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THE HUMAN ENDOTHELIAL CELL IN TISSUE CULTURE*

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With 25 Figures in the Text

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Introduction

In spite of many painstaking studies on endothelial cells in vitro since LEWIS (1921) first reported the culture of these elements from liver sinusoids, their morphology and identification have not yet been consolidated to reach a definite conclusion.

According to most previous reports (RIENHOFF 1922, MAXIMOW 1925, SCHOPPER 1929, SILBERBERG 1930, BENEWOLENSKAJA 1930, HERZOG and SCHOPPER 1931, LEWIS 1931, SCRIBA 1935, TÖRÖ 1937, CAMERON and CHAMBERS 1937, MURRAY and STOUT 1944, WHITE and PARSHLEY 1951) endothelial cells grew in capillary-like formations. But other authors reported that they developed loose reticular formations (LEWIS 1922, NISHIBE 1928—1929) and rarely formed a sheet. MURRAY and STOUT (1944) considered the cells in sheets as atypical ones, while KHLOPIN (1957), KHLOPIN and CHISTOVA (1958) regarded endothelial cells as highly specialized elements which differed from and never transformed into connective tissue cells in vitro.

Judging from the observations of many investigators there is a rather common belief that it is difficult to distinguish endothelial cells from fibroblasts in tissue culture and also that endothelial cells may turn into fibroblasts or fibroblast-like cells (ALTSCHUL 1954).

The present experiment was undertaken to investigate morphological characteristics and behavior of endothelial cells in tissue culture. Accordingly, a pure culture of these elements was indispensable, and therefore, special care was taken to get endothelial cells without any contamination with other cells.

Material and Methods

Endothelial cells from the human umbilical vein isolated by the action of trypsin were cultivated. Human umbilical cords ligated at both ends in order that the vein would retain

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blood in its lumen were stored in the ice-box immediately after delivery. Cords which had been stored for more than twelve hours were discarded.

Isolation of cells and culture methods. The umbilical vein was exposed at both cut ends of the cord. A short vinyl tube was inserted through each opening of the vein and fixed tightly. Ca- und Mg-free balanced salt solution was gently perfused and then an 0.2% solution of trypsin (1:300, National Biochemical Co.) was employed to fill the vein. The umbilical cord ligated at both ends was rinsed in a balanced salt solution at 37° C for forty-five minutes. The solution in the vein was poured in a tube together with 20—30 ml of balanced salt solution perfused through the vein in order to collect all dissociated cells in its lumen. The solution was centrifuged twice. The supernatant was discarded and a nutrient medium was added to the sediment which was pipetted gently. Cells suspended in the fluid medium were cultivated in slightly slanting test tubes with narrow coverslips sunk at the bottom, in flat culture tubes and also in Rose chambers, all in the stationary state. At the bottom of the flat tubes cross lines were previously drawn to follow particular cells continuously. Reconstituted rat-tail collagen (BORNSTEIN 1958) was used as a substrate for coverslip culture.

Culture media. Fluid medium consisted of human umbilical cord serum or horse serum 20% and YLH 80% with 200 μg/ml of streptomycin. The YLH medium contained lactoalbumin hydrolysate 0.5% and yeast extract 0.1% in HANKS' balanced salt solution.

Methods of observations. The behavior of the cells and their cytological characteristics were studied for cell identification by phase contrast microscopy and with stained specimens.

JACOBSON's stain and silver nitrate impregnation were usually employed together with neutral red vital-staining and Sudan black B stain for lipoid granules. GOMORI's method for alkaline phosphatase was applied with incubations carried out for 2—4 hours at a p_H of 9.0.

Preparations of umbilical cords. In order to examine the removal of the endothelium and not of subendothelial elements, several segments of the umbilical cord of which venous endothelium had been subjected to trypsin were fixed for sectioning. Deparaffinized sections were usually stained by concentrated aldehyde-fuchsin (FUJITA 1959) followed by the Masson-Goldner method. Preparations of untreated umbilical cord were prepared as controls. Localization of alkaline phosphatase activity in the endothelium of the umbilical vein was examined histochemically following GOMORI's method. Frozen sections fixed with cold acetone were incubated for 40—90 minutes at a p_H of 9.0. β-glycerophosphate was used as a substrate. The shortest interval between delivery and fixation of cords was two hours.

Culture of WHARTON's *jelly.* Explants cut from the WHARTON's jelly were cultured with MAXIMOW's double coverslip and the roller tube methods. Plasma clots were made of equal parts of heparinized cockerel plasma and chicken embryonic extract. Additional nutrient media for roller tube culture consisted of human ascites fluid 50%, GEY's balanced salt solution 45% and chicken embryonic extract 5%. The same media as used for culture of endothelial cells were also employed.

Results

Observations on cultured cells

General characteristics. In the early stage of culture, round cells in which the nucleus occupied its large part were scattered or formed clumps (Fig. 1). After 3—4 days in culture they became flat gradually, sending fine processes to adhere to each other (Fig. 2). In the second stage, 7—14 days in culture, they tended to lie closely and formed an epithelial sheet (Figs. 3 and 4). Some of these fused to form a larger one. In another part of the same culture, cells became loose followed by disintegration of the sheet (Figs. 7—9). Mitotic divisions were rarely observed. After about 7 days many lipoid granules and vacuoles accumulated at the perinuclear region. As this tendency proceeded, the perinuclear zone was completely filled with granules and vacuoles after 14—21 days and the cells showed the tendency of degeneration (Figs. 5 and 6). There were no marked morphological differ-

ences in the living state between the cells on glass and on collagen films (Figs. 1 to 6, Figs. 7—9). Cellular defects in the epithelial sheet became larger. Scattered cells did not retain epithelial arrangement as noted in later observations (Fig. 6).

Fig. 1

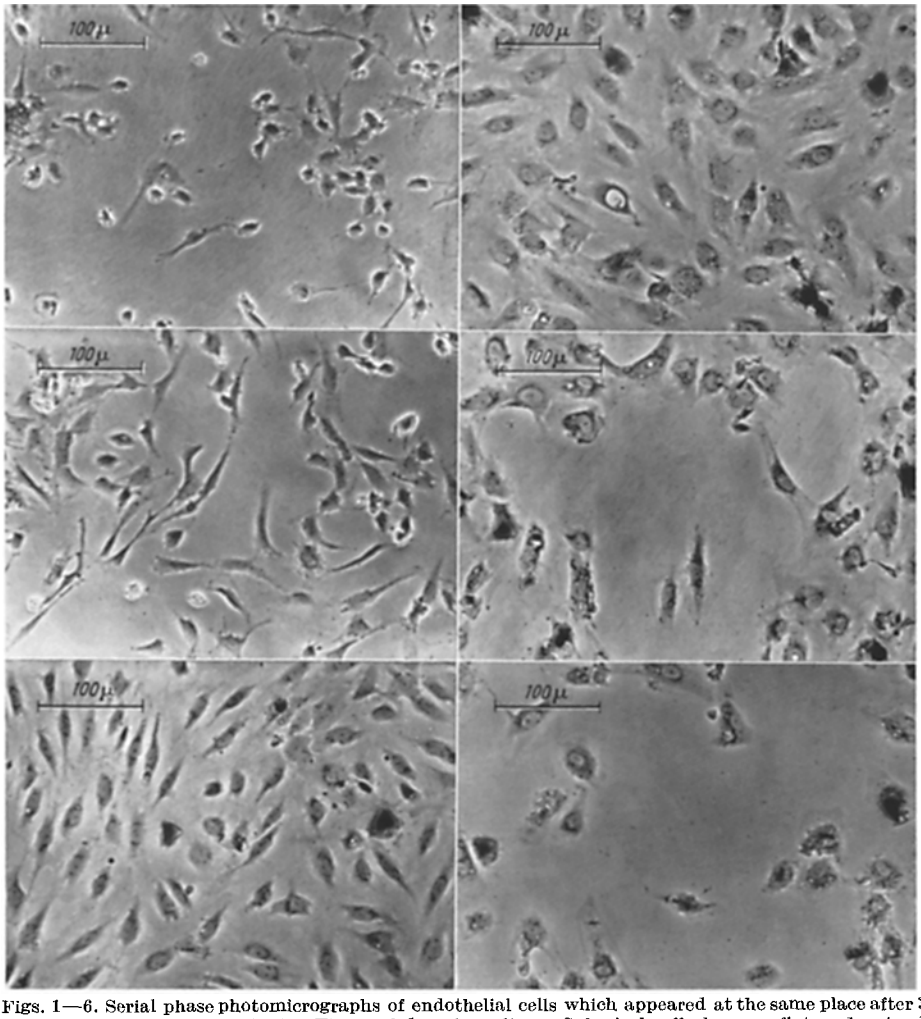

Fig. 4

Fig. 2

Fig. 5

Fig. 3

Fig. 6

Figs. 1—6. Serial phase photomicrographs of endothelial cells which appeared at the same place after 3 to 21 days in culture (see text). Fig. 1. 3 days in culture. Spherical cells become flat and extend processes. Fig. 2. 4 days in culture. Cells are in contact with each other by processes. Fig. 3. 8 days in culture. Formation of a sheet. Fig. 4. 13 days in culture. Perinuclear elevation decreases. Fig. 5. 18 days in culture. Lipoid granules and vacuoles accumulate at the perinuclear region. Some cells show the tendency to degenerate. Note disintegration of a sheet. Fig. 6. 21 days in culture. Freed cells with degenerative appearance resemble macrophages but do not show phagocytosis of indian ink. The magnification is indicated by the scale in the upper left of each figure

During the entire stage of culture neither capillary-like nor reticular formations of the cells could be observed.

Cytological findings. There were great variations in size and shape of the cells. In general they were rectangular, rhombic or oval in shape and had no long, slender processes (Figs. 10—12) except at the initial stage of culture (Figs. 1 and 2). The nucleus was oval and contained one or two nucleoli. The perinuclear

region contained fine granules which protruded and was moderately refractile. Granules often localized at one side of the nucleus. The cells gave rise to broad membranous expansions where fine fibrillar structures, probably tension striae,

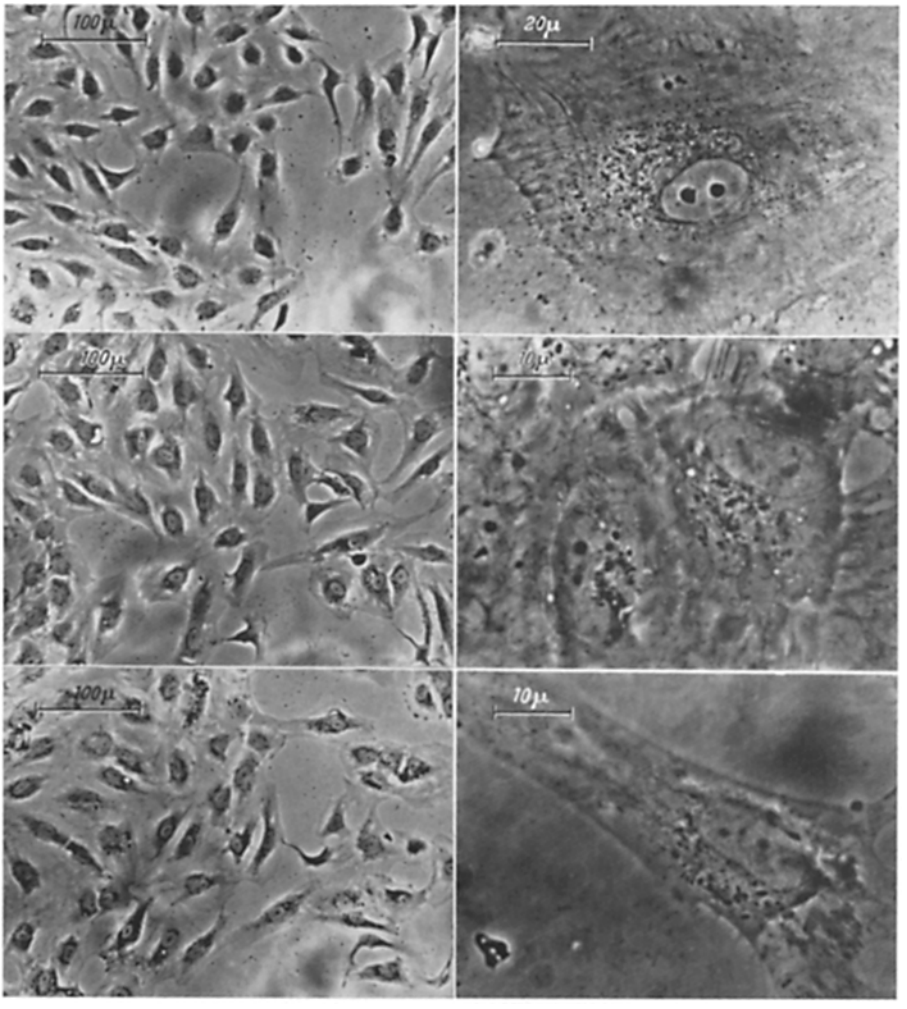

Fig. 7 Fig. 10

Fig. 8 Fig. 11

Fig. 9 Fig. 12

Figs. 7—9. Serial phase photomicrographs of endothelial cells which appeared at the same place after 9, 13 and 15 days in culture on collagen film. Sheets fuse into a larger one (Fig. 7). Disintegration of a sheet at the peripheral zone (Fig. 9)

Figs. 10—12. Phase photomicrographs of different types of endothelial cells after 5 to 7 days in culture. Granules are at one side of the oval nucleus. Fig. 10. A cell after 7 days shows filamentous mitochondria, fine fibrillar structures in a broad membranous expansion and indented edges. Fig. 11. Cells after 5 days in culture. Note cellular bridges and mitochrondria. Fig. 12. A cell at the periphery of a sheet after 5 days. The magnification is indicated by the scale in the upper left of each figure

were often observed (Fig. 10). Mitochondria were filamentous (Fig. 11). Pinocytosis was frequently observed in many cells (Fig. 13). Many binucleate cells and sometimes multinucleate cells were seen. Round or oval, small or large intercellular gaps formed by the cellular bridges often appeared both in the living state (Figs. 11 and 13) and in fixed materials (Fig. 14) which were so-called

"stigmata". The edges of cells at the periphery of a sheet as well as those of isolated cells were smooth or indented (Figs. 10 and 12). The nucleus was rich in fine chromatin and stained deeply with basic dye. The perinuclear, elevated region had fine granules and was basophilic, while the thin membranous part was slightly acidophilic (Figs. 18 and 19). The cellular contour was hardly revealed

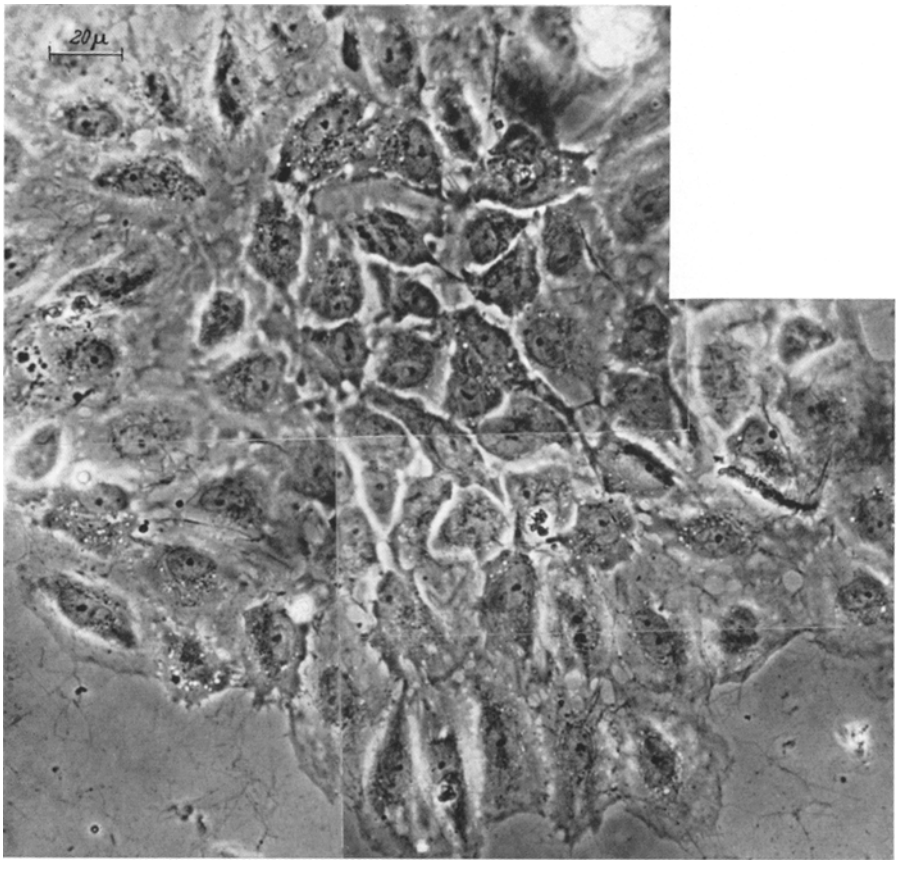

Fig. 13. A montage of phase photomicrographs of living endothelial cells arranged in a sheet after 3 days in culture on collagen film. Notice pinocytosis. The magnification is indicated by the scale in the upper left

by JACOBSON's stain, while silver nitrate impregnation demonstrated the mosaic of cement lines showing the epithelial arrangement of the cells (Figs. 14 and 15). PAS positive materials and alkaline phosphatase could not be demonstrated. Granules were neutral red and sudanophilic positive.

The umbilical vein

In a stained section of the control umbilical vein (Figs. 22 and 23), the internal elastic layer appeared to be in contact with the endothelium; fibroblasts could not be found between the two layers. The internal elastic layer was stained faintly compared with that of the umbilical artery. The endothelial cells showed no alkaline phosphatase activity.

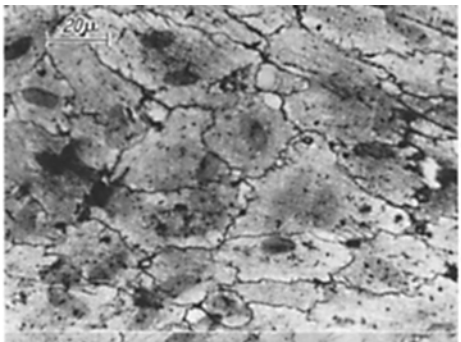

Fig. 14

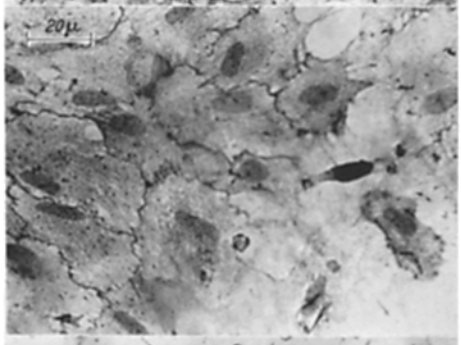

Fig. 15

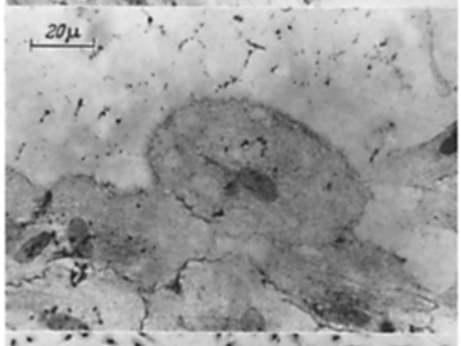

Fig. 16

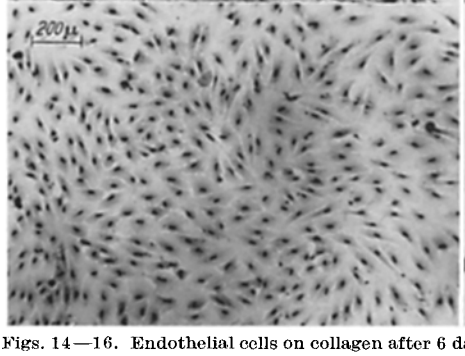

Fig. 17

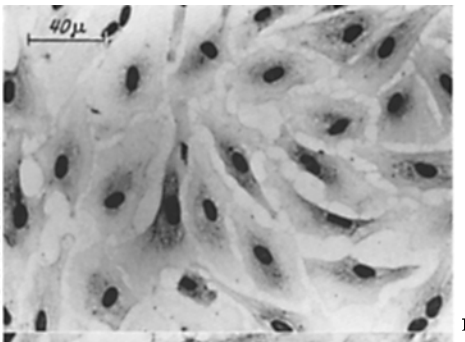

Fig. 18

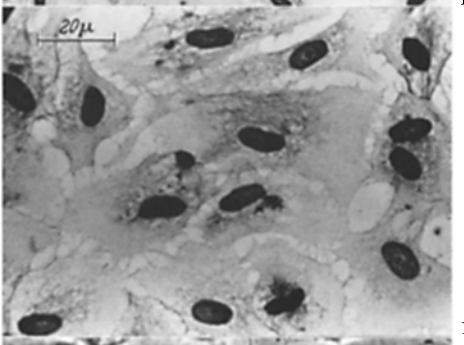

Fig. 19

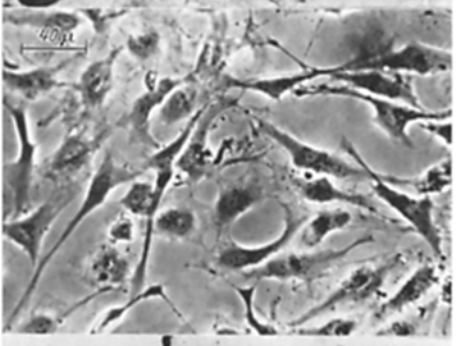

Fig. 20

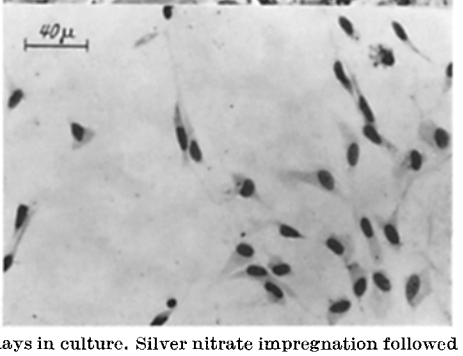

Fig. 21

Figs. 14—16. Endothelial cells on collagen after 6 days in culture. Silver nitrate impregnation followed by Giemsa stain demonstrates a mosaic of cementing lines and stigmata (Figs. 14 and 15). Lines are less marked at the periphery (Figs. 15 and 16)

Figs. 17—19. Endothelial cells after 5 days in culture without collagen as a substrate. Silver nitrate, and JACOBSON's stain. Notice intercellular gaps and no cementing lines (Fig. 17). Higher magnification (Figs. 18 and 19). Perinuclear regions are basophilic

Figs. 20 and 21. Loose reticular form of endothelial cells. Fig. 20. After 12 days in culture. Phase photomicrograph. Fig. 21. After 10 days in culture. JACOBSON's stain. The magnification is indicated by the scale in the upper left of each figure

The preparations of umbilical veins which were trypsinized demonstrated the removal of the endothelium (Figs. 24 and 25). Although all umbilical veins did not show the same degree of desquamation of the endothelium, trypsin appeared

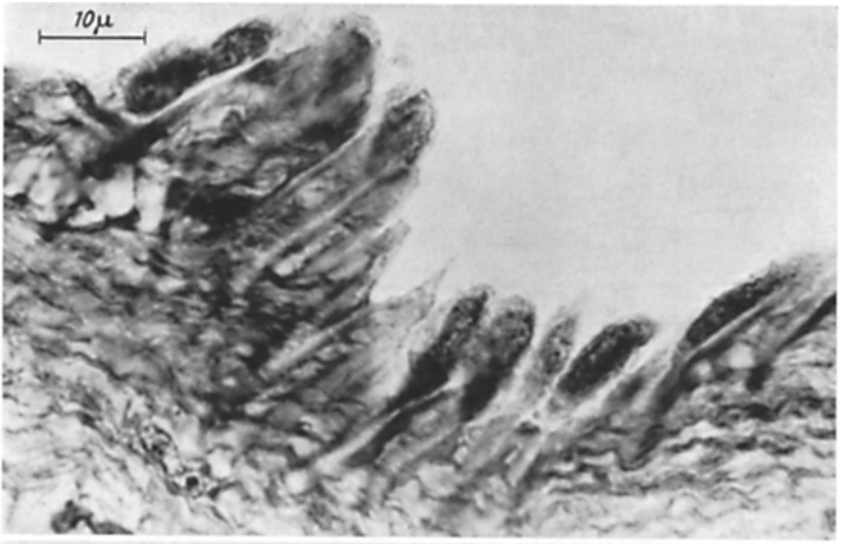

Fig. 22

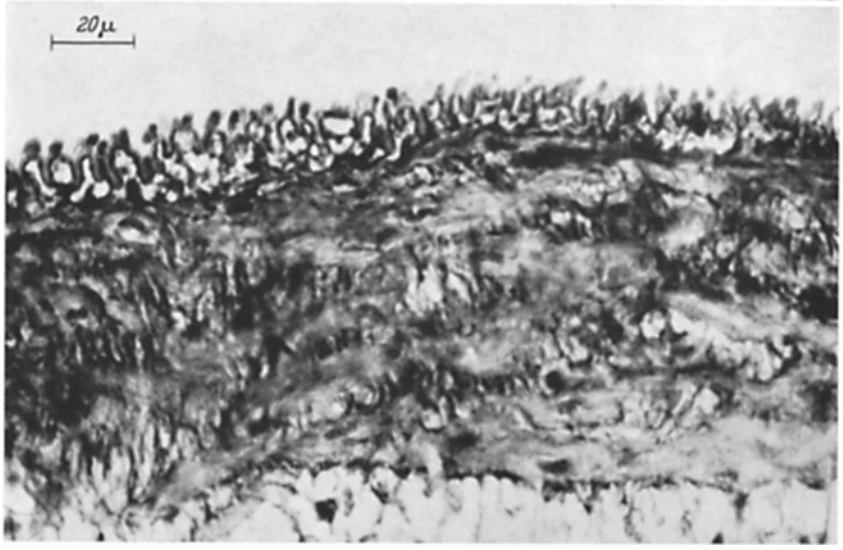

Fig. 23

Figs. 22 and 23. Sections of umbilical veins. Control. Fig. 22. Oblique section. Masson-Goldner stain. Fig. 23. Cross section. Concentrated Aldehyde-Fuchsin, Masson-Goldner. The magnification is indicated by the scale in the upper left of each figure

to have acted homogeneously on the endothelium of different segments of each vein.

Dissociation was considered to be satisfactory when the elastic layer was found to be intact (Fig. 24) or when the underlying tissue was kept intact in spite of obscurity of the elastic layer (Fig. 25).

Transformation of "endothelial" cells

Infrequently some cells transformed into fibroblast-like elements mostly in test tube cultures. For example in certain series of cultures made from an umbilical vein, there were no fibroblast-like cells after 2—4 days in culture but they appeared after 10 and 12 days (Figs. 21 and 20). These cells were spindle-shaped with slender processes and grew in a loose reticular form. They were

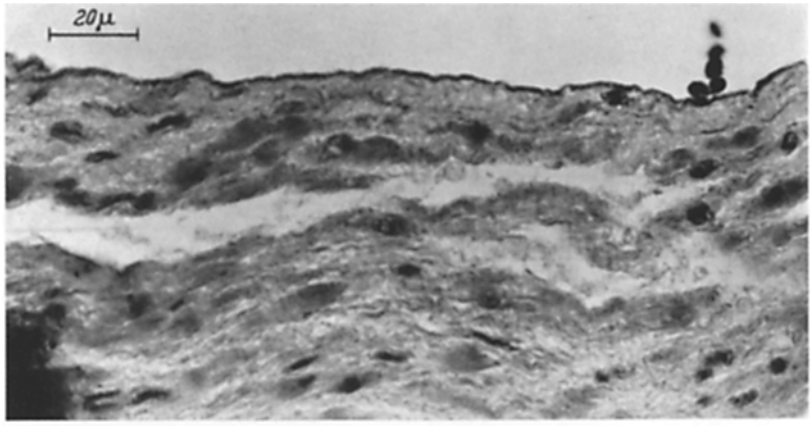

Fig. 24

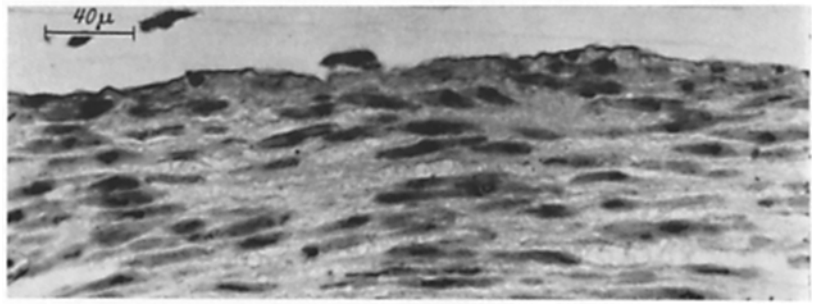

Fig. 25

Figs. 24 and 25. Sections of umbilical veins which were trypsinized show the removal of the endothelium. The internal elastic layer is intact (Fig. 24). The cells in Figs. 11—16 were isolated from this vein. Though a part of internal elastic layer is obscure the underlying tissue is intact (Fig. 25)

observed more easily at the peripheral zone of the culture where disintegration of a sheet was remarkable. There were no definite differences in staining properties between the "endothelial" cells and fibroblast-like elements.

Culture from the WHARTON'S *jelly*

No outgrowth was observed from explants of the jelly tissue both with MAXIMOW's and the roller-tube methods, although comparison of endothelial cells with fibroblasts from the umbilical cord was expected.

Discussion

Umbilical cord as materials used

In order to obtain endothelial cells, previous authors cultivated the tissues which contained capillaries or sinusoids, or a part of blood vessel wall especially of great vessels. The former were used more often by many workers. In pre-

liminary experiments the present author also cultured chicken embryonic liver, great vessels, chorio-alantoic membrane as well as vena cava of young rabbits and dogs by MAXIMOW's double coverslip and the roller-tube methods. It was not possible to distinguish endothelial cells from other cellular elements in cultures of blood vessels and of the tissue containing sinusoids or capillaries.

Subsequently both the endocardial surface of the heart and the intimal surface of blood vessels of young animals were curetted and cultured. The endothelium was peeled off with ease but it survived only for a short time probably due to cellular damage caused by mechanical action. Thus, the dissociation technique with trypsin was employed in the present experiment.

The human umbilical cord was useful material for the present purpose, because it is of fetal origin; it is obtained with ease in a fresh condition; furthermore, the umbilical vein has no tributaries and its lumen has an adequate capacity for trypsinization. The culture of human endothelial cells obtained from normal blood vessels has not been reported. BENEWOLENSKAJA (1930) used the human liver and MURRAY and STOUT (1944) hemangioendothelioma.

Identification of endothelial cells previously reported

Although it is generally believed that the cells originating from a culture of the heart tissue are fibroblasts, they must contain epithelial cells of the endocardial and epicardial origin. However, they do not show any of the characteristic morphological features of epithelial cells.

According to previous reports many investigators stated that endothelial cells grew in capillary formation in vitro. MAXIMOW (1925) and SILBERBERG (1930) observed a parallel bundle of endothelial cells which had a direct connection with the cut-end of an artery in the explant. Sooner or later they migrated out and could not be distinguished from fibroblasts. According to BENEWOLENSKAJA (1930), LEWIS (1931) and WHITE and PARSHLEY (1951), a capillary formation contained blood cells or fluid, but it was disorganized mostly within two weeks. But a capillary formation is not necessarily a product of endothelial cells since HUEPER and RUSSELL (1932) and PARKER (1933) observed such a structure in the culture of blood cells.

LEWIS (1922) and NISHIBE (1928—1929), who worked at LEWIS' laboratory, reported endothelial cells arranged in reticular forms. They considered that the materials consisted of two kinds of elements; endothelial and liver cells (LEWIS) or heart muscle cells (NISHIBE), and that liver or heart muscle cells were identified with ease, therefore, the rest of the elements in cultures were of endothelial origin. There still remains some question whether or not fibroblasts in the liver and the heart were mixed, or whether there were fibroblasts transformed from cardiac muscle cells.

MURRAY and STOUT (1944) and KHLOPIN and CHISTOVA (1958) demonstrated cell boundaries, i.e. cementing lines, by silver impregnation in cultures of hemangioendothelioma or vena cava. They did not describe other cells than those in sheets.

Endothelial cells in the present study

In the previous reports as well as in the preliminary observations by the present author, there was no definite way to identify the isolated cells with those

of endothelial origin. It was, therefore, our first consideration to obtain only endothelial cells without contamination with other cellular elements.

1. Observations of stained sections from selected segments of every umbilical vein of which endothelium was used for culture were made to check the removal of endothelial cells and not of other elements.

2. When a defect of the subendothelial tissue was found in section, the cultures from that material were considered to be contaminated with fibroblasts.

When cells formed a sheet, silver impregnation to reveal cementing lines was positive. This was associated with the cells of endothelial origin. Since the materials were carefully prepared, isolated cells also could be identified with endothelial elements. And it was an initial goal to determine whether some morphological characteristics in those cells differed from fibroblasts.

1. WHARTON's jelly was cultivated for the purpose of comparing isolated endothelial cells with fibroblasts from the same material, but this was unsuccessful.

2. Embryonic fibroblasts from other tissues showed no marked differences from those isolated endothelial cells which developed a spindle shape.

3. Histochemical demonstration of alkaline phosphatase in the isolated endothelial cells, it was hoped, might provide a method of identification. Some authors demonstrated this enzyme in the endothelium of small blood vessels and capillaries but not of large vessels (TAKAMATSU 1939; GOMORI 1939, 1941; KABAT and FURTH 1941; LANDOW et al. 1942; HONJIN et al. 1962). The present author could not demonstrate it in the endothelium of large blood vessels of adult ratr (unpublished data), nor in the present materials.

Summary

Endothelial cells of the human umbilical vein were isolated by trypsin and cultured. Histological preparations of those umbilical cords were made to check the removal of the endothelium and not of other tissues.

The cells in culture grew in sheets which were identified with endothelial cells. Isolated cells rarely showed a spindle shape. Further investigation is needed to distinguish spindle-shaped cells from fibroblasts or other cellular elements.

Identification of endothelial cells in the previous reports and materials used are discussed.

Bibliography

ALTSCHUL, R.: Endothelium, its development, morphology, function, and pathology. New York: Macmillan 1954.

BENEWOLENSKAJA, S. W.: Hematopoiesis in cultures of embryonic liver of man. Arch. exp. Zellforsch. **9**, 128—141 (1930).

BORNSTEIN, M. B.: Reconstituted rat-tail collagen used as substrate for tissue cultures on coverslips in Maximow slides and roller tubes. Lab. Invest. **7**, 134—137 (1958).

CAMERON, G., and R. CHAMBERS: Neoplasm studies III. Organization of cells of human tumors in tissue culture. Amer. J. Cancer **30**, 115—129 (1937).

FUJITA, T.: Histological studies on the neuro-insular complex in the pancreas of some mammals. Z. Zellforsch. **50**, 94—109 (1959).

GOMORI, G.: Microtechnical demonstration of phosphatase in tissue sections. Proc. Soc. exp. Biol. Med. (N. Y.) **42**, 23—26 (1939).

— The distribution of phosphatase in normal organs and tissues. J. cell. comp. Physiol. **17**, 71—83 (1941).

HERZOG, G., u. W. SCHOPPER: Über das Verhalten der Blutgefäße in der Kultur. Arch. exp. Zellforsch. **11**, 202—218 (1931).

HONJIN, R., T. NAKAMURA, Y. OKAYAMA, Z. NUNOGAMI and T. WAZIMA: Histochemistry of alkaline phosphatase. Report 2. A new histochemical procedure for alkaline phosphatase with alizarin red. Acta Anat. Nippon **37**, 126—133 (1962).

HUEPER, W. C., and M. A. RUSSELL: "Capillary-like formations" in tissue cultures of leucocytes. Arch. exp. Zellforsch. **12**, 407—424 (1932).

KABAT, E. A., and J. FURTH: A histochemical study of the distribution of alkaline phosphatase in various normal and neoplastic tissues. Amer. J. Path. **17**, 303—318 (1941).

KHLOPIN, N. G.: Changes in the endothelium of the arteries, veins and heart in experimental conditions. Voprosy Etiologii I Pathogeneza Opukholei (Leningrad) 1957, 21—25 (Abst.). Excerpta med. (Amst.), Sect. I, **14**, 425 (1960).

—, and N. M. CHISTOVA: Endothelial tissue culture. Doklady Biol. Sci. Sect. **119**, 217—220 (1958) (Abst.). Excerpta med. (Amst.), Sect. I, **14**, 596 (1960).

LANDOW, H., E. A. KABAT and W. NEWMAN: Distribution of alkaline phosphatase in normal and in neoplastic tissues of the nervous system, a histochemical study. Arch. Neurol. Psychiat. (Chic.) **48**, 518—530 (1942).

LEWIS, W. H.: Smooth muscle and endothelium in tissue culture. Anat. Rec. **21**, 72 (1921).

— Endothelium in tissue culture. Amer. J. Anat. **30**, 39—59 (1922).

— The outgrowth of endothelium and capillaries in tissue culture. Bull. Johns Hopk. Hosp. **48**, 242—253 (1931).

MAXIMOW, A. A.: Behavior of endothelium of blood vessels in tissue culture. Anat. Rec. **29**, 369 (1925).

MURRAY, M. R., and A. P. STOUT: Cultural characteristics of a hemangioendothelioma. Amer. J. Path. **20**, 277—283 (1944).

NISHIBE, M.: Growth of endocardial cells from the chick embryo heart in vitro. Arch. exp. Zellforsch. **7**, 333—343 (1928—1929).

PARKER, R. C.: The development of organized vessels in cultures of blood cells. Science **77**, 544—546 (1933).

RIENHOFF jr., W. F.: Development and growth of the metanephros or permanent kidney in chick embryos (8 to 10 days incubation). Bull. Johns Hopk. Hosp. **33**, 392—406 (1922).

SCHOPPER, W.: Netzexplantation. Verh. dtsch. path. Ges. **24**, 25—28 (1929).

SCRIBA, K.: Explantationsstudien über das Gefäßwachstum bei 9 Tage alten Hühnerembryonen. Arch. exp. Zellforsch. **17**, 68—77 (1935).

SILBERBERG, M.: Endothel in der Gewebskultur. Arch. exp. Zellforsch. **9**, 36—53 (1930).

TAKAMATSU, H.: Histologische und biochemische Studien über die Phosphatase. I. Histochemische Untersuchungsmethodik der Phosphatase und deren Verteilung in verschiedenen Organen und Geweben. Trans. Soc. Path. Jap. **29**, 492—498 (1939).

TÖRÖ, E.: Untersuchungen über die Potenz der Endothelzellen bei der Gefäßbildung in der Gewebekultur. Arch. exp. Zellforsch. **20**, 156—171 (1937).

WHITE, J. F., and M. S. PARSHLEY: Growth in vitro of blood vessels from bone marrow of adult chicken. Amer. J. Anat. **89**, 321—345 (1951).

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