Alterations in the Surface Morphology of Synchronized Cells of Hamster Kidney Carcinoma During the Cell Cycle

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(Received February 25, 1980)

ABSTRACT

To correlate changes of cell surface structure with the cell cycle phases, scanning electron microscopic observation was made on culture cell line of renal carcinoma of Syrian hamster (HKC-400) which was synchronized by double thymidine block. Cell surface morphology could be classified into three major categories depending on the cell cycle phases i.e. flattening with few excrescences in the S phase, many blebs and filaments in the M phase and active ruffling in early G1. Further study is needed to characterize G2 and late-G1 cells. Functional significance was also discussed.

Key words: surface morphology; cell cycle; synchronous culture

INTRODUCTION

The cell cycle traverse is known to be accompanied by cyclic changes of various structural and biochemical characteristics such as DNA replication in a discrete phase, the condensation and de-condensation of chromatin, increase in the number of mitochondria[1-3]. Cell surface which plays a role in mediating messages from outside and inhibiting or triggering initiation of cell proliferation also changes during the cell cycle[4]. Contact inhibition, changes in cell motility, and rounding of cells during mitosis are features that are relevant both to the cell surface and to the cell cycle progression[4-6].

Although there are many reports on the scanning electron microscopic observation of cell surface, a few are concerned with cell cycle phases or cell proliferative capacity[7-10]. The present paper describes the changes of cell surface morphology as observed by the scanning electron microscopy (S.E.M.) of synchronous culture of renal carcinoma of Syrian

hamster in various phases of the cell cycle. The functional significance will also be discussed briefly.

MATERIALS AND METHODS

Two series of experiments were performed using asynchronous cells in exponential phase of growth and those synchronized by thymidine double block.

Cell: Hamster kidney carcinoma (HKC-400) which was used in this experiment was originally induced in a male Syrian hamster by repeated subcutaneous injection of diethylstilbestrol, a synthetic estrogen, and then transformed to a tissue culture strain. This culture has been maintained in our laboratory for more than 7 years before use. The culture medium was Ham's F-12[11] (Nissui Co., Tokyo) supplemented with 10% calf serum and 0.06 mg/ml of Keflin (Cefalotin sodium, Shionogi Co., Osaka). The cells grew in monolayer and were subcultured weekly using 0.04% trypsin and 0.02% EDTA in phosphate buffered saline.

Asynchronous culture: Cells were subcultured on plastic cover slip in Petri dishes and incubated in a CO_2 incubator for 2 days before fixation and observation with S.E.M.

Synchronous culture: Two days prior to synchronization procedure, the cells were transplanted to Petri dishes which contained plastic cover slips or slide glass for later observation with S.E.M. and with autoradiography. The cultures were placed in a CO_2 incubator. Synchronization was then performed by double thymidine block according to the protocol of Galavazi et al[12]. The first and second block was made 7 h apart each block lasting for 13 h based on the cell cycle parameters (T_{G1}=7.4 h, T_S=6.0 h, T_{G2}=4.9 h, T_M=0.2 h) measured in a preliminary experiment by the fraction of labeled mitoses method. The block was released by washing twice in pre-warmed and CO_2-incubated medium. A special caution was exercised during washing in order not to lower the temperature, otherwise many cells were dislodged from the cover slip. After release from the second block, cover slips and slide glass were taken out at hourly interval and processed for S.E.M. and autoradiography.

Autoradiography: In order to examine the effect of synchronization procedure, ^{3}H-thymidine labeling indices were measured at hourly interval for 7 h after the release from block as follws. The cells were pulse-labeled for 20 min in culture medium containing 1 μCi/ml of ^{3}H-thymidine (methyl-^{3}H-thymidine, The Radiochemical Center, Amersham,

specific activity 2.0 Ci/mM) prepared from thymidine-free modification of F-12 (Nissui Co., Tokyo). Then, the culture was washed once in pre-warmed F-12 medium without serum before being fixed in ethanol-acetic acid (3 : 1) for 1 h. The fixed samples were washed in 70% ethanol and distilled water. The slides were coated with photographic emulsion (ET2F, generously supplied by Fuji Photo Film Co. Ltd., Tokyo) according to a dipping method, exposed for 1 week in a refrigerator (at 4°C), developed and stained with hematoxylin. Labeled cells were counted under high power view (×400). Mitotic index was also measured on the same preparation.

S.E.M.: Cells on the plastic cover slip were rinsed once with pre-warmed F-12 medium without serum and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer and post-fixed with 1% OsO_4 in the above buffer. They were then dehydrated in graded alcohol, passed through rising concentration of amyl acetate in alcohol and dried in desiccator. The dried specimens were coated with gold-paradium and observed by a scanning electron microscope JSM-S1 (Japan Electron Optics Laboratory, Tokyo) at 10kV.

RESULTS

〔1〕 Cell Synchrony

A state of partial synchrony was obtained by the above described procedure. Fig. 1 showes changes in pulse labeling index (LI) and mitotic index (MI). The LI was practically zero at the time of release and rose quickly for 2 h to attain a level of about 80% which was maintained for the succeeding 4 h. The LI fell to about 60% at the 7th h after the release. Mitotic figures were first found at the 6th h and increased gradually and progressively to reach 5% level by the end of observation. The arrested cells showed no degenerative changes such as cell shrinkage, vacuolization and desquamation.

〔2〕 S.E.M.

Observation was made on synchronous and asynchronous cultures. Synchronous culture: Materials were divided into two groups depending on the time of sampling after release of the second block. The cells that were taken within 6 h after release were considered to belong to the S phase and the cells that were taken more than 6 h after release contain other phases. They will be called "DNA synthetic" and "miscellaneous phase" group, respectively.

i) DNA synthetic group: Majority of cells (approximately 90%) in this group were ovoid, measuring approximately 50×80 μm (Fig. 2). The

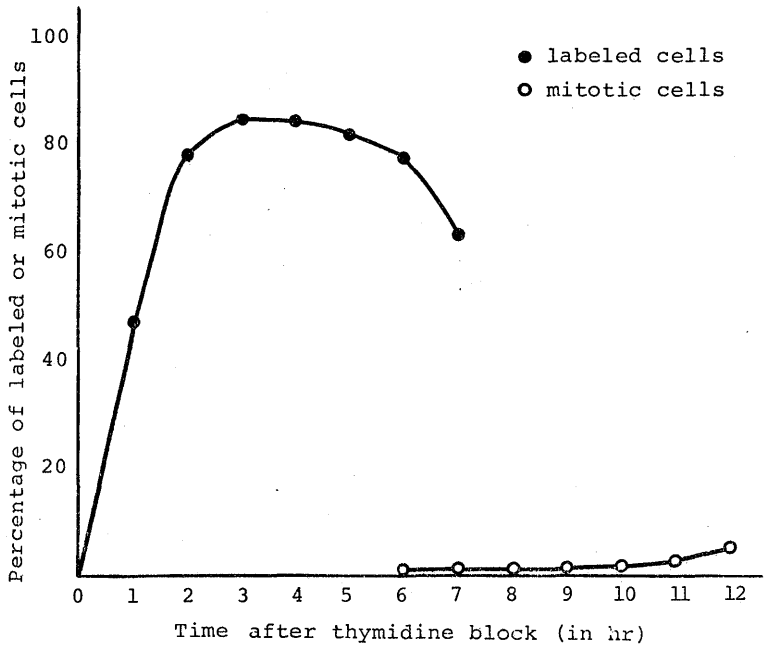

Fig. 1. The percentages of labeled cells and mtotic cells are plotted against time after release from thymidine block.

nucleus was centrally located and the overlying cytoplasm was slightly elevated. Microvilli were short and sparsely distributed. A few cytoplasmic blebs of spherical shape (1-5 μm in diameter) were on the perimeter of nucleus. They had a thick stalk and gave a polypoid appearance. Ruffles of cytoplasmic margin were few in number and small in size. The remaining cells (about 10%) were like a folding fan in shape and measured approximately 30×80 μm (Fig. 3). The nucleus was at the site that corresponded to the rivet. The cytoplasmic margin was curved like an arc and extended laterally from the rivet. The cytoplasm tended thicker toward this arc. Other features including blebs and ruffles were the same as observed in the ovoid cells. These fan-shaped cells were not necessarily representing quiescent cells, for they were labeled.

ii) Miscellaneous phase group: The cells sampled more than 6 h after the second block were not strikingly different morphologically from those described above and were either ovoid and fan-shaped. However, a few percentages of cells exhibited very peculiar features (Fig. 4). Their cytoplasm was contracted and spherical. In reference to light microscopic features, they were considered as metaphase cells. The

surface was covered with numerous blebs of variable sizes giving a coarse granular appearance. In addition, they had a number of long filopodia radially extending from the base or from their outer margin but no microvilli were observed. Telophase cells could be judged from their existence in pairs. They looked like metaphase cells except for their smaller size (Fig. 5). The cells that were considered as early G1 were small with pseudopodia and thick cytoplasm. They were also found in doublet (Fig. 6). Morphological transition from telophase cells was noted (Fig. 7). A trace of filopodia was observed at the periphery of some pseudopodia and the cells were densely covered with many microvilli. Characteristic to this phase was the presence of cytoplasmic ruffling at the periphery of most pseudopodia. Least in number were the cells which differed from metaphase cells. They were flat with a number of microvilli and filopodia (Fig. 8). It was difficult to assign the stage of cell cycle of these cells, but we tentatively considered them as in the prophase.

Asynchronous culture: Every type of cell surface observed in the synchronized culture had their counterparts in the asynchronous population, indicating that none of them was a direct morphological manifestation of thymidine toxicity. However, the cells with features similar to those of the S-phase cells outnumbered the others and it will provide another evidence to indicate that there is no clearcut distinction between G1 and S phase cells.

DISCUSSION

Cell surface morphology was studied in association with the cell cycle traverse using cultures which were partially synchronized by thymidine block. For the purpose of studying cells in the S phase or thereafter, the present method is advantageous over mitotic selection method because by the latter technique cell synchrony deteriolates rapidly in G1. A question may be raised whether the observed changes are cell cycle-dependent or simply representing thymidine effects on cell metabolism other than DNA[13,14]. The fact that each morphologic type was also found in the untreated asynchronous culture, may be an answer in favor of the former possibility.

The surface changes were generally expressed in various forms of cytoplasmic excrescences which include microvilli, blebs, filopodia, pseudopodia and ruffles. The present observation were in fair agreement with the reports by other investigators[7-10] in that there exists correlation

between cell surface morphology and cell cycle phases. The S phase cells are characteristically flat and ovoid. The cytoplasm is smooth-surfaced and extremely thinned out toward periphery with only a few small ruffles. Microvilli, even if present, are few in number and sparsely distributed over the entire surface. The number of microvilli is not increased during the S phase and in this respect the present result differs from the report of Lundgren and Roos[7]. The cells in the G2 phase are difficult to be identified because the culture is desynchronized to a large extent before this phase is reached. However, in the prophase, numerous microvilli appear rather abruptly. The most dramatic and outstanding changes occur successively in the phases that follow. During mitosis the cells are rounded and contracted with the cytoplasm being retracted. Presence of long filopodia is the feature that characterizes metaphase. It is not clear, however, whether it is produced as a result of retraction of cytoplasm that was anchored to the surface of cover slip or it is projected from inside[8,9]. Other features of importance in this phase are the blebs that cover the entire surface and the abscence of microvilli. This may suggest a possibility that the blebs are transient transformation of microvilli. Actually, the same surface morphology as in the prophase is restored in telophase. Several hypotheses[5,10] have been proposed as regard to the significance of blebs and microvilli: Pasternak[15] considered that microvilli serve as membrane resorvoir that can provide extra surface area for cell division. In early G1, many large ruffles are observed at the end of pseudopodia. Since they are formed in all directions, the cells may remain in the place but their functional significance may lie in increasing surface area and accelerating exchange of substance to cope with the increasing metabolic demand[9,16]. In contrast, fan-shaped cells have ruffles only on one side and they are in a state of active locomotion[17].

Minor discrepancy of results among investigators may be due to difference of cell lines, different culture conditions such as cell density and different methods of preparing sample for S.E.M. According to our experience, the surface morphology is very unstable and subject to rapid fluctuations depending on the environmental conditions. Yet, it was possible to classify the cell surface characteristics into three major categories i.e. cell flattening with few excrescences in the S phase, many blebs and filamentous structures in the M phase and active ruffling with many microvilli in early G1. Further study is required to characterize cells in G2 and after mid-G1 phases.

Relevance of fewer microvilli on the S phase cells to the intracellular events is unclear. Lundgren and Roos[7] demonstrated that cell surface

morphology characteristic to the S phase did not appear when DNA synthesis was arrested by 1-β-D-arabinofuranosylcytosine, hydroxyurea, mitomycin C, or bleomycin in spite of a continued cytoplasmic growth. This result suggests strong influence of nuclear activity to cell surface change. However, according to Allred et al. a large fraction of cells of WI-38, a slow growing cell line of human origin, were either in G1 or G0 state but their surface morphology was indistinguishable from the S-phase cells[5]. Therefore, it must be admitted that the transition of cell surface structure from G1 to S is gradual and does not coincide with the initiation of DNA synthesis.

ACKNOWLEDGEMENT

The author wishes to thank Professor M. Takahashi for valuable discussion and data analysis.

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EXPLANATION OF PLATES

Fig. 2. Ovoid cells in the S phase cells with few excrescences are extremely flat. ×600.
Fig. 3. Fan-shaped S-phase cell migrating upwards in this figure. Note ruffles at the leading edge. ×1, 000.
Fig. 4. Round metaphase cell with many long filopodia. Microvilli observed in prophase cell are replaced by numerous small blebs. ×3, 000.
Fig. 5. Telophase cell in doublet. Surface characteristics are similar to those of metaphase cell. ×2, 000.
Fig. 6. Two early G1 cells with prominet ruffles. Microvilli are restored. Blebs and filopodia are diminished. ×1, 500.
Fig. 7. Cells of transitional type from telophase to early Gl. ×3, 000.
Fig. 8. Probable prophase cell with many filopodia and microvilli. ×1, 500.

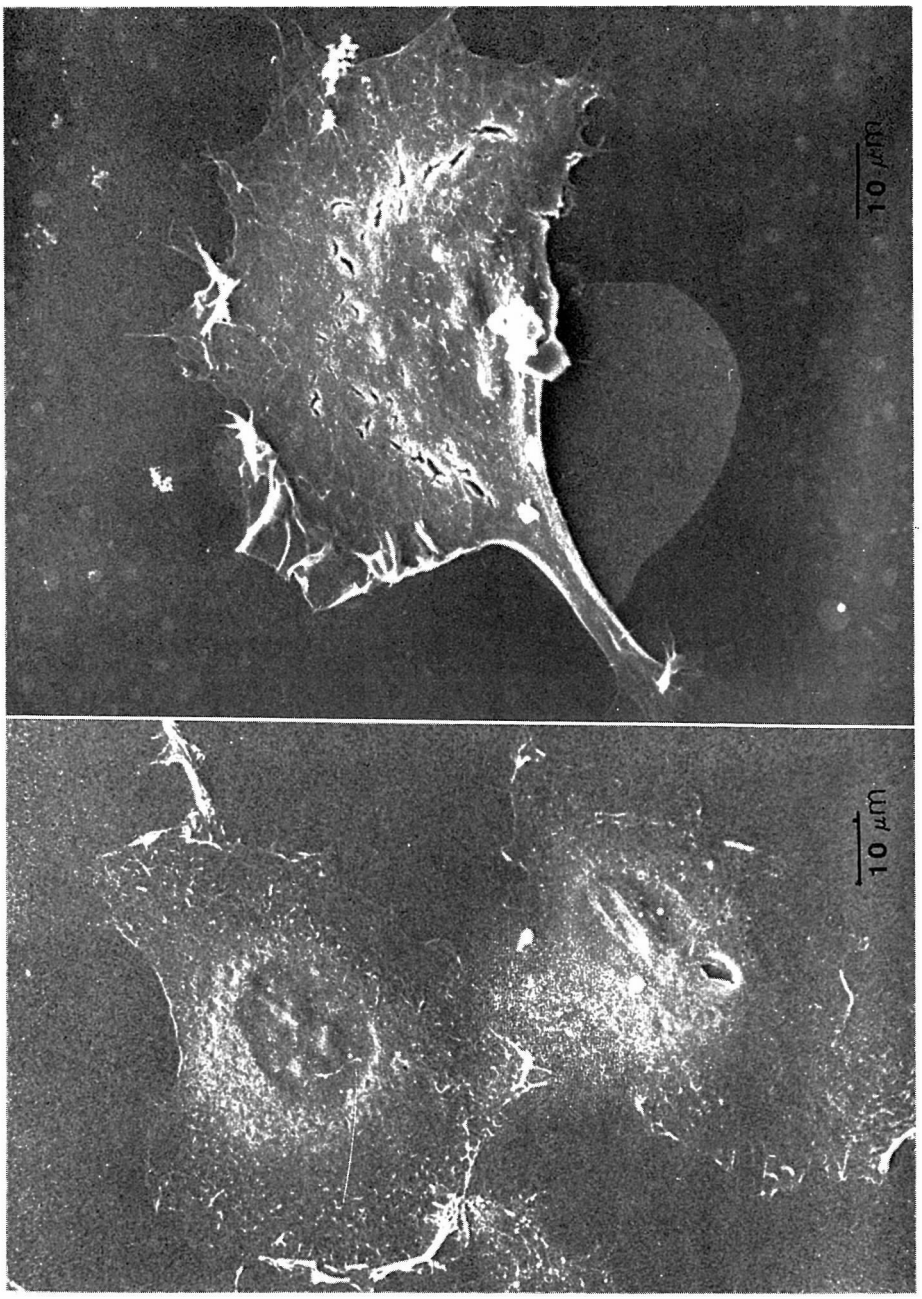

Fig. 3

Fig. 2

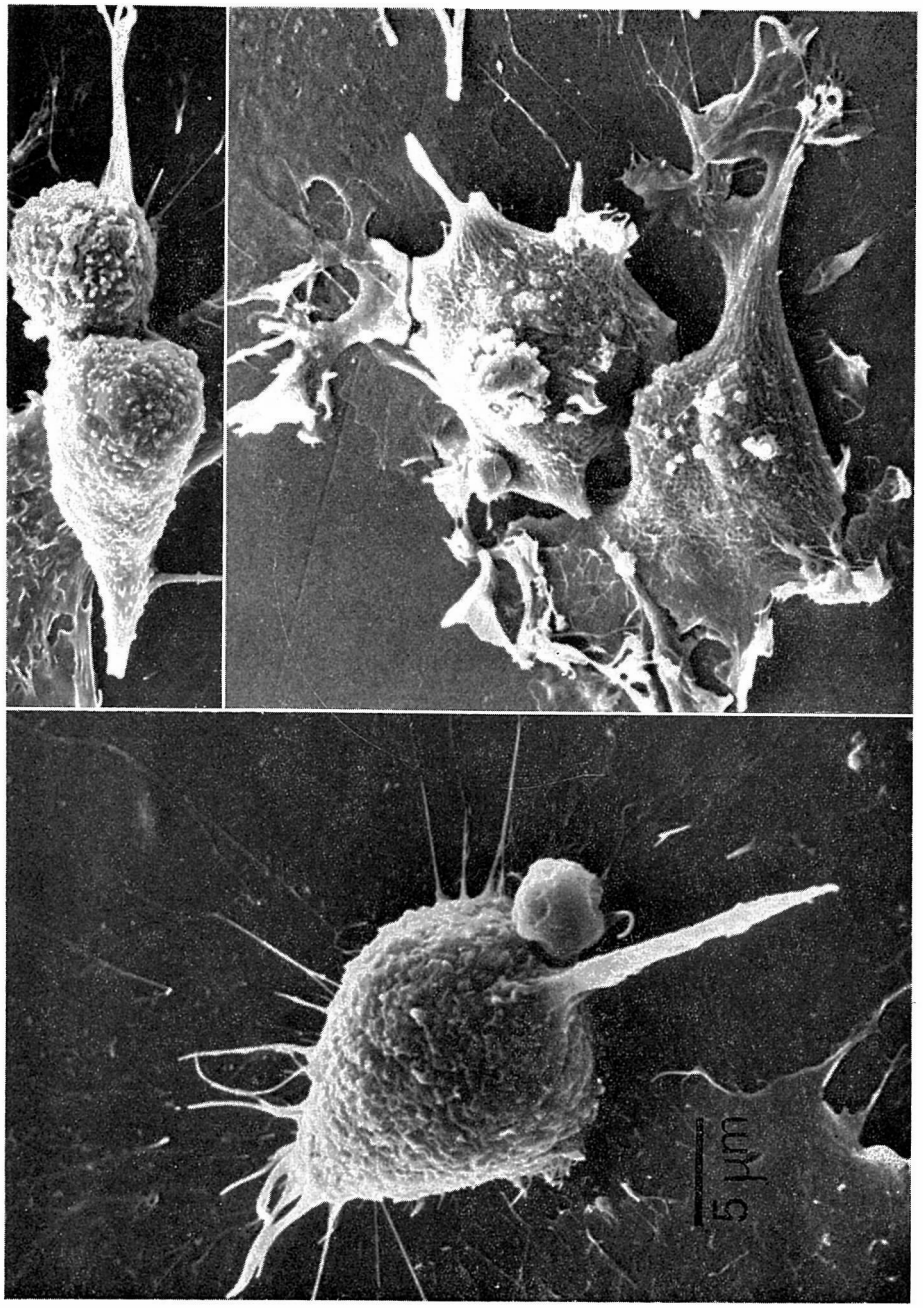

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Fig. 4

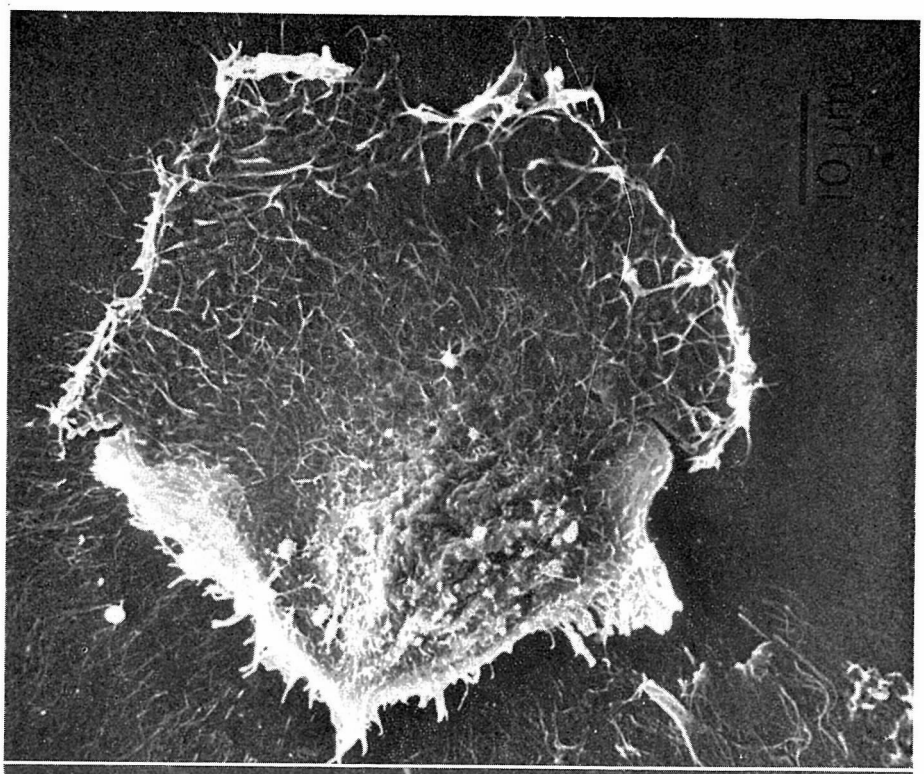

Fig. 8

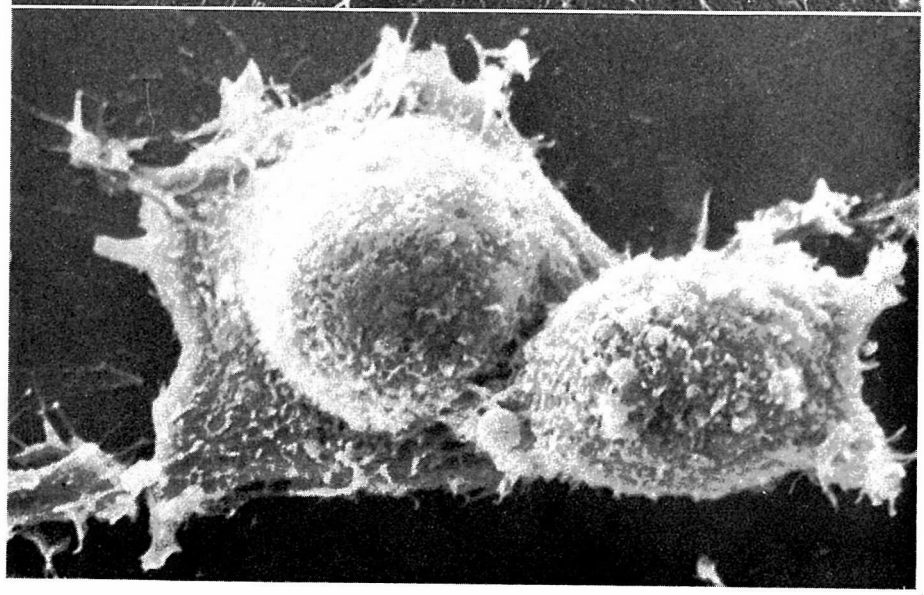

Fig. 7