Replication of Human Endothelial Cells in Culture

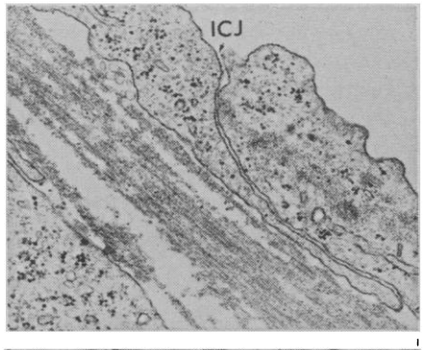
Abstract. *Investigative studies dealing with the properties and functions of endothelial cells have been hampered because there has been little or no success in the isolation, growth, and passage of individual cells in large numbers. We have developed a system whereby pure cultures of endothelial cells derived from umbilical veins can be subcultured for at least five serial passages. Many facets of endothelial function and interaction can be evaluated with the use of this new adaptive system of isolation and culture.*

The endothelium performs many functions related to the exchange of cells and materials across the vessel wall. Alterations of endothelial integrity may represent a key component in the pathogenesis of disorders related to inflammation, thrombosis, and atherosclerosis. Numerous references to short-term organotypic cultures of endothelium are recorded, but most of them reported little success (*1*). Although Maruyama (*2*) was able to culture endothelial cells from umbilical cord veins, he claimed that mitotic divisions were rarely observed. In fact, after 14 to 21 days the cells actually showed a tendency to degenerate. In addition, his cultures were in quantities infinitely small and obviously could not be serially subcultured since the sheet disintegrated after the cells were in culture for about 13 days. By use of our method of isolation, culture, and passage, it is now possible to propagate endothelial cells from human umbilical veins in sufficient numbers for many studies. To date, only endothelial cells derived from veins have been used.

Endothelial cells were obtained from human endothelial veins treated with a 0.125 percent trypsin solution in normal saline at room temperature. All the steps up to seeding the cultures were carried out at room temperature. With the use of aseptic techniques, the vein was excised and placed in a large Pyrex dish or cut up into sections and the sections were placed in large petri dishes. The cord or sections were then washed three times with Hanks balanced salt solution. After washing, the vein was incised with scissors to expose the lumen. A small curved ophthalmic scalpel was then used to scrape off the inner lining of cells. The cells were harvested by irrigating the scrapings, drawing them up with a Pasteur pipette, and depositing them into a small erlenmeyer flask.

The pool of cells was then treated with 30 ml of a 0.125 percent trypsin solution for a total of 40 minutes. After the first 20-minute period, most of the turbid suspension was withdrawn with a Pasteur pipette and held in a conical test tube. It is advisable to remove those cells which have become loosened and thus not permit their possible destruction by lengthy contact with the trypsin solution. At the end of 40 minutes, the balance of the turbid suspension was drawn off in the same manner as described above and added to the first sample. After 8 minutes of centrifugation at 1000 rev/min, the supernatant fluid was decanted and the pellet was suspended in 5 ml of a culture medium consisting of a mixture of medium 199 supplemented with 20 ml of 50 percent glucose, 10 ml of a 100× amino acid mixture, 10 ml of a 100× vitamin mixture, 10 ml of a 100× glutamine solution, 0.5 g of Bacto peptone, and 100 ml of inactivated fetal calf serum. The medium was sterilized by filtration and the *p*H was adjusted to 6.8 to 7.2 with sodium bicarbonate. Attempts to use other commercial media and medium 199 without supplements have met with little success.

Cultures were seeded with 15×10^3 cells per milliliter of medium and equilibrated with gaseous CO_2 to a *p*H of 6.8 to 7.2. The number of cells one is able to obtain from one vein will usually be sufficient to seed six T-75 Falcon flasks containing 5 ml of medium. A confluent sheet was attained in approximately 10 to 14 days when the cultures were incubated at 37.5°C. Since it is possible to serially subcul-



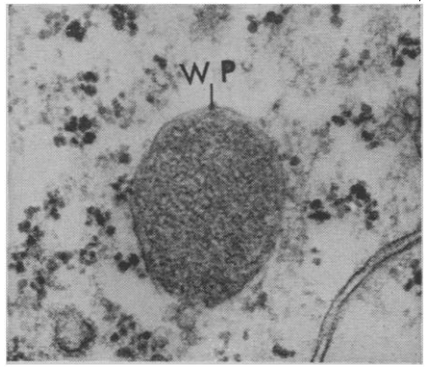


Fig. 1 (top left). Electron micrograph of cultured endothelial cells after fourth passage, which demonstrates an intercellular junction (*ICJ*) (× 19,100). Fig. 2 (bottom left). Transverse section of a Weibel-Palade body (*WP*) in an endothelial cell after fourth passage (× 56,500). Fig. 3 (right). Freeze-etch preparation of endothelial cells after fourth passage which shows caveolae (*C*) in the plasma membrane (*PM*) (× 9,900).

ture endothelial cells isolated and grown in this manner, the establishment of strains is not difficult and has been accomplished. Once established, the strains can be preserved in liquid nitrogen and thus provide a reliable source of the same cells for investigational purposes. Each split can be made at a 6 : 1 ratio with a doubling time of approximately 40 hours. Although the primary cultures may contain a few cells which display a fibroblastic morphology, these are soon overgrown by the great predominance of endothelial cells which are present. In fact, by the third serial subculture the cultures appear to be composed of pure endothelial cells, and when compared to fibroblasts, the cultured endothelial cells are morphologically different.

Although replication is indicated by our ability to subculture these cells, it was also shown that cells cultured in this manner when treated with [^{3}H]-thymidine labeled 80 percent of the nuclei, providing further evidence that the cells were actively dividing.

When tested by the indirect fluorescent-antibody technique, these cultures of endothelial cells stained brilliantly with antibody against human thrombosthenin. Cultures of human foreskin fibroblasts did not. These results suggest that the endothelial cells produce a substance which reacts like thrombosthenin, but might not be monospecific. In addition, these endothelial cells were found to possess ABO blood group antigens, as demonstrated by rosette formation and absorption techniques. If the cultured endothelial cells from the umbilical vein of a type A infant were treated with anti-A serum, washed thoroughly, and type A erythrocytes were added, rosettes formed. No such rosettes were found when type B erythrocytes were added to type A endothelial cells after incubation of the type A endothelial cells with anti-B serum. Similarly, fibroblasts did not react with absorption of anti-A or anti-B antibodies. It had been observed earlier that human endothelial cells had ABO antigens and immunologic evidence of thrombosthenin (*3, 4*).

Electron microscopy of the cultured cells demonstrated tight intercellular junctions and typical morphologic features of endothelial cells, including Weibel-Palade bodies (*5*) (Figs. 1 and 2). Further evidence that the cells obtained after serial subculture were endothelial cells was provided by freeze-etch preparations which demonstrated caveolae in the plasma membrane (Fig. 3). These features are characteristics of endothelial cells prepared from kidney, pituitary, and lung tissues (*6*).

The results of the present study indicate that human endothelial cells can grow and replicate in vitro. This growth occurs at a rate which facilitates the harvest of adequate numbers of cells for investigative studies which, heretofore, had been considered improbable, if not impossible.

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References and Notes

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27 April 1973

Feline Leukemia and RD-114 Virus Group-Specific Proteins: Comparison of Amino Terminal Sequence

Abstract. *The major internal virion polypeptide from feline and RD-114 type C viruses has been subjected to amino terminal sequence analyses with the Beckman automated sequencer. These proteins, as well as their homologs in rat and mouse viruses, begin with the sequence prolylleucylarginyl (Pro-Leu-Arg). Virus RD-114 differs from conventional feline type C viruses that show about 80 percent relatedness based on calculation of the minimum number of base changes to give equivalent coding for the protein segments analyzed. In addition, insertion of a gap in the RD-114 sequence is necessary to maintain positional homology. The difference between RD-114 and feline leukemia virus appears as great as the difference between mouse type C viruses and either of these two viruses. Thus, even though current evidence suggests that RD-114 is of feline origin, the sequence differences between RD-114 and conventional feline virus group-specific proteins is well beyond that based on one or a few point mutations.*

The type C viruses of mice, cats, rats, and hamsters are related both morphologically and immunochemically. The major internal polypeptide (gs protein) of viruses from these species (molecular weight about 30,000) contains a cross-reactive antigenic determinant (*1, 2*), originally designed group-specific protein-3 (gs-3) (*3*), in addition to species-specific antigenic determinants (*1*), originally designated gs-1 (*4*). Current evidence (*2, 5*) indicates that these designations will require modification to include results both from more detailed analyses of subprimate gs proteins and from studies of gs proteins of two primate isolates, woolly monkey and gibbon ape.

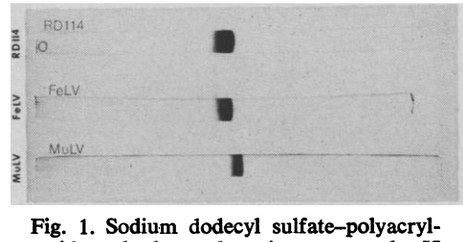

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis at neutral *p*H (*17*) of gs proteins purified by isoelectric focusing as described earlier for mouse (*18*), cat (*19*), and RD-114 (*8*) viruses. In the final electrofocusing, the gradients were *p*H 5 to *p*H 8 for mouse, and *p*H 7 to *p*H 10 for cat and RD-114 virus. Ampholine was removed by gel filtration through Bio-Gel P-10 or Bio-Gel P-100 (Bio-Rad), equilibrated with 0.33*M* ammonium acetate, and proteins were recovered by lyophilization; *O* indicates origin.

In order to more specifically define relationships among the various viruses, we have initiated NH_2-terminal sequence analysis of the gs protein from several viruses. In the course of this work a new virus, designated RD-114, was recovered from human rhabdomyosarcoma cells passaged through a fetal cat (*6, 7*). This virus was distinguished from known conventional feline type C viruses (FeLV) on the basis of the absence of feline gs-1 (*7, 8*) and the