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Identification of the connective tissues synthesized by the venous and arterial endothelia of the human umbilical cord: a comparative study

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Summary. Immunocytochemistry has been used to identify endothelial cells in sections of human umbilical cord and in cultures of the venous and arterial endothelium, using Factor VIII and Ulex europaeus as endothelial markers. The connective tissue components, including various collagen types, fibronectin and laminin, were identified and localized in the cord and in both venous and arterial cultured endothelium. Interstitial collagens synthesized by the cultured cells were isolated and quantified. Angiogenic ability was examined. The effect of a noxious stimulus, 24 h hypoxia, was quantified in cultured venous endothelium. The results showed that cultured arterial endothelium possesses a vacuolated cytoplasm which is absent in venous endothelium. The major collagens observed in venous culture were types III and V; the latter was found mainly in the cell layer. Venous endothelium was angiogenic. It responded to hypoxia by producing fewer cells, more protein/10^6 cells but less collagen, both in absolute terms and as a percentage of protein/10^6 cells, thus behaving like cultured porcine and bovine aortic endothelium. Fibronectin was the major 'glue' associated with endothelium. We conclude that culture can reveal the synthetic potential of endothelium which the cord itself does not often show; moreover culture appears essential to demonstrate that arterial and venous endothelium behave differently from each other.

Keywords: endothelium, collagens, fibronectin, angiogenesis, hypoxia

Following our original finding that porcine and bovine endothelium in culture can produce interstitial collagens, almost always a component of atherosclerotic plaques (Levene & Poole 1962), as well as basement membrane collagen and three of the collagen marker enzymes, proline and lysine hydroxylases and lysine oxidase (Levene & Heslop 1977), the collagen types were identified by SDS–PAGE in the cell layer and in the supernatant medium, and quantified by fluorography (Levene *et al.* 1984). The results demonstrated considerable species difference (Table 1).

A similar species difference may be seen in sprouting endothelial cultures showing angiogenesis; these can be desprouted by treatment with 8-bromocyclic-AMP or cholera toxin, following which they revert to the original cobblestone appearance (Makarski 1982). The pig, subjected to this procedure, shows little change in its collagen profile,

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Table 1. Comparison of the proportions of collagens I, III and V synthesized by porcine and by bovine aortic endothelium in culture; the table also compares the amounts present in the cell layer with the amounts released into the overlying culture medium

	Cells			Medium		
	I	III	V	I	III	V
Porcine (%)	73	0	27	95	3	2
Bovine (%)	0	2	98	0	72	28

whereas bovine endothelium undergoes considerable variation. Clearly, phenotypic changes in the collagen polymorphic profile is also species specific under angiogenic circumstances (Levene *et al.* 1984).

Finally, we have shown that endothelium of pig and cow subjected to two of the components of cigarette smoke, hypoxia and carbon monoxide, also demonstrated species specificity; endothelium subjected to hypoxia, for example, produces a massive change in the collagen profile in the cow but very little in the pig, whereas carbon monoxide affects the collagen profile in neither species (Levene *et al.* 1985).

Our findings have led us to conclude that since porcine and bovine aortic endothelium show species specificity, human vascular endothelium should also be examined. Furthermore, a comparison should be made between human arterial, venous and capillary endothelium, using specific immunocytochemical and biochemical markers. Is human endothelium capable of synthesizing collagen and, if so, what types? What factors regulate the phenotype? Is human endothelium capable of angiogenesis in culture and, if so, which type(s) possess the capacity? How does human endothelium respond to hypoxia? A fundamental study of this nature should provide a baseline to inform us whether all human endothelia behave identically or not; this baseline should eventually enable us to define abnormal endothelial behaviour in particular disease processes.

Materials and methods

Endothelial cell markers

As well as using paraffin sections of human umbilical cord and cultured human umbilical arterial and venous endothelial cells, 3T6 fibroblasts were grown on slides to act as negative controls for these cell markers.

Factor VIII. Methods identical to those described later under Peroxidase–anti-Peroxidase were employed with the following variations: the primary antibody was mouse monoclonal antibody to human Factor VIII (a gift from Dr Alison Goodall, Academic Department of Immunology, Royal Free Hospital School of Medicine) at a dilution of 1/500–1/1000; the second antibody was sheep anti-mouse(1/10)(Sera-Lab) and finally mouse PAP(1/1000)(Sera-Lab).

Ulex-europaeus-I (UEA-I). Sections of cells previously treated as above were incubated with Ulex-europaeus-I (EY Labs) for 60 min. After washing, the colour was developed with Diamino-benzidine (DAB, Sigma).

Immunohistochemical localization of collagen types, fibronectin and laminin

Tissues and cells. Umbilical cords from term pregnancies were obtained from the Rosie Maternity Unit, Addenbrooke's Hospital, Cambridge and fixed within 48 h of birth in formol saline for 24 h at room temperature. After embedding in paraffin wax 6 μm sections were cut. They were then dewaxed and brought to water before immunostaining. Endothelial cells, cultured on 8-well microscope slides (Flow Labs) were washed twice in phosphate buffered saline pH 7.2 (PBS), fixed in methanol and air-dried. At this stage slides can be stored at −20°C prior to rehydrating for immunostaining.

Endothelial cell culture. Human umbilical cords were collected and stored in cold PBS, containing 50 μg/ml. gentamicin and used

within 48 h of delivery. The method for isolation of endothelial cells was essentially that of Jaffe *et al.* (1973). Briefly, the vessels were washed free of blood with PBS and the endothelial cells detached by a 15 min incubation with 0·15% collagenase (Worthington CLS II).

The cells were grown in 25 cm^2 flasks or 35 mm plates (Costar), precoated with 1% gelatin (Sigma). The medium used was Iscove's modification of Dulbecco's medium, without bovine serum albumin, pure human transferrin and soybean lecithin (Flow Labs). The cells were fed every 2–3 days and used in primary culture or first passage. Their endothelial identity was established by the presence of Factor VIII or by positive Ulex staining.

For subculture, 0.05% trypsin–0.02% EDTA (Gibco) was used to detach the cells, usually requiring less than 5 min at 37°C. Seeding density was $2–4 \times 10/cm^2$.

For histology, cells were grown on gelatin-coated coverslips and fixed in methanol before staining with the May–Grunwald modification of the Giemsa stain.

Localization of collagen types, fibronectin and laminin. Antisera raised in rabbits to human collagens types I and IV were obtained from the Laboratoire de Pathologie Cellulaire, Institut Pasteur de Lyon. Rabbit anti-human type III collagen was a gift from Dr George Martin, NIDR, NIH. Rabbit anti-fibronectin and anti-laminin were a gift from Dr Antonio Martinez, Department of Pathology, Jefferson Medical College.

Peroxidase-anti-peroxidase (PAP). All washes between stages were in Tris Buffered Saline pH 7.4 (TBS) and all antibody dilutions were made in TBS containing 1% bovine serum albumin (BSA, Sigma) and 1% normal swine serum (NSwS, Dako) in order to reduce non-specific background staining. All washes and incubations were carried out at room temperature.

All slides were treated in the same way following either trypsin treatment (paraffin sections) or rehydration after air-drying (cells grown on slides).

Pretreatment with trypsin. Having been rehydrated, formalin-fixed sections were washed for 5 min in PBS at room temperature and then 5 min at 37°C prior to incubation in 1% trypsin (Difco) (in 1% $CaCl_2$ pH 7.8) for 20 min shaking at 37°C. The reaction was stopped by washing in PBS at room temperature. Sections and cells were then treated with 3% hydrogen peroxide to block endogenous peroxidase, then washed and incubated with NSwS (1/10) for 30 min; this was tipped off carefully and without washing; the primary antibody was then applied. The following dilutions were used for 60 min:

Rabbit anti-Type I collagen	1/100–1/200
Rabbit anti-Type III collagen	1/100–1/200
Rabbit anti-Type IV collagen	1/200–1/400
Rabbit anti-Fibronectin	1/50–1/100
Rabbit anti-Laminin	1/50–1/100

Normal rabbit serum (NSR, Dako) at the appropriate dilution was used as the negative control. The second antibody, Swine anti-rabbit (Dako) was applied at 1/20 for 90 min. Slides were then washed, developed with DAB (Diaminobenzidine, Sigma), washed, counterstained with Carazzi's haematoxylin for 8 min, cleared, dehydrated and mounted.

Chemical procedures

Cell numbers. These were estimated by direct counting following trypsination and/or by DNA measurements (Burton 1956).

Collagen synthesis. This was studied by the addition to cultures for a period of 24 h, of 20 μCi/60 mm plate of L-(5-^{3}H)-proline, a precursor for ^{3}H-hydroxyproline, which is a marker for collagen; ascorbic acid, an essential cofactor, is simultaneously added at 50 μg/ml. medium. Collagen was estimated in both the cell layer and the medium by incubation within a dialysis bag with a protease-free, highly purified collagenase.

The hydrolysate was dried down *in vacuo*, hydrolysed in 6 M HCl and ^{3}H-proline and ^{3}H-hydroxyproline separated by paper chromatography; they were then eluted from the paper and counted in a Beckman liquid scintillation counter, thus permitting quantification of collagen synthesis during the 24 h pulse (Levene & Bates 1975).

Isolation and quantification of collagen types. During the experimental period when half of the cultures were subjected to hypoxia, all the cultures, including normal controls, received 100 μCi L-(5-^{3}H)-proline (Amersham International) per 60 mm plate plus 50 μg ascorbic acid per ml of medium, plus 100 μg/ml medium of β-aminopropionitrile; the latter was added to increase the yield of extractable collagen for typing.

Collagens were isolated and the relative proportions of collagen types I, III and V assayed by SDS–PAGE (Laemmli 1970; Sykes *et al.* 1978) followed by fluorography (Bonner & Laskey 1974; Laskey & Mills 1975) as previously described (Levene *et al.* 1984).

Production of hypoxic environment. The Fildes' jars containing the experimental dishes in which the human umbilical venous endothelium had been grown to confluence, were gassed for the appropriate time with a mixture of 95% N_2 and 5% CO_2 and incubated for 24 h at 37°C. This mixture is hypoxic rather than anoxic since it is known to contain traces of O_2. The control plates received the normal mixture of 75% N_2, 20% O_2 and 5% CO_2.

Results

Identification of endothelium

Cord

Factor VIII. This was found to be present in the endothelium lining the umbilical artery and vein: it had diffused sub-endothelially in the artery. In the vein we found that what we considered to be the internal elastic lamina

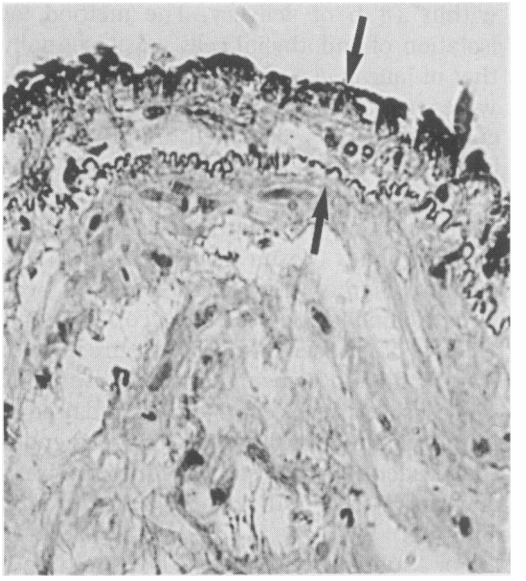

Fig. 1. Human umbilical cord stained with PAP method with anti-Factor VIII antiserum. The venous endothelium stains positively; the internal elastic lamina also appears to stain positively (see arrows).

was also clearly stained by the anti-Factor VIII antiserum (Fig. 1).

Ulex europaeus. The venous and arterial endothelial cells were stained strongly by this lectin (Fig. 2).

Cultured endothelia

Factor VIII. The cytoplasm of cultured venous endothelium showed a strong positive staining for Factor VIII (Fig. 3). Arterial endothelium in primary culture however stained negatively.

Ulex europaeus. The cytoplasm of cultured venous and arterial endothelium stained positively and strongly (Fig. 4).

Morphological difference between cultured venous and arterial endothelium. Arterial endothelium grown on coverslips and stained after methanol fixation with the Giemsa stain, was always noted to have a

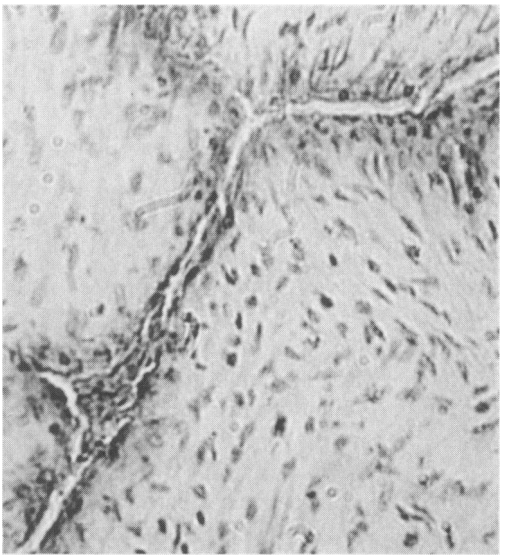

Fig. 2. Human umbilical cord stained with the lectin Ulex europaeus to demonstrate positive staining of the arterial endothelium.

highly vacuolated cytoplasm; this was never observed in similarly treated cultures of venous endothelium.

Immunocytochemical localization of various collagen types, fibronectin and laminin

Cord

Type I collagen. Venous endothelium showed no type I collagen subendothelially; arterial endothelium showed a faintly positive result. This antiserum, however, stained the blood vessel walls a faintly positive colour.

Type III collagen. No staining was observed beneath venous or arterial endothelium: however, like type I, anti-type III collagen stained the blood vessel walls a faintly positive colour.

Type IV collagen. A strongly positive reaction was observed beneath the venous endothelium and to a lesser extent, beneath the arterial endothelium; curiously, it also stained the elastica in vein and artery, very

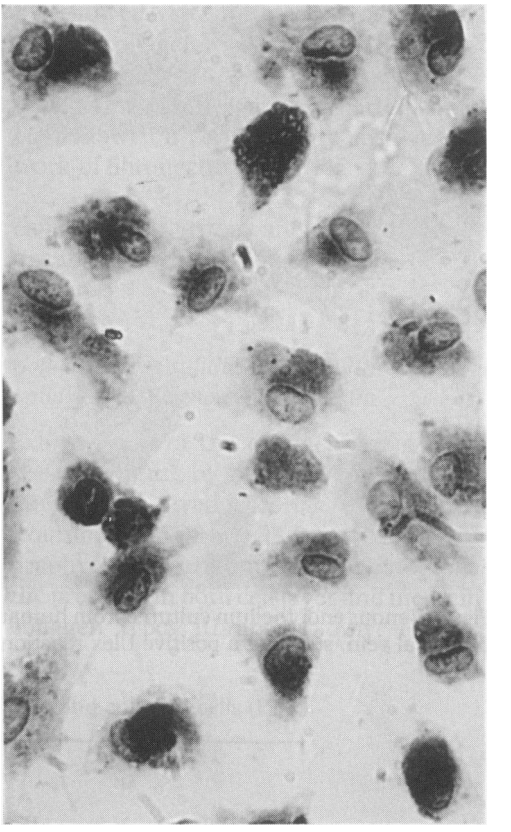

Fig. 3. Venous endothelium cultured from human umbilical vein and stained with anti-Factor VIII antiserum using the PAP method, to show positively stained cytoplasm.

like the effect of anti-Factor VIII antiserum on the vein (Fig. 5). In view of the considerable thickness of the arterial intima, as opposed to the venous intima, it is necessary to look for elastin at the medial-intimal junction fairly deep inside the vessel wall.

Type V collagen. No antiserum was available to test for this collagen.

Laminin. Venous and arterial subendothelium stained negatively.

Fibronectin. A strong positive stain was seen subendothelially in both vein and artery. The artery and vein both showed a faintly posi-

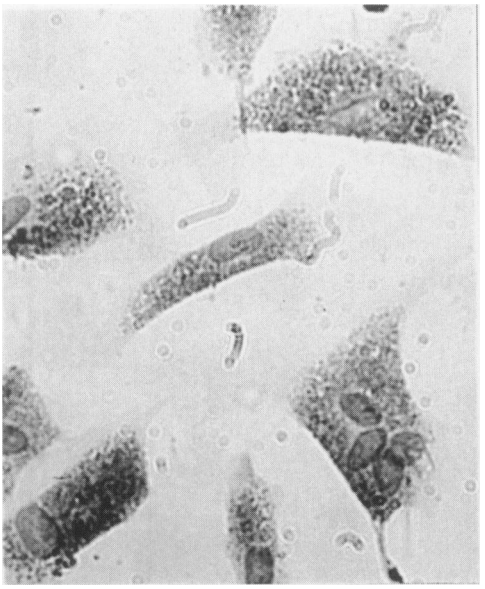

Fig. 4. Venous endothelium cultured from human umbilical vein, showing a positive Ulex reaction.

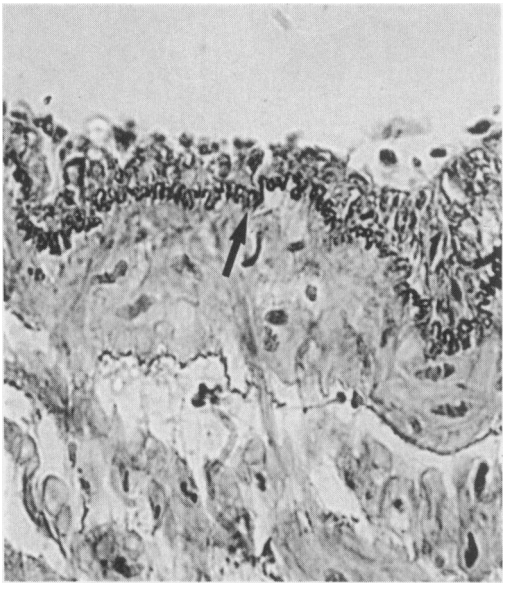

Fig. 5. Human umbilical cord stained with the PAP method, using anti-type IV antiserum to demonstrate subendothelial localisation; the internal elastic lamina also stains positively (see arrow).

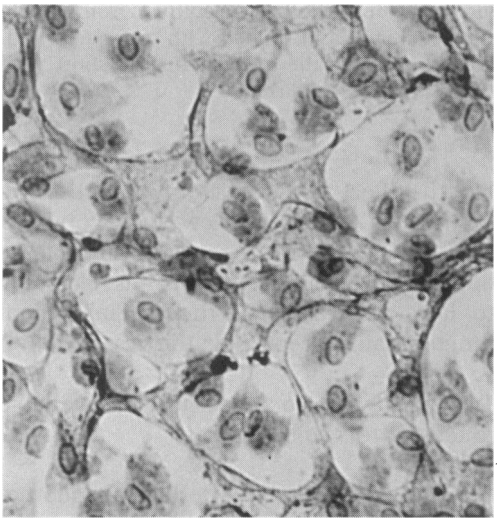

Fig. 6. Venous endothelium cultured from human umbilical cord and stained with the PAP method, using anti-type IV collagen antiserum; a positively stained fibrillar network enmeshes the cells.

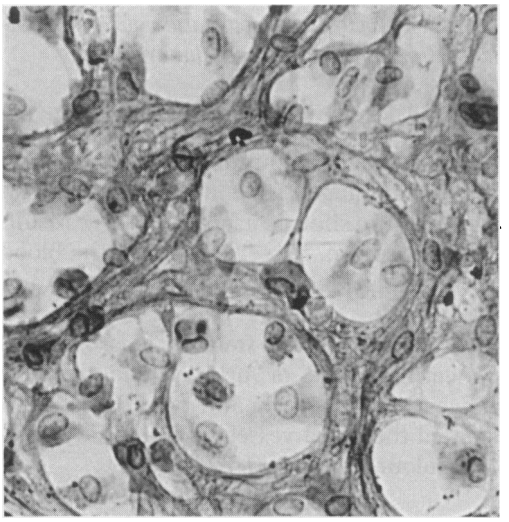

Fig. 7. Venous endothelium cultured from human umbilical cord and stained with the PAP method, using anti-fibronectin antiserum; a positively stained fibrillar network is clearly seen.

tive background throughout the vessel which was, however, absent in the Wharton's jelly part of the cord.

Cultured endothelia

Type I collagen. Venous endothelium stained positively but the arterial endothelium was negative. The venous collagen was seen as a thin positive network enmeshing the cells.

Type III collagen. Venous cells showed a strongly positive network of fibres enmeshing the cells but arterial cells stained negatively.

Type IV collagen. This also showed a strongly positive fibrillar collagen network surrounding and enmeshing the venous cells (Fig. 6). The arterial cells, which grew more slowly and had not reached confluence, nevertheless showed a strongly positive cytoplasm for type IV collagen.

Fibronectin. Both vein and artery-derived cells showed a very strongly positive meshwork of fibronectin fibres (Fig. 7).

Laminin. Both venous and arterial cells were negative—no fibrillar network was observed (Table 2).

Collagen types synthesized in culture by human umbilical cord venous endothelium

Examination of the collagen types synthesized in culture by human venous endothelium, when given ascorbic acid daily and examined in SDS–PAGE followed by fluorography before and after reduction, showed the presence in both cell layer and medium of collagen types III and V only. However,

Table 2. Immunocytochemical and lectin staining of cord and cultured cells (PAP)

CORD

Stain	Vein	Artery
Factor VIII	Positive (also elastin)	Positive
Ulex europaeus	Positive	Positive
Collagen type I*	Negative	Faint positive
Collagen type III*	Negative	Negative
Collagen type IV	Positive (also elastin)	Modest positive
Laminin	Negative	Negative
Fibronectin	Positive	Positive

* Both I and III showed weak diffuse staining of all the vessels.

CULTURED CELLS

	Venous	Arterial
Cytoplasmic vacuolation	Negative	Positive
Factor VIII	Positive	Negative
Ulex europaeus	Positive	Positive
Collagen type I	Positive network	Negative
Collagen type III	Positive network	Negative
Collagen type IV	Positive network	Positive cytoplasm
Fibronectin	Positive network	Positive network
Laminin	Negative	Negative

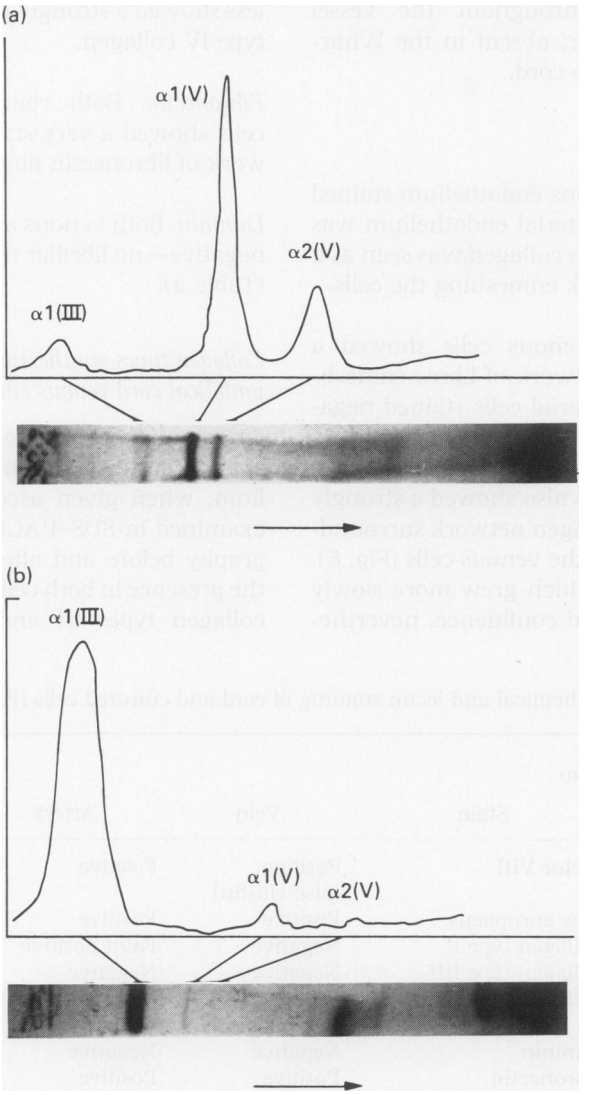

Fig. 8. SDS–PAGE gels (*a*) of collagens isolated from venous endothelium cultured from human umbilical cord and (*b*) of collagens isolated from the culture medium. Types III and V are present; quantitative scanning of the gels following fluorography showed that type V collagen predominates in the cell layer whilst type III predominates in the medium.

whereas type III was quantitatively minimal in the cell layer and type V maximal, the opposite was found to be true for the medium. Clearly, the cell layer retained type V preferentially and released type III into the overyling medium (Fig. 8).

Angiogenesis

As previously shown, monolayers of bovine aortic cells formed everted capillaries in culture (Fig. 9) which grew in the correct conformation when grown in a three-dimen-

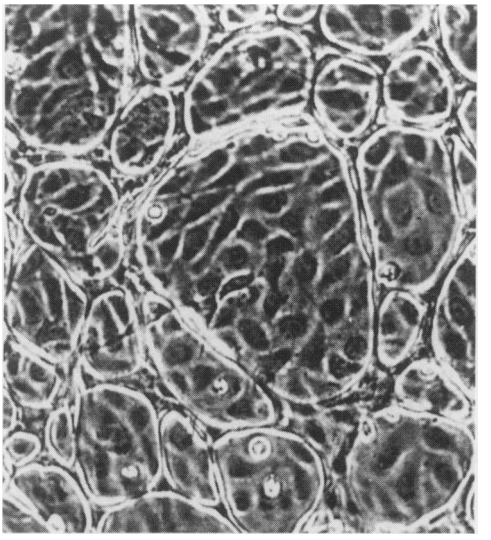

Fig. 9. Bovine aortic endothelium in culture showing in-vitro angiogenesis; the capillaries are growing 'inside out'.

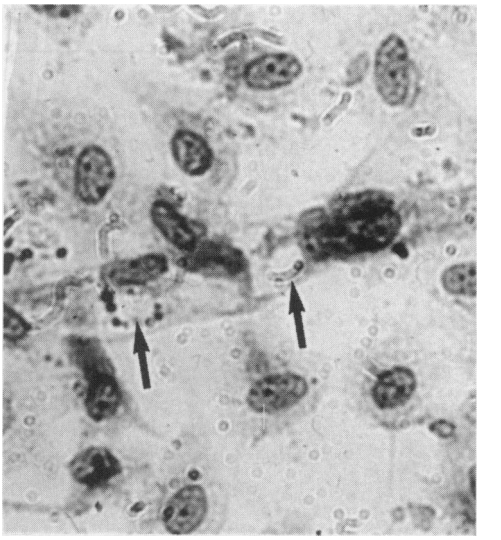

Fig. 10. Human umbilical venous endothelium in monolayer culture showing early angiogenesis; note the partial vacuolation of the lumen.

sional matrix of collagen gel. In this study, old cultures of human umbilical venous endothelium in monolayers were also seen to exhibit the earliest signs of angiogenesis, i.e. vacuolation occurring in what appeared to be a blocked early 'precapillary' (Fig. 10).

Effect of 24 h hypoxia on cultured umbilical venous endothelium

Twenty-four hour growth in an hypoxic environment resulted in:

(a) A modest diminution in cell numbers.
(b) A rise in total protein synthesized/10^6 cells.
(c) A modest fall in collagen synthesized/10^6 cells.
(d) A decrease of approximately 30% in collagen as a percentage of the total protein synthesized in 24 h/10^6 cells (Table 3).

These findings correlate well with previous findings of the effect of hypoxia on cultured *porcine* aortic endothelium and, to a large extent, with the results on cultured *bovine* aortic endothelium (Levene *et al.* 1985).

Discussion

Examination of cord sections showed Factor VIII to be present in both venous and arterial endothelium whereas in culture only venous endothelium revealed its presence. The explanation may be either that culture results in the loss of Factor VIII from the arterial cells or that arterial cells lose the capacity to synthesize Factor VIII; we believe the former to be the likelier explanation. We cannot explain why the antiserum to Factor VIII stained venous internal elastic lamina. The antiserum was monoclonal and raises the question of a possible antigen common to endothelium and an elastin component.

Ulex gave positive results which were consistent in both cord and cultured cells. Morphologically, arterial cells consistently showed cytoplasmic vacuolation; venous endothelium did not; such vacuolation may prove to be a marker for cultured arterial endothelium.

Table 3. Effect of 24 h hypoxia on human umbilical venous endothelium cultured for 3 days

	$+O_2$	$-O_2$
Cell no./60 mm plate*	1.04×10^6	0.89×10^6
Total protein synthesized in 24 h/10^6 cells – dpm†	352.3×10^3	433.4×10^3
Total collagen synthesized in 24 h/10^6 cells – dpm†	23.8×10^3	20.0×10^3
Collagen as a per cent of protein per 10^6 cells†	6.7	4.7

* Cells (in passage 1) plated at 670 000/60 mm dish and at 230 000/35 mm dish containing a coverslip.

† These radioactive assays were made on the total contents of the dish, i.e. cell layer + overlying medium.

Apparent inconsistencies were found in the identification of collagens when comparing cord with cultured cells, e.g. collagens I and III gave a general background stain in the vessels in the cord, but there was no particular staining observed in the region of the vascular endothelium in these sections. In culture, however, both I and III showed an elegant collagenous network enmeshing the venous cells whereas the cultured arterial cells were negative. We do not know why. Culture clearly changes the cells' behaviour in regard to collagen synthesis, i.e. it clearly shows the cells' potential for collagen synthesis but does not appear to reflect their actual behaviour *in vivo*.

Collagen type IV gave a strong positive reaction in the cord beneath the vascular endothelium, but it also stained the elastica, just as Factor VIII stained the elastica. However, in culture, type IV collagen was seen as an elegant network surrounding the venous cells, but present only in the cytoplasm of the cultured arterial cells where no extracellular network was observed. We cannot, as yet, explain this observation.

Against this, however, we found that fibronectin was present as a clear network around both venous and arterial cells in culture.

Laminin was never clearly observed in either blood vessel.

It is also clear from the SDS–PAGE study that the cell layer in culture retains type V collagen whereas the bulk of type III collagen is released into the medium. The answer probably lies in the functions of these two collagens, which are as yet unknown; our finding described above may eventually provide a clue as to the function of type V collagen. Also of interest is the failure to detect type I collagen in the SDS gels, which had originated from the collagen of cultured endothelia, whereas type I was evident in cultured venous cells immunocytochemically; a likely explanation is that immunocytochemistry is a more sensitive method than SDS–PAGE.

Old cultures of porcine and bovine aortic endothelium have been shown to possess the property of developing 'sprouts' which are eventually considered to become capillaries. We have also found this angiogenic capacity to occur in older cultures of human venous endothelium as described by Folkman and Haudenschild (1980); in older cultures of human arterial endothelium we have found many crescent-shaped cells which Folkman and Haudenschild (1982) have implied are capillary precursors.

Finally, we have examined the behaviour of cultured venous endothelium under pathological conditions. When subjected to hypoxia, one of the noxious factors asso-

ciated with cigarette smoking *in vivo*, where lesions have already been observed in the human cord of mothers who smoke heavily (Asmussen 1982), the venous cells responded in the same manner as previously shown for cultured pig aortic endothelium and to a large extent for cultured bovine aortic endothelium, i.e. diminution in cell numbers, increase in total protein synthesis per 10^6 cells but an absolute fall in collagen synthesis, both on a per 10^6 cell basis and as a percentage of total protein synthesized/10^6 cells (Levene *et al.* 1985).

This study leads us to conclude firstly that arterial and venous endothelium derived from the human umbilical cord look and behave differently from each other and secondly, that culture of these cells can reveal the potential synthetic abilities of endothelium which the vessels, as examined in the cord *in situ*, do not often show.

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