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Fluorescence and Electron Microscopic Studies of the Cytoskeletal Organi-zation of Normal, Established and Transformed Chick Embryo Cells

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Abstract

The cytoskeletons of two established chick embryo cell (CEC) lines were examined by fluorescence and electron microscopy and compared with those of control cells and cells transformed by Rous sarcoma virus (RSV). In normal CEC, many stress fibers were observed. On the other hand, stress fibers were disorganized in nontransformed spontaneously established CEC, non-tumorigenic CEC partially transformed with a chemical carcinogen, and tumorigenic RSV-transformed CEC. In the normal CEC, actin filaments formed several bundles along the processes of the cell. Stereo-images of the peripheral region revealed bundles of filaments which were located along the attached side to the substrate. A fine well preserved network of filaments was also observed. On the other hand, in spontaneously established, partially transformed and RSV-transformed CEC, a fine network of filaments, but no actin cables, was found. These results support previous evidence that the cytoskeletal changes themselves are not directly related to the transformation or tumorigenicity of cells.

KEYWORDS: chick embryo cells, cytoskeleton, actin, Triton X-100, Rous sarcoma virus.

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The cytoskeletons of two established chick embryo cell (CEC) lines were examined by fluorescence and electron microscopy and compared with those of control cells and cells transformed by Rous sarcoma virus (RSV). In normal CEC, many stress fibers were observed. On the other hand, stress fibers were disorganized in non-transformed spontaneously established CEC, non-tumorigenic CEC partially transformed with a chemical carcinogen, and tumorigenic RSV-transformed CEC. In the normal CEC, actin filaments formed several bundles along the processes of the cell. Stereo-images of the peripheral region revealed bundles of filaments which were located along the attached side to the substrate. A fine well preserved network of filaments was also observed. On the other hand, in spontaneously established, partially transformed and RSV-transformed CEC, a fine network of filaments, but no actin cables, was found. These results support previous evidence that the cytoskeletal changes themselves are not directly related to the transformation or tumorigenicity of cells.

Key words : **chick embryo cells, cytoskeleton, actin, Triton X-100, Rous sarcoma virus.**

Stress fibers, a major cytoskeletal component first described by Lewis *et al.* (1), consist of microfilament bundles (2). By the use of immunofluorescence microscopy, they have been shown to contain several contractile proteins such as actin (3), myosin (4), alpha-actinin (5) and tropomyosin (6). The organization of these stress fibers was shown to change following transformation, and disorganized microfilaments were observed following transformation by RSV. Intensive studies have been made to elucidate the correlation of transformation by RSV and intracellular cytoskeletal changes, especially concerning the organization of stress fibers (7). Cytoskeletal changes are thought to result from the direct interaction of $pp60^{src}$, a single viral oncogene product of RSV, with microfilaments or microfilament-associated proteins (7-12). On the other hand, Notter and Balduzzi (13) recently reported that the organization of actin and tubulin was unaffected in CEC transformed by certain strains of RSV. More recently,

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Nigg *et al.* (14) reported that the morphology of a transformed cell is not determined simply by pp60^{src} but by a combination of several factors. In systems other than RSV, some research groups have observed the loss of actin organization in a benign-to-malignant tumor transition (15) while other groups have shown that the loss of microfilament bundles was not related to tumorigenicity (16-18). Thus, there are discrepancies about the correlation between cytoskeletal changes and transformation or tumorigenicity.

To study systematically the correlation of cytoskeletal structure and cellular transformation, we used two avian fibroblastic cell lines established by Ogura *et al.* (19), their normal counterparts and RSV-transformed cells. Particular attention was paid to the changes of stress fibers in these cells.

Materials and Methods

Cells and virus. Embryonated eggs were obtained from Kanonji Institute, the Research Foundation for Microbial Disease of Osaka University. The CEC culture was prepared by the routine method. The Schmidt-Ruppin strain of RSV was used for transformation of CEC. The non-transformed spontaneously established CEC line (SPCC-OU1) and the CEC line (CHCC-OU1) partially transformed by treament with N-methyl-N'-nitro-N-nitrosoguanidine were described previously (19). The cultures were maintained in a CO_2-incubator at 41°C.

Fluorescence microscopy. Cells of the last passage were grown on coverslips. The cells were fixed with 3.7% paraformaldehyde, treated with absolute acetone at −20°C for 5 min, and then air dried. The cells on coverslips were stained with a few drops of rhodamine-labeled phalloidin, a cyclic peptide that specifically binds to F-actin (20, 21), in a moist chamber at room temperature for 20 min, washed with a buffer solution and kept in 50% glycerin solution. The samples were observed with an Olympus fluorescence microscope (BH-2). Photographs were taken with Kodak Tri-X film.

Electron microscopy. Gold grids, sandwiched between a thin layer of Formvar and coverslips, were coated with carbon and sterilized by ultraviolet irradiation (12). Chick embryo cells were grown on these carbon- and Formvar-coated EM grids on coverslips. These coverslips were treated with 0.15% Triton X-100 dissolved in a PHEM buffer solution containing 60 mM piperazine-N, N'-bis (2-ethanesulfonic acid), 25 mM N-2-hydroxy ethylpiperazine-N'-2-ethanesulfonic acid, 10 mM ethylene glycol bis-(β-aminoethyl-ether) N, N, N', N'-tetraacetic acid (EGTA) and 2 mM $MgCl_2$ (22) for 1 min at room temperature. They were fixed with 2.5% glutaraldehyde solution for 10-20 min and postfixed with 0.5% osmium tetroxide in 0.05 M phosphate buffer, pH 6.0, for 1 min at 0°C. They were dehydrated with a graded ethanol series, immersed in isoamyl acetate and dried by critical point drying, and observed without staining with a transmission electron microscope (JEM 100 CX) at an accelerating voltage of 80 kV.

Results

Fig. 1a is a fluorescence micrograph showing the distribution of actin stained with rhodamine-labeled phalloidin in a normal C-EC. Many stress fibers extended along the long axis of normal cells, and some fibers crossed them to give a lattice appearance. These fibers extended into the ruffles and terminated at the edges or a few micrometers from the edges of the ruffles. The submembranous region without membrane ruffles was also stained intensely so that the concave of the cell border was delimited. Both spontaneously established SPCC-OU1 cells and partially transformed CHCC-OU1 cells showed similar structures. Stress fibers were disorganized and the background fluorescent intensity decreased. Although stress

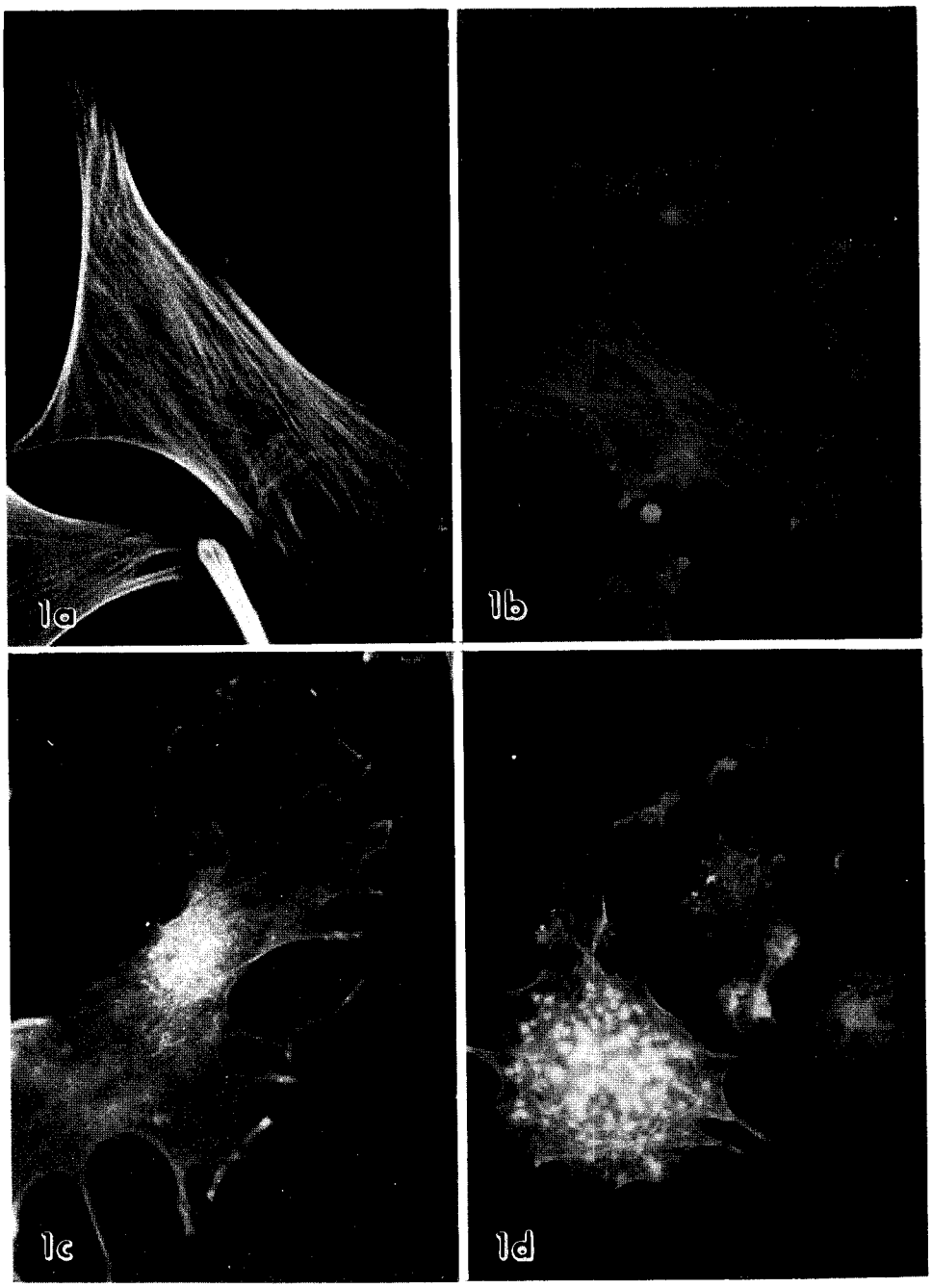

Fig. 1 Fluorescence micrographs of chick embryo cells (CEC) stained with rhodamin (rh)-phalloidin. (a) Normal CEC. Many stress fibers run along the long axis of the cells and into the ruffles. The cell cortex without membrane ruffles is also stained intensely. ×600. (b) Spontaneuosly established CEC. Stress fibers are disorganized although filaments remain in the cytoplasm. Fluorescent intensity is decreased. ×600. (c) Partially transformed CEC. Fluorescent intensity in the cytoplasm is decreased. Intense fluorescence is seen in the center of the cell. ×600. (d) RSV-transformed CEC. The cells are round and have fine filaments in the cytoplasm, but stress fibers are not observed. Actin is concentrated at the ruffle and around the nucleus. Actin aggregates (actin patches) are present. ×500.

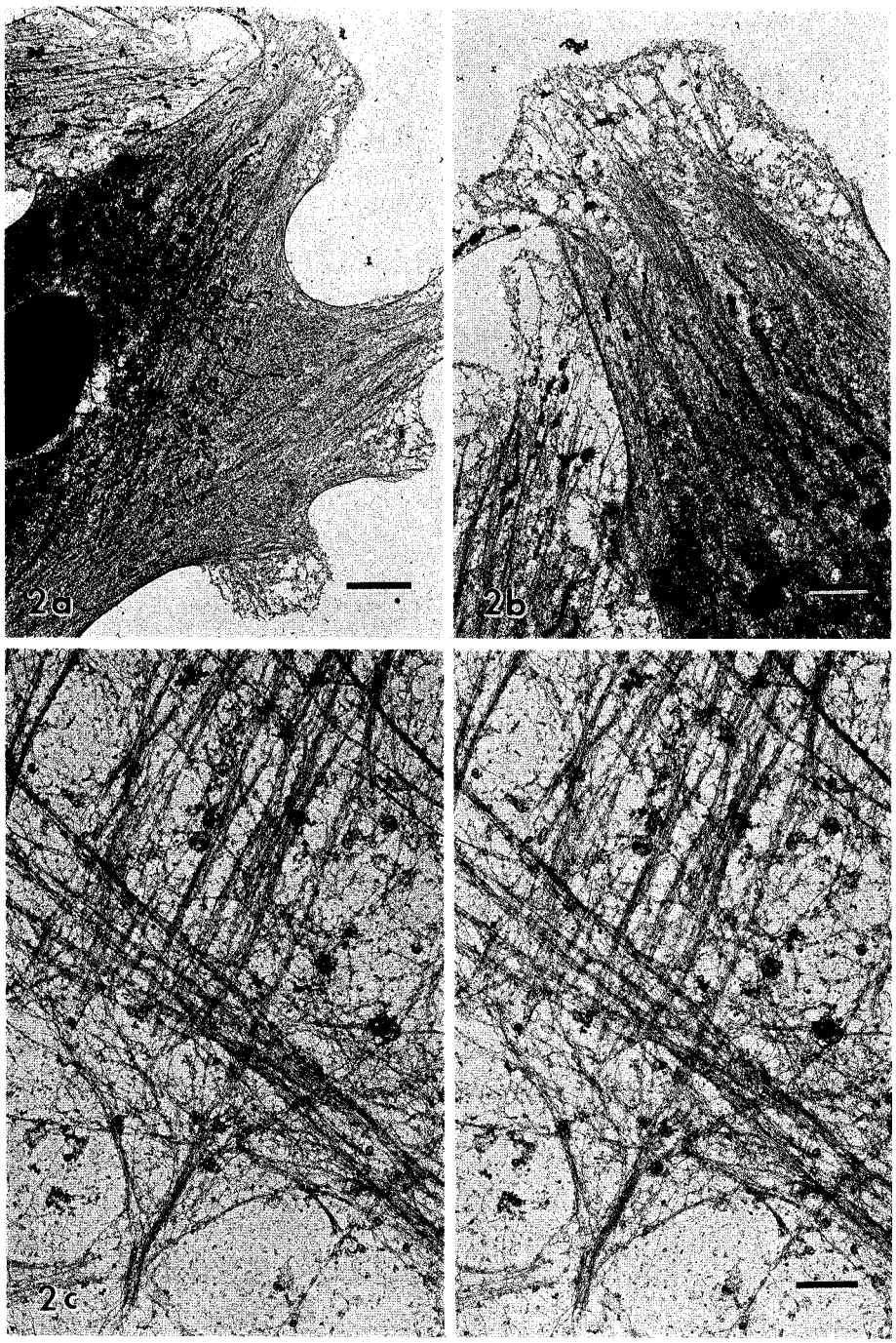

Fig. 2 Transmission electron micrographs of normal CEC treated with 0.15% Triton X-100. (a) Many filaments form several bundles identified as stress fibers by fluorescent microscopy. Bar=5 μm. (b) These bundles terminate before the edges of the cell and form focal patches where these cells adhered to the substratum. Bar=2 μm. (c) Stereopair of micrographs showing radiating filament bundles. Granular bodies exist in an area more dorsal than these filaments. Bar=1 μm.

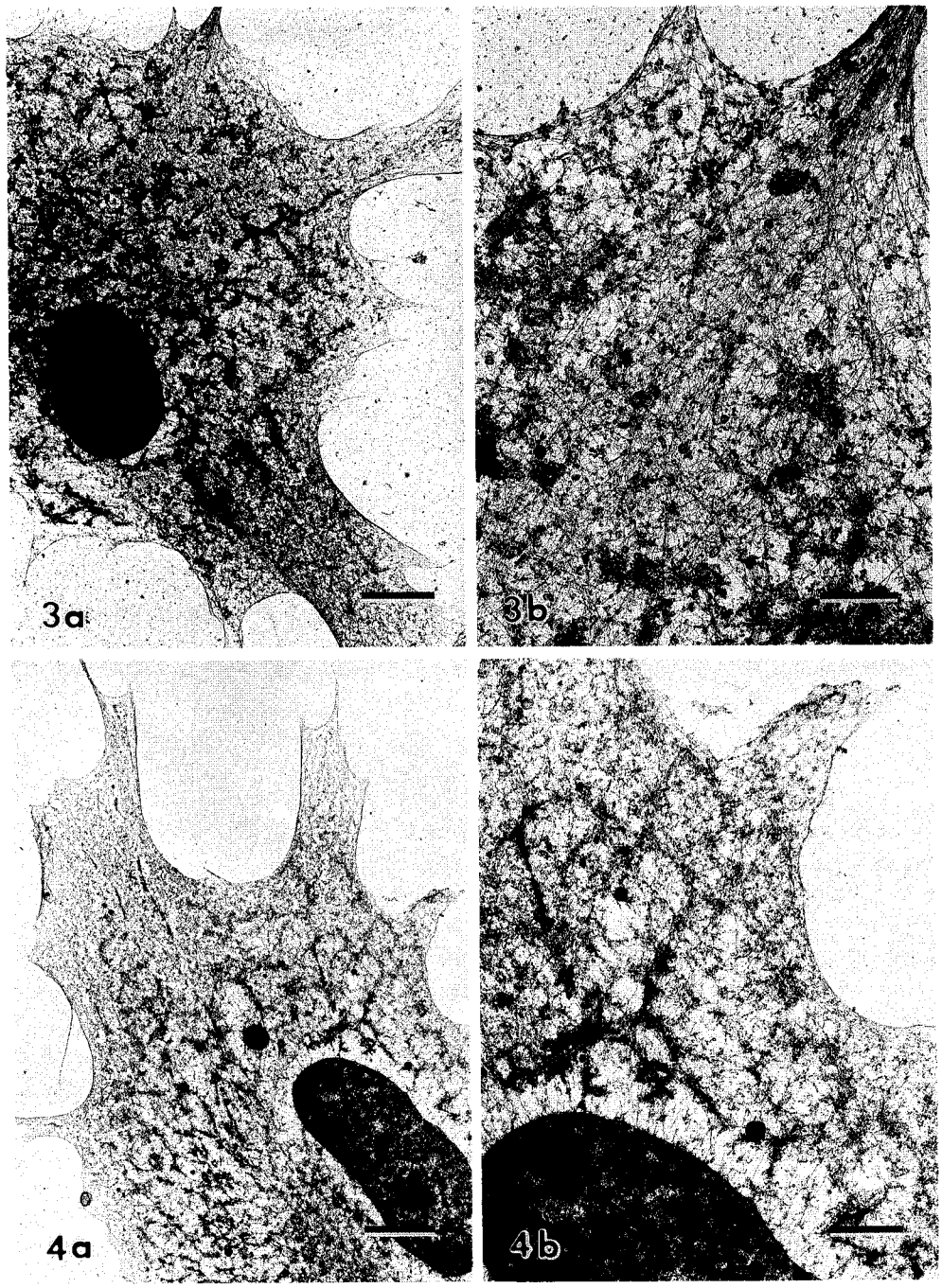

Fig. 3 Transmission electron micrographs of spontaneously established CEC treated with detergent. (a) Flattened cells have several processes whose shapes are the same as observed by fluorescence microscopy. Bar=5 μm (b) In the processes, thicker filaments are observed. Bar=1 μm.

Fig. 4 Transmission electron micrographs of partially transformed CEC treated with detergent. (a) The morphology is quite similar to spontaneously established CEC. Bar=5 μm. (b) Thicker filaments are observed around the nucleus. Bar=2 μm.

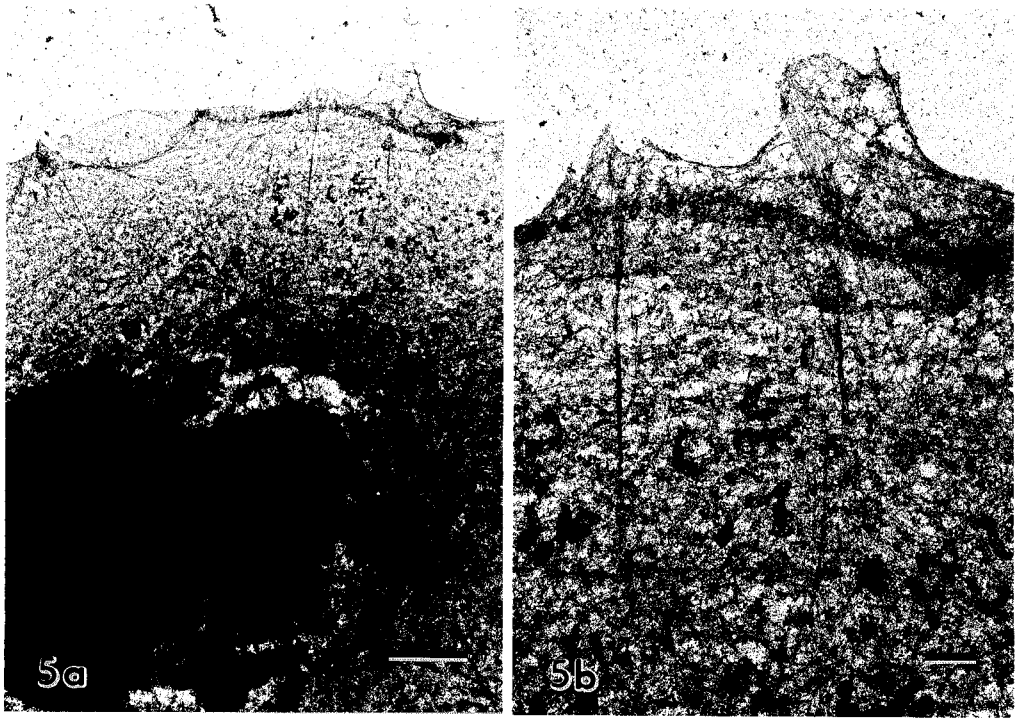

Fig. 5 Transmission electron micrographs of RSV-transformed CEC treated with detergent. (a) Stress fibers are not found in these cells, but fine filaments run radially from the nucleus to the periphery. Bar=5 μm. (b) Some filaments run along the cell periphery. Bar=1 μm.

fibers were no more visible, weakly stained fine filaments were present in the cytoplasm (Fig. 1 b). Actin often was concentrated at the periphery of the cell and around the nucleus (Fig. 1 c). RSV-transformed CEC became round with fine filaments in the cytoplasm, but stress fibers were not ovserved. Actin was concentrated at the ruffle or around the nucleus. Actin aggregates (actin patches) were often present near the ventral surface of the cells (Fig. 1 d).

The treatment of the cells with a detergent solution removed the cytoplasmic materials satisfactorily and revealed the cytoskeletal organization of these cells (Figs. 2-5). Figs. 2 a-c show normal CEC treated with detergent. Many filaments formed several bundles identified as stress fibers by fluorescence microscopy. These bundles terminated before the edges of the cell where these cells adhered to the substratum, forming focal patches. Fine filaments splayed to the edges and formed a network at the ruffles where cytoplasmic materials were scarce. Stereo-pairs of micrographs showed that radiating filament bundles ran along the attached side to the substratum, and fibers which delimited the cell edges crossed over these filament bundles. Granular bodies existed in an area more dorsal to these filaments. The network of filaments was well preserved (Fig. 2 c). In spontaneously established SPCC-OU1 cells (Figs. 3 a, b) and partially transformed CHCC-OU1 cells (Figs. 4 a, b), the shape of the cell margin was the same as that seen by fluorescence microscopy. Stress fibers were not observed, and thicker filaments were often seen around the nucleus. A network of filaments was present in the cell body. RSV-

transformed CEC (Figs. 5 a,b) were round, and stress fibers were not formed in these cells, but fine filaments radiated from the nucleus region to the periphery. Some filaments ran along the cell periphery.

Discussion

Previous reports about the correlation between cytoskeletal changes and transformation (7, 11) led us to investigate systematically the cytoskeletal structures in CEC. Transformed cells contained far fewer stress fibers than did their normal counterparts. The cells investigated in this study were: a) normal, b) spontaneously established but not transformed (SPCC-OU1), c) partially transformed by a chemical carcinogen but not tumorigenic and d) tumorigenic RSV-transformed CECs. The SPCC-OU1 cells are morphologically normal and form no colony in soft agar, while CHCC-OU1 cells pile up and form colonies in soft agar, but do not produce tumors either in the syngeneic chicken or in nude mice (19).

To study the fine structure of the cytoskeleton, we applied fluorescence microscopy using rhodamine-labeled phalloidin and electron microscopy using a detergent (23, 24). Typical stress fibers were observed by fluorescence microscopy only in normal CEC. In spontaneously established non-transformed SPCC-OU1, partially transformed, non-tumorigenic CHCC-OU1 and tumorigenic RSV-transformed CEC, stress fibers were disorganized. Instead of the bundles of filaments which constitute stress fibers, a fine network of filaments was observed in these cells by electron microscopy. The fluorescence microscopic findings correlated well with the electron microscopic findings.

These observations are in agreement with the reports which state that there is no direct correlation between cytoskeletal changes and transformation (13, 14), and, accordingly, disagree with the proposal that a correlation exists between stress fibers and transformation (8, 9, 12). The idea that the cytoskeletal change causes a loss of adhesiveness to the substratum and allows cells to deform and invade neighboring tissues (15) is simple and tempting, but not certain (16-18).

The cytoplasmic aggregates described as a flower (7, 11) or patch (25) were observed in CEC transformed by RSV but not in CEC transformed by a chemical carcinogen. At present, the role and meaning of these structures in RSV-transformed cells are unknown.

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