Intracellular diaphragmed fenestrae in cultured capillary endothelial cells

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Summary

The endothelium of visceral capillaries is characterized by the occurrence of numerous fenestrae, which are usually bridged by a thin, single-layered diaphragm. Both in vivo and in vitro, diaphragmed fenestrae perforate the endothelial cell cytoplasm in the most attenuated regions of the cell. We report here that in capillary endothelial cells grown under experimental conditions promoting the development of intracellular lumina (for example, suspension within a three-dimensional collagen matrix), diaphragmed fenestrae can form in a unique, previously undescribed intracellular location – that is, within thin cytoplasmic septa separating contiguous luminal compartments.

Key words: endothelial cells, fenestrae, extracellular matrix.

Introduction

A characteristic feature of the endothelium of visceral capillaries is the presence of numerous circular windows, or fenestrae, about 70 nm in diameter, that traverse the cytoplasm of endothelial cells and participate in the exchange of substances between blood and tissues (Rhodin, 1962; Maul, 1971; Simionescu et al. 1982; Simionescu, 1983; Bearer & Orci, 1985). The fenestrae are closed (except in glomerular endothelium) by a thin, single-layered diaphragm, and usually occur in clusters in the most attenuated regions of the endothelial cells (Simionescu et al. 1974; Orci & Perrelet, 1975). In addition to this typical location, diaphragmed fenestrae have also been observed in the thin cytoplasmic folds that project into the capillary lumen as dome-like structures delimiting an endothelial pocket (Wolff & Merker, 1966; Milici et al. 1986).

We (Montesano & Orci, 1985; Lombardi et al. 1986) and others (Milici et al. 1985) have recently demonstrated that diaphragmed fenestrae are formed in capillary endothelial cells grown in vitro, and that their number can be modulated by environmental signals (Milici et al. 1985; Lombardi et al. 1986). In this report we show that under certain experimental conditions, endothelial fenestrae may occur in a peculiar, hitherto unrecognized intracellular location.

Materials and methods

Cloned capillary endothelial cells derived from the bovine adrenal cortex (Furie et al. 1984) were a generous gift from Dr M. B. Furie and Dr S. C. Silverstein (Columbia University, New York). The cells were routinely subcultured in gelatin-coated tissue-culture flasks (Falcon, Becton Dickinson Labware, Oxnard, CA) in complete medium consisting of minimal essential medium, alpha modification (n'-MEM) (Gibco Laboratories, Grand Island, New York) supplemented with 15% heat-inactivated donor calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland), penicillin (500 U ml⁻¹) and streptomycin (100 μg ml⁻¹).

Suspensions of single cells in collagen gels were obtained as follows: freshly trypsinized endothelial cells were centrifuged in a plastic tube and resuspended at a density of 2–8×10⁶ cells ml⁻¹ in a polymerizing collagen solution, which was prepared by quickly mixing 8 vol. of type I collagen (extracted from rat tail tendons as previously described, Montesano et al. 1983) with 1 vol. of 10X concentrated minimal essential medium (MEM, GIBCO) and 1 vol. of sodium bicarbonate (11.76 mg ml⁻¹). The cold mixture was then dispensed into 35-mm plastic dishes (Falcon) (2 ml per dish) and allowed to gel for about 10 min at 37°C before adding complete culture medium.

To obtain suspensions of single cells in fibrin gels, endothelial cells were resuspended at a density of 2×10⁶ cells ml⁻¹ in 1.8 ml of a freshly made solution of bovine fibrinogen (Calbiochem AG, Lucerne, Switzerland) (2.5 mg protein ml⁻¹ in calcium-free MEM). Clotting was produced as
described (Montesano et al. 1987) by the addition of 0·2 ml of 10X concentrated MEM containing 25 U ml\(^{-1}\) human plasma thrombin (Sigma Chemical Co., St Louis, MO). The mixture was immediately transferred into a 35-mm plastic Petri dish and allowed to gel at 37°C for about 2 min before adding complete culture medium.

In other experiments, capillary endothelial cells were seeded onto preformed collagen gels in 35-mm dishes and grown to confluency. The cells were then stimulated to invade the underlying collagen matrix by treatment with either phorbol esters, fibroblast growth factor, or sodium orthovanadate, as described in detail in previous studies (Montesano & Orci, 1985; Montesano et al. 1986, 1988).

For electron microscopy, the cultures were fixed in situ with 2·5% glutaraldehyde–1% tannic acid in 0·1M sodium cacodylate buffer, pI 7·4. After several rinses in cacodylate buffer, the collagen disks were gently detached from the bottom of the Petri dishes and trimmed into squares of about 3X3 mm. These were postfixed in 1% osmium tetroxide in veronal acetate buffer for 60 min, stained en bloc with 2·5% uranyl acetate in 50% ethanol, dehydrated in graded ethanol, and embedded in Epon 812 in flat moulds. Dehydration and embedding times were from Grinnell & Bennett (1981). For ruthenium red staining (Luft, 1971), collagen gels prefixed with 2·5% glutaraldehyde were minced into very small fragments, which were incubated overnight in the same fixative containing 0·5% ruthenium red (Fluka AG, Buchs, Switzerland) and postfixed in 1% osmium tetroxide in 0·1M cacodylate buffer containing 0·1% ruthenium red. Thin sections were cut perpendicularly to the culture plane with an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips EM 410 LS electron microscope.

Results

Our first observations of intracellular diaphragmed fenestrae were made during the course of studies aimed at investigating factors that might control endothelial cell invasiveness. As described in detail in previous reports, capillary endothelial cells grown to confluency on the surface of three-dimensional collagen gels were induced to invade the underlying fibrillar matrix and to form capillary-like tubular structures in response to either tumour-promoting phorbol esters (Montesano & Orci, 1985), the angiogenic agent, basic fibroblast growth factor (Montesano et al. 1986), or sodium orthovanadate, a potent phosphotyrosine phosphatase inhibitor that mimics the action of certain growth factors by increasing the level of phosphotyrosine in cellular proteins (for a more detailed discussion of vanadate effects, see Montesano et al. 1988). Although the cells invading the collagen matrix in response to these stimuli were usually organized so as to form multicellular tubes, isolated endothelial cells containing an intracellular lumen were also occasionally seen. Many of the intracellular lumina were characterized by the presence of thin cytoplasmic septa of quite uniform thickness (about 30–50 nm) subdividing the lumen itself into several compartments (a representative example from a culture treated with vanadate is shown in Fig. 1A, but similar observations were made in cultures treated with phorbol esters or fibroblast growth factor). These septa had a striking, unique feature: they appeared to be perforated by numerous fenestrae, about 70 nm in diameter, bridged by a thin, single-layered diaphragm (the circular profile of the fenestrae was clearly evident in tangential sections) (Fig. 1B). These findings suggested that the assembly of diaphragmed fenestrae in this unusual location might be associated with the formation of an intracellular lumen.

However, in these experiments the phenomenon was observed in endothelial cells that had migrated into the collagen matrix following exposure to specific stimuli, which might themselves have induced the formation of intracellular fenestrae (phorbol myristate acetate, for example, has been shown to increase the number of fenestrae in monolayer cultures of these cells, Lombardi et al. 1986). We therefore sought culture conditions that might promote formation of intracellular lumina in the absence of any pharmacological treatment. We found that suspending endothelial cells within collagen or fibrin gels at a density preventing intercellular contact did induce the formation of intracellular lumina. A quantitative evaluation carried out on 89 semithin sections showed that 52% of single cells suspended in collagen gels had a clearly identifiable intracellular lumen (total number of cells examined = 1157). This value represents an underestimation, since in serial semithin sections, cells that were apparently devoid of a lumen in a given section frequently showed a lumen in adjacent sections. That the observed lumina had an intracellular location was confirmed in electron microscopy by: (1) post-fixation in the presence of ruthenium red (Luft, 1971), which revealed a staining of the endothelial cell surface, but not of the membrane limiting the luminal spaces, and (2) examination of serial thin sections, in which no communication was seen between the lumen and the extracellular space (data not shown). In thin sections, approximately 41% of the intracellular lumina present in endothelial cells suspended in collagen gels were traversed by thin septa containing numerous diaphragmed fenestrae (Fig. 2) (observations made in

*Collagen and fibrin gels represent physiologically relevant substrata for endothelial cells, since formation of new capillary lumina during angiogenesis is associated with the migration of sprouting endothelial cells through the perivascular stroma, whose major component is type I collagen, or through a fibrin-rich matrix, as in the process of wound healing (for a more detailed discussion of this point, see Montesano et al. 1987).
Fig. 1. Thin section perpendicular to the plane of a confluent culture of capillary endothelial cells grown on a three-dimensional collagen gel in the presence of 5 μM-sodium orthovanadate. A. An endothelial cell has migrated from the surface monolayer (ml) into the underlying collagen gel (cg). The cell contains an intracellular lumen subdivided into several compartments by thin cytoplasmic septa (arrows). B. Higher magnification of the area outlined in A. The luminal septa are crowded with fenestrae, which are recognized as either circular windows, about 70 nm in diameter (arrows) in tangential sections (left portion of the micrograph) or discrete discontinuities spanned by a thin diaphragm (arrowheads) in cross sections (right portion of the micrograph). Most of the dense cytoplasmic bodies are mitochondria. A, ×7500; B, ×18 500.
Fig. 2. Thin sections of endothelial cells suspended within collagen gels. A, D. The intracellular lumina, which contain a slightly osmiophilic flocculent material, appear to be compartmentalized by thin cytoplasmic septa. cg, collagen gel. B, C, E. Higher magnification of the areas outlined in A and D showing the diaphragmed fenestrae that perforate the septa (arrows). The arrowheads in B indicate an electron-dense line running longitudinally through the centre of the cytoplasmic septum. This 'midline' structure appears to be in register with the fenestral diaphragms. A, ×4500; B, C, ×42000; D, ×10000; E, ×44000.

Discussion

The endothelium of capillary blood vessels consists of a highly curved monolayer of flattened cells that fold their cytoplasm to encircle the capillary lumen. Although the lumen is usually delimited by one or two endothelial cells, the existence of true intracellular lumina ('seamless' endothelia) has been documented in various capillary beds in vivo (Wolff & Bar, 1972; Wolff et al. 1972; Güldner & Wolff, 1973). Capillary endothelial cells also have the capacity to form lumina in vitro, and this potential can be expressed under suitable culture conditions. In this respect, we have shown previously that capillary endothelial cells grown as a monolayer on the surface of a collagen gel can be induced to organize into correctly polarized capillary-like tubes either by overlaying them with a second layer of collagen (Montesano et al. 1983), or by stimulating them to invade the underlying matrix with appropriate signals (Montesano & Orci, 1985; Montesano et al. 1986) four separate experiments). Similar results were obtained by suspending endothelial cells within fibrin gels (two experiments) (Fig. 3). In rare instances, dia-phragmed fenestrae were observed in both luminal septa and attenuated cytoplasmic regions separating the intracellular lumen from the extracellular space (cf. Fig. 1B). Even in this situation, however, the lumen was segregated from the extracellular milieu: the fenestrae perforating the septa had, therefore, an intracellular location, in contrast to the situation observed in endothelial pockets (Milici et al. 1986). A thin electron-dense line running along the axis of the cytoplasmic septa on either side of the fenestrae was frequently observed, and appeared to be in register with the fenestral diaphragms themselves (see, for example, Figs 2B, 3D). A similar 'midline' structure, whose nature remains to be determined, has also been observed in attenuated cytoplasmic regions of capillary endothelial cells in monolayer culture (unpublished results).
have shown that formation of intracellular lumina is reproducibly induced by suspending endothelial cells at low density within three-dimensional collagen or fibrin gels. A common feature shared by all of the experimental conditions promoting the development of intracellular lumina, is that single endothelial cells were entirely surrounded by extracellular matrix, either because they had been stimulated to migrate from the matrix–medium interface to the interior of the matrix, or because they had been suspended at low density within the matrix. This situation is unusual for endothelial cells, which normally possess distinct apical and basal plasma membrane domains (Lombardi et al. 1985; Muller & Gimbrone, 1986) that, in vivo, are in contact with the circulating blood and extracellular matrix, respectively. The formation of an intracellular lumen under these conditions may therefore be viewed as an attempt of the cell to re-establish its original polarity by creating a new intracellular ‘apical’ compartment. A similar interpretation has been proposed to explain the formation of lumen-like intracellular compartments in epithelial cells (Remy, 1986; Vega-Salas et al. 1987).

A striking feature observed in many of the intracellular endothelial lumina was the presence of thin cytoplasmic septa perforated by numerous diaphragmed fenestrae. Although these were morphologically indistinguishable from fenestrae occurring in attenuated regions of the capillary endothelium in vivo, it remains to be determined whether they also have similar charge properties and lectin-binding specificities (Simionescu, 1983). Compartmentalization of lumen-like intracellular vacuoles by thin cytoplasmic septa similar to those described in the present study has recently been observed in a human angiosarcoma (Shimizu et al. 1986). However, due to the low magnification of the published micrographs, it cannot be ascertained whether the septa contain diaphragmed fenestrae.

The reason for the formation of fenestrated intraluminal septa in endothelial cells is at present unclear, as are the mechanisms by which these structures are assembled in this unique intracellular location. It is clear from these experiments that the formation of intracellular endothelial fenestrae

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**Fig. 3.** Thin section of an endothelial cell suspended within a fibrin gel. A. A system of anastomosing thin cytoplasmic septa subdivides the intracellular lumen into several contiguous compartments filled with electron-dense flocculent material, fg, fibrin gel. B. Higher magnification of the area outlined in A revealing the occurrence of numerous diaphragmed fenestrae. C,D. Details of the areas outlined in B. The arrows indicate the central knob that is characteristic of fenestral diaphragms. Also notice the cytoplasmic ‘midline’ structure (arrowheads) in register with the diaphragms. The surface of the cytoplasmic septa is covered by a fuzzy coating clearly visualized with tannic acid. A, ×7500; B, ×42,000; C,D, ×137,000.
intracellular fenestrae can occur in the absence of an exogenously added pharmacological stimulus. Neither is formation of fenestrae induced by the collagen or fibrin substrate itself, since this phenomenon is not observed in endothelial cells grown on the surface of these gels. A tentative hypothesis to explain the formation of intracellular fenestrae is suggested by the particular geometry of endothelial cells grown within a three-dimensional matrix: under these culture conditions, the cells do not show the characteristic flattened configuration observed either in vivo or in conventional monolayer culture, and usually lack highly attenuated cytoplasmic regions. One may therefore speculate that in the absence of the appropriate site of insertion, diaphragmed fenestrae are still assembled, but accumulate in an intracellular compartment.

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