. In SF culture condition, two new major bands (50 and 56 kd) were expressed with the five major bands (Fig. 8, lane 2). The profile under PF condition was identical with that under SF condition (Fig. 8, lane 3). In western blot analysis, PKK1 antibodies reacted with polypeptides of 40, 45 and 52 kd, and MA-903 antibodies with 40, 47, 50, 56 and 58 kd respectively. This revealed that these seven proteins were cytokeratins (Fig. 9).

Restoration of morphology and keratin profiles

The cells were re-fed with the serum containing medium after 20 passages in PF medium. Immediately, many cells showed polygonal shape and cobblestone growth patterns (Fig. 10). The focal stratifications decreased in number and ultrastructural analysis revealed the reduced expression of tonofilament bundles and desmosomes (data not shown). Keratin profiles were not drastically changed at once, but gradual decrease in 50 and 56 kd keratin bands was observed, resulting in complete restoration of keratin profiles to those of parent OSC-19 in serum supplemented culture within a few passages. (Fig. 11).

DISCUSSION

Animal cell culture has been widely adapted in many biological studies. It is generally accepted that serum is required to maintain animal cells in culture. The major roles of serum are to provide hormonal and growth factors, attachment and spreading factors, and binding proteins (1). However, there are several disadvantages of using serum in culture (variations among different lots, virus and micoplasma contamination, costliness, etc.). Recently, with the development of culture technique, various types of cells have been successfully maintained in SF chemically defined media(4, 8, 21). In SF culture, several factors which support cell growth are generally added to culture media. Barnes and Sato reported that insulin and transferrin were required to support the growth of most cell lines examined in SF culture condition (2). We also employed these two factors and selenium to support the growth of a cell line, OSC-19 described in this paper. OSC-19 cells showed favorable growth in the SF medium and over

60 passages were carried out to date. Furthermore, the cells maintained in SF culture medium could continuously grow in PF medium which contained no exogenous factors. This indicated that OSC-19 cells possessed excellent adaptability to unfavorable growth conditions. There are several reports that transformed cells secrete growth factors which stimulate their own growth (10, 11, 17). OSC-19 cells might also secrete growth factors which support its own growth in PF medium.

When cultured in the serum depleted media, OSC-19 cells showed morphological changes as compared with the cells in serum containing culture. In serum depleted culture condition, the cells had a tendency to elongate and grew in scrollwork-like patterns. Although the exact mechanism to form the scrollwork-like growth patterns is not known, the development of desmosomes might play an important role in the scrollwork-like growth pattern, for, as shown in Fig. 2, many desmosomes were detected even before the confluent culture.

With the change of cell shape, focal cellular stratified structure was observed and ultrastructural examination revealed the well developed tonofilament bundles and desmosomes. In addition, immunofluorescence microscopy using antibody against keratin of human epidermal stratum corneum revealed more developed fibrillar structure of the cells in serum depleted culture. These findings suggested that serum depletion induced differentiation of OSC-19 cells. However, this stratified structure was somewhat different from that of normal keratinocyte (9), because the unidirectional differentiation from the bottom to top layer was not observed in our experiment. The cornified cell envelopes and horny cells, both of which are phenotypic markers of terminal differentiation, were not detected in the present study. Considering these observations, it may be reasonable to suppose that OSC-19 cells in SF or PF medium do not have the ability to terminal differentiation.

Cultured normal epidermal cells are known to expressed four major (46, 50, 56 and 58 kd) and three minor keratins (40, 48 and 52 kd) (3, 18). The cell lines established from human squamous cell carcinomas also express similar keratin classes, but have a more increased level of 40 kd keratin (22). OSC-19 cells in serum containing culture expressed five major keratins (40, 45, 47, 52 and 58 kd). When cultured in serum depleted culture media, two new major keratins (50 and 56 kd) were observed and 58 kd keratin was significantly increased. The 50 and 58 kd keratin classes (No. 14 and 5, according to Moll *et al.*) (14) are thought to be a marker for stratified squamous epithelia (15). This may be true in our experiment, since OSC-19 cells in serum depleted culture showed focal stratification and increased expression of 50 and 58 kd keratins. The 56.5 kd keratin (No. 10) is shown to be a marker for keratinization (19). If the 56

kd keratin seen in our experiment was the same keratin as the marker keratin (No. 10), the expression of the 56 kd keratin was in accord with a more differentiated state of OSC-19 cells in serum depleted culture. The analysis using two-dimensional electrophoresis and specific antibodies might provide the probe to clarify these problems. High molecular weight keratins (65-68 kd) which are characteristic of terminal squamous differentiation were not detected even in SF or PF cultures. This correlated well with the morphological analysis in which terminal squamous differentiation was not observed in SF or PF culture.

There was a possibility that the selection of the cells capable of growing in serum depleted media caused the morphological and cytoskeletal alteration of OSC-19. To examine whether such selection resulted in these alterations or not, the cells maintained in PF medium were re-fed with serum containing medium. Immediately after medium changes, the cell shape and growth patterns became similar to those of parent OSC-19 cells which were continuously maintained in serum containing medium. Within a few passages in serum supplemeted medium, the stratifications were not detected. These changes correlated well with the changes in keratin profiles (Fig. 11). These results suggest strongly that the morphological and cytoskeletal alterations are not due to the selection, and that serum contains some factors which inhibit squamous differentiation.

It is well known that vitamin A has an important function in the epithelial differentiation and inhibits complete differentiation of cultured human keratinocytes (6, 16). The more differentiated state in chemically defined media was supposed to be mainly due to the deprivation of vitamin A, but it was impossible to deny that the serum contains other factors which inhibit the differentiation of keratinocytes. The analysis of the effect of vitamin A on OSC -19 cells is now underway.

OSC-19 cell line capable of growing in SF and PF medium should prove to be a valuable tool for studies relating not only to the effects of hormones and growth factors, but also to the mechanism of differentiation and, if it produces some growth factors, autocrine secretion.

ACKNOWLEDGMENTS

We wish to thank Prof. Yohichi Mochizuki and Prof. Gen-iku Kohama for their valuable suggestions, and Ms. Minako Kuwano for technical assistance. This work was supported by a Grant-in-Aid from Hokkaido Geriatric Research Institute.

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