Br. J. exp. Path. (1984) **65**, 165–170

Ring formation by human variant endothelial cells *in vitro*

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Received for publication 17 November 1983

Summary. Variant endothelial cells were cultured from the healthy carotid arteries of a 19-year-old woman who was killed accidentally. The cells were grown and subcultured in Medium 199 supplemented with 20% heat-inactivated fetal calf serum. The cells are still viable after 20 passages. They were recognized as variant endothelial cells by their morphology which included ring formation, and by the existence of factor VIII-related surface antigen on the cell membrane. The cultured endothelial cells produced prostacyclin when the cells were incubated with arachidonic acid in smaller amounts than does typical endothelium, but far in excess of that produced by vascular smooth muscle cells.

Keywords: ring formation, variant endothelial cells, in-vitro culture, factor VIII-related surface antigen

In recent years, cultures of vascular endothelial cells have proved useful in elucidating the pathophysiological and biochemical aspects of arteriosclerosis (Gimbrone 1976). There has been considerable interest also in the development of atherosclerotic lesions after intimal thickening and smooth muscle cell proliferation which follows endothelial injury (Ross & Glomset 1976; Schwartz *et al.*, 1981).

In the past, most cultured endothelial cells have been derived from the aortas of rabbits, dogs, pigs or cattle (Slater & Sloan 1975; Gimbrone 1976; Tokunaga & Nakashima 1978), or from human umbilical veins or capillaries, or from iliac and pulmonary arteries (Jaffe *et al.* 1973; Johnson 1980; Glassberg *et al.* 1982). There were no significant differences in the morphological or physiological characteristics of such cultures except that the capillary-derived endothelial cells exhibited angiogenesis *in vitro*, were more spindle shaped and some of them formed ring structures (Folkman & Hauden 1980).

The cells derived from the human carotid artery, although this is a large artery as opposed to a capillary, also formed rings in culture. Single-cell rings resembled capillary lumina; rings formed from two to five cells resembled the lumina of small arteries. This ring formation was the most characteristic feature of these cultured arterial cells. The nature and derivation of these cells is discussed in this paper.

Materials and methods

Isolation and culture of endothelial cells. A pair of carotid arteries, 10 cm long, was removed aseptically at autopsy within 1 h of the accidental death of a 19-year-old woman who had no detectable disease. Using gentle surgical techniques to minimize trauma to the endothelial lining, the artery was rinsed extensively with cold phosphate-buffered

saline (PBS) and blood was washed away. One end of the vessel was clamped and the vessel lumen was filled with 0·2% trypsin and 0·01% EDTA (GIBCO) dissolved in PBS; the other end was then clamped. The vessel was incubated at 37°C for 45 min and the medium containing a suspension of detached cells was transferred into a 10-ml centrifuge tube and the cells washed twice with culture medium. The pooled cells were spun down at 800 *g* for 10 min at room temperature and the sediment was resuspended in 1·5 ml of Medium 199 (Chibaken Kessei Laboratory) containing 0·29 g/l L-glutamine and 1·4 g/l sodium bicarbonate. This medium was supplemented with 20% heat-inactivated fetal calf serum (Filton, Australia) plus 30 μg/ml cefmetazol (Sankyo). The collected cells were seeded in a 35-mm Falcon tissue culture dish and incubated at 37°C in a humidified, 5% CO_2 incubator. The medium was changed every 3 days and the cells observed daily with a phase-contrast microscope. When the cells became confluent, they were subcultured at a ratio of 1:2 by using a 0·2% trypsin and 0·01% EDTA treatment.

Electron microscopy. The cell cultures were fixed with 2·5% glutaraldehyde in PBS, pH 7·4, for 60 min. The cells were washed twice with the same buffer, post-fixed with 2% osmium tetroxide, and embedded in Epon 812. En-face ultrathin sections were cut with an LKB ultra-microtome in the plane of the culture dish and double stained with uranyl acetate and lead citrate, and examined on a Hitachi JEM as described previously (Tokunaga & Nakashima 1978).

Results

Endothelial cells were observed to attach to the culture dish within 2 days. Thereafter the medium was changed every 3 days.

In primary culture, the number of cells surviving was exceedingly small and the cell growth slow. The cells were subcultured when the primary culture became confluent after 14 days. The confluent culture consisted of a monolayer of ovoid cells with no visible cellular overgrowth. The growth rate of the cells was rapid, with doubling times of approximately 48 h up to 10 passages; it then gradually reduced to about 60 h after 15 passages. To date there have been 22 passages, but the morphology of the cultured cells has not changed since the primary culture.

The most striking feature of the cultured cells was their propensity for ring formation. The ovoid cells extended bipolar cytoplasmic processes along their long axes; the tips of the processes then curved round thus bringing their tips together to form a ring resembling a capillary lumen (Fig. 1). Sometimes two to five cells formed a composite ring bigger than those formed by a single cell (Fig. 2).

The rings ranged in size from 20 μm to 50 μm for a single cell ring and from 50 μm to 120 μm for a composite cell ring.

Ring formation was more frequent when the cells were subcultured at low cell density.

When the cells came in contact with adjoining cells they stopped growing and did not overgrow each other. They resembled typical cultured endothelial cells derived from umbilical vein, but had slightly more cytoplasm and when viewed by phase-contrast could be seen to contain microtubules arranged parallel to the axis of the cell.

When viewed by electron microscopy the inner surface of the rings appeared to have a smooth profile and carried a few small microvilli. In contrast, the outer surface was irregular. Small accumulations of microfibrils, so called 'myofilamentous dense patches', were scattered in the peripheral cytoplasm adjacent to the cell membrane. Mitochondria were fewer in number than in normal cells but the amount of endoplasmic reticulum was increased (Fig. 3). Weibel–Palade bodies which are specific for endothelial cells, were not seen in this study.

Discussion

The vascular intima, a single layer of endothelial cells covering the internal elastic

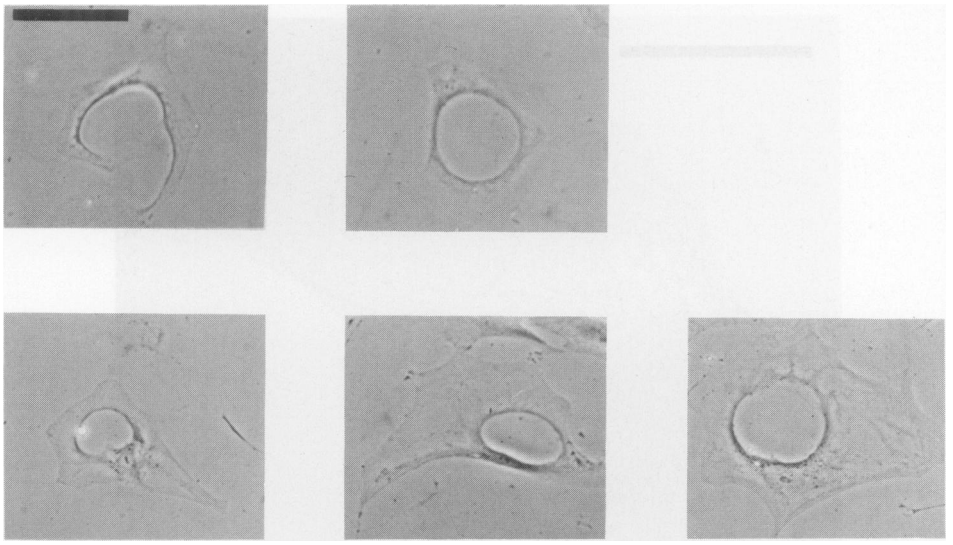

Fig. 1. Ring formation by a single human endothelial cell (phase-contrast). Each plate shows a different stage of this process at low cell density. The extended cytoplasmic processes on the bottom of the culture dish join to form a ring resembling capillary lumen. Bar = 50 μm.

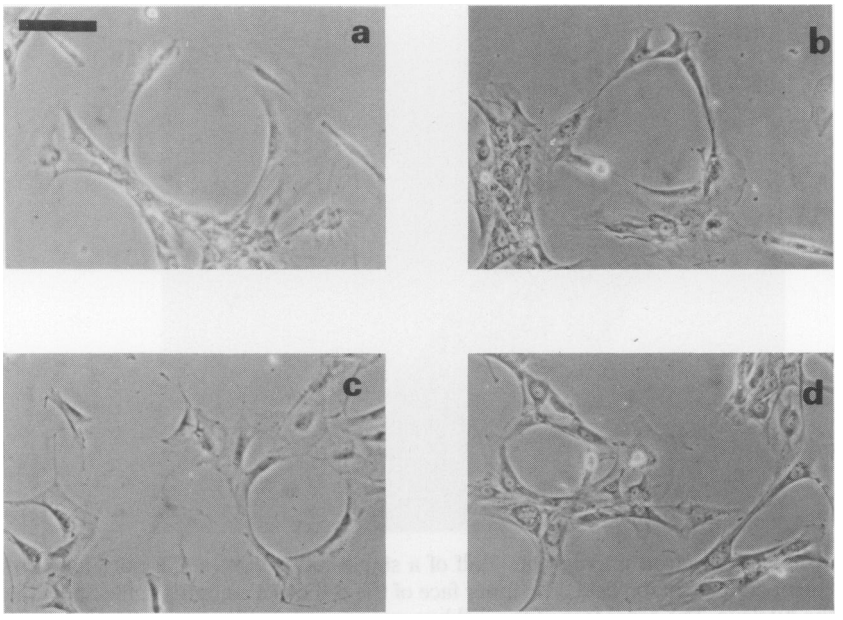

Fig. 2. Composite cell ring (phase-contrast). Two to five endothelial cells form a larger ring. The inner side of the ring has denser cytoplasm and a smoother profile than the outer edge. (*a*), Early stage of ring formation. Cytoplasmic processes extend from two cells and grow towards each other; (*b*) processes just joined; (*c*) a completed ring and another incomplete ring; (*d*) advanced stage. At least three composite rings and one incomplete ring are present in this field. Bar = 50 μm.

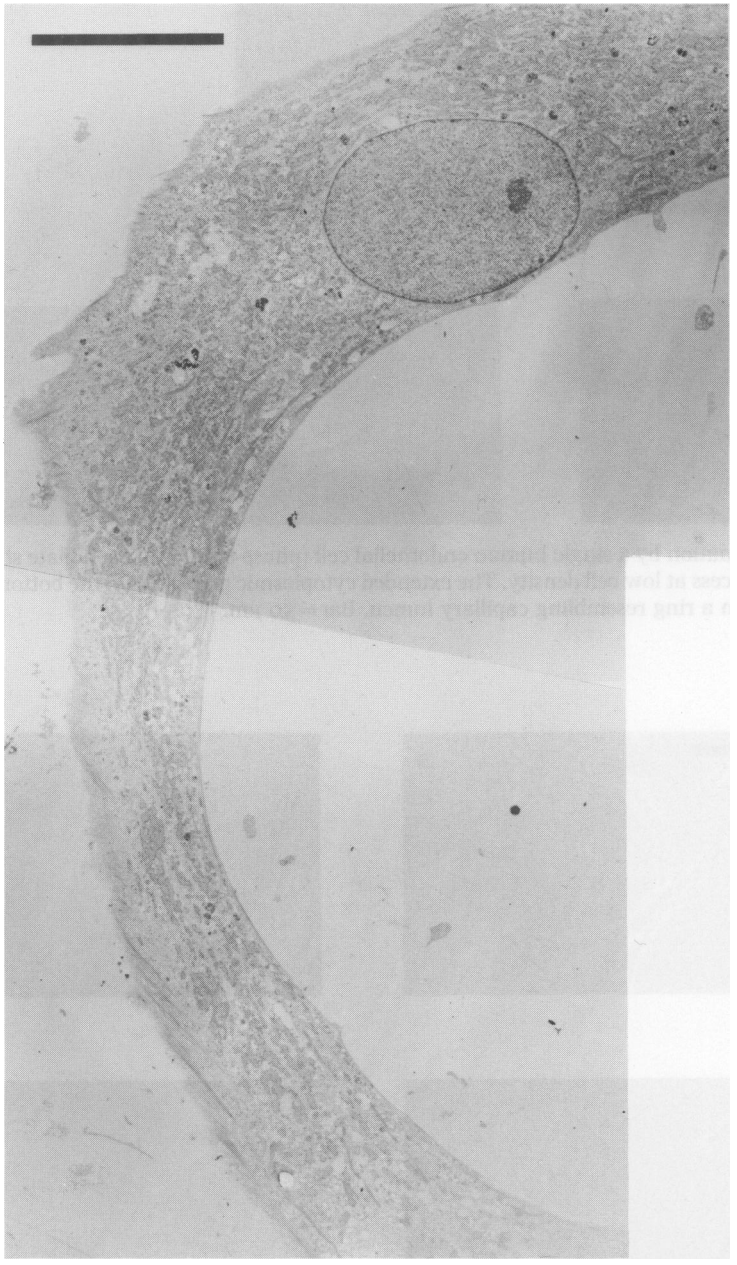

Fig. 3. Transmission electron micrograph. Half of a single cell ring. A small nucleus with nucleoli is present in the upper part of the field. The inner face of the cell has a smooth profile and a microvillus is seen near the nucleus. The outer face of the cell has an irregular profile. The cell is rich in endoplasmic reticulum. No Weibel–Palade bodies can be seen. Bar = 10μm.

lamina at birth, is gradually thickened to a varying degree with aging. The endothelial cells covering such a thickened intima may differ functionally and morphologically from the endothelial cells of the neonatal artery.

Glassberg *et al.* (1982) cultured endothelial cells derived from a 68-year-old man with a history of diabetes mellitus whose iliac artery had advanced atherosclerotic lesions. The confluent cultures they obtained consisted of a monolayer of closely apposed polygonal cells with no visible cellular overgrowth. Our endothelial cells from human carotid arteries have survived in culture for 22 passages without requiring any specific culture conditions, though after 15 passages the growth rate was slightly reduced. These cells were probably derived from the thickened intima and were not typical endothelium because although they have factor VIII-related surface antigen specific for endothelium (Jaffe 1977) and cease to grow when they have formed a confluent monolayer thus demonstrating contact inhibition, they lack some other endothelial cell characteristics, such as angiotensin-converting enzyme production.

The most characteristic feature of these cultured cells from the carotid artery was ring formation both by individual and by groups of two to five cells.

Folkman & Hauden (1980) described ring formation by single capillary endothelial cells which had been stimulated by tumour-conditioned medium. They regarded ring formation as one of the identifying criteria for capillary endothelial cells. In the current investigation, these variant endothelial cells formed rings even though they were derived from the carotid artery and not from a capillary. Ring formation is thus not exclusive to capillary endothelium but may be a universal attribute of vascular endothelium. By contrast smooth muscle cells cultured from arterial media never form ring structures (O. Tokunaga, unpublished data).

These cultured cells from human carotid artery differed slightly from cultured endothelium derived from human umbilical vein or human diabetic or atherosclerotic iliac artery. Other investigators also have described atypical endothelial cells (Fryer *et al.* 1964) and cultured aberrant endothelium (Gospodarowicz *et al.* 1978; Schwartz 1978). McAuslan *et al.* (1980) reported variant endothelial cells whose characteristics included fibroblastic morphology, lack of contact-inhibition, the capacity to synthesise factor VIII antigen and the ability to bind more concanavalin A to the cell surface than normal cells.

We describe here variant endothelial cells which exhibit some endothelial-specific characteristics such as factor VIII-related antigen, atypical sheet arrangement in culture, contact inhibition and the ability to convert arachidonic acid to prostacyclin (Tokunaga *et al.* 1983), but lack others including the ability to synthese angiotensin-converting enzyme and to produce Weibel–Palade bodies.

We suggest that these cells could be either modified smooth muscle cells, or subendothelial cells which are convertible to endothelial and smooth muscle cells. When subendothelial cells are exposed to blood flow and pressure following loss of the overlying endothelial cells, it is possible that they both function as an endothelium and express some endothelial functions but are unable to develop all the properties of normal endothelium *in vivo* or as seen in umbilical endothelial cell culture. Such cells might also be able to be transformed to ring-forming cells thus producing a capillary-like lumen.

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