Analysis of polypeptides induced by hydrocortisone-21-sodium succinate (HCS). Five pairs of salivary glands were dissected in insect Ringer and incubated for 90 min at 24 °C in 50 µl of Poel's medium[10] containing appropriate concentrations of HCS or no HCS (controls); HCS was used because it is soluble in the incubation medium at higher concentrations than hydrocortisone or most other hydrocortisone salts. In some experiments 5 µg/ml α-amanitin was added to the HCS containing incubation medium. Glands were labeled for 45 min at 24 °C in fresh medium containing 400 µCi/ml ^{35}S-methionine (sp. act. 600–1300 Ci/mM, Amersham), then transferred to buffer containing 50 µl 0.0625 M Tris-HCl pH 6.8,2% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol, 0.001% bromophenol blue, 10% glycerol and were heated in boiling water for 10 min. After centrifugation the supernatant was run on 11% SDS-polyacrylamide gels[11]. Gels were stained with Coomassie blue and dried; autoradiography was performed using Kodak No-Screen X-ray Film.

Proteins from similarly treated glands were also analyzed on two/dimensional gels according to O'Farrell[12].

Analysis of heat-shock induced polypeptides. Larvae were shocked at 36.5 °C for 30 min; the salivary glands were dissected in insect Ringer, and proteins were immediately labeled with ^{35}S-methionine and analyzed as described above.

Results and discussion. Isolated salivary glands from 3rd instar *Drosophila melanogaster* larvae responded to incubation in presence of hydrocortisone-21-sodium succinate by synthesizing a protein significantly less represented in glands incubated in the same medium without HCS (compare lane a with lanes b–c in fig. 1); this protein was also absent in glands treated with comparable concentrations of sodium succinate, and its synthesis is inhibited by 5 µg/ml α-amanitin (data not shown). Maximum induction occurred at about 7 mM HCS; at higher concentrations, total protein synthesis rapidly diminished and was almost absent at 13 mM HCS (fig. 1, d). In 11% SDS-polyacrylamide gels, the protein induced by HCS migrated to the position corresponding to the 70K; the heat-shock response, however, is clearly much stronger (fig. 1, f). Proteins at the positions of hsp26, hsp68 and hsp83 were also present in small amounts in HCS-treated glands; it should be pointed out that traces of these proteins were also often present in our controls.

To confirm the identification of the major HCS-induced protein as the 70K hsp, ^{35}S-methionine labeled proteins from HCS-treated glands were run on 2-dimensional gels and compared with autoradiograms of similarly labeled proteins from heat-shocked glands (fig. 2, A). In the HCS treated sample, the major newly synthesized protein also migrated to the position of the 70K hsp (fig. 2, B). Moreover, we observed cytologically in HCS treated glands significant induction of the puffs at 87A and 87C, the loci of hsp70 synthesis[13,14].

It is difficult to assess the biological significance, if any, of induction of hsp70 (and possibly other hsp's) by a mammalian steroid hormone such as hydrocortisone. We cannot exclude a specific hormone-mediated response, but, because of the high concentration necessary for induction (compare with 10 µM ecdysone sufficient for hsp stimulation in *Drosophila* cells lines[5]), and of the fact that still higher doses abolished total protein synthesis, we favor the hypothesis that incubation in HCS at the doses employed represents per se a stress situation. If this is the case, HCS is to be added to the list of agents capable of eliciting the 'heatshock' response, at least in isolated salivary glands of *Drosophila melanogaster.*

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Cultivation of arterial endothelial cells from human umbilical cord

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Summary. We have developed a simple method for the isolation of endothelial cells from human umbilical artery. The method provides a sufficient number of cells to be of experimental value. The presence of factor VIII antigen specific for endothelium has been demonstrated by immunofluorescence as well as by the peroxidase-antiperoxidase immune complex method.

Valuable information on the physiological functions of endothelium has been obtained using the method of Maruyama[1] and Jaffe et al.[2] developed for the cultivation of endothelial cells from the human umbilical vein. This method has also been utilized by several investigators for the cultivation of umbilical arterial endothelium, but isolation procedures have not been reported in detail[3-5]. From our experience arterial endothelial cells in large numbers

have hitherto not been obtainable. The umbilical artery is much smaller than the vein and, due to marked vasoconstriction, infusion of an enzyme solution into the vascular lumen is difficult. We therefore attempted to prepare cultures of endothelial cells from umbilical arteries in numbers sufficient to be of experimental value. In this report, the simple and reproducible technique for isolating and growing arterial endothelial cells from human umbilical cord is described, and the presence of endothelium-specific factor VIII antigen is demonstrated using immunofluorescent and immunoperoxidase staining methods.

Materials and methods. The umbilical cord was severed from the placenta immediately after delivery, placed in Ca^{++}-Mg^{++}-free phosphate buffered saline (PBS), and held at 4 °C until processing. For isolation of arterial endothelial cells, we separated the arterial vessels from the cord in the following way: A fine catheter (diameter 1.3 mm) was inserted into the cord tissue and pushed forward along the arterial vessel. Since the cord tissue tears longitudinally with ease, the intact artery was easily separated from the cord (fig. 1, a and b). The isolated arterial vessel was then ligated at one end and was cannulated with a catheter (diameter 1.3 mm) fitted with a syringe. Then 2–3 ml of nifedipine (BAY a 1040, 0.2 mg/l, Bayer), a potent vasodilator, was infused into the lumen under gentle pressure. After 2–3 min the ligated end was cut, and the artery was perfused with PBS to wash out residual blood and nifedipine. The relaxed artery was again ligated at one end; dispase II (500 PU/ml, Godo-shusei) in Medium 199 containing 5% newborn calf serum (GIBCO) was then infused into the vessel, and the other end was clamped with a hemostat. After a 20-min incubation at 37 °C the enzyme solution containing the cells was flushed from the vascular lumen by twice perfusing it with Medium 199. The effluent was collected in a centrifuge tube. The preparation of endothelial cells from human umbilical vein was performed by the method of Jaffe et al., except that dispase was used instead of collagenase[2]. The endothelial cells were cultivated with Medium 199 supplemented with 20% fetal bovine serum, 100 µg/ml streptomycin sulfate, 100 IU/ml penicillin and 2 mM L-glutamine at 37 °C under 5% CO_2.

The presence of factor VIII antigen in endothelial cells was demonstrated by both immunofluorescent staining[6,7] and by peroxidase-antiperoxidase (PAP) staining using DAKO PAP KIT (K510). Human vascular smooth muscle cells derived from the umbilical vein and human fetal fibroblasts were used as controls.

Results and discussion. The average yield of endothelial cells from 1 artery was 2.38×10^5 cells/10 cm cord, corresponding to about 60% of that from a vein (4.24×10^5 cells/10 cm cord). Since 2 arteries but only 1 vein are obtainable from 1 cord, the cell yield from arteries is comparable to that from the vein.

Arterial endothelial cells initially assume a characteristic oval or polygonal shape and tend to grow in well-defined colonies. At confluence they exhibit a cobble-stone morphology typical of the endothelium, indistinguishable from that of venous provenance (fig. 2).

We used a neutral bacterial protease, dispase, as a dissociating agent to isolate endothelial cells. The enzyme is active in a culture medium supplemented with serum, and cell damage is minimal even after prolonged exposure[8,9]. It has been reported that dispase detaches epithelial cells in sheets[10]. In our experience, dispase digestion caused exfoliation of endothelial cells from the inner lining of arterial

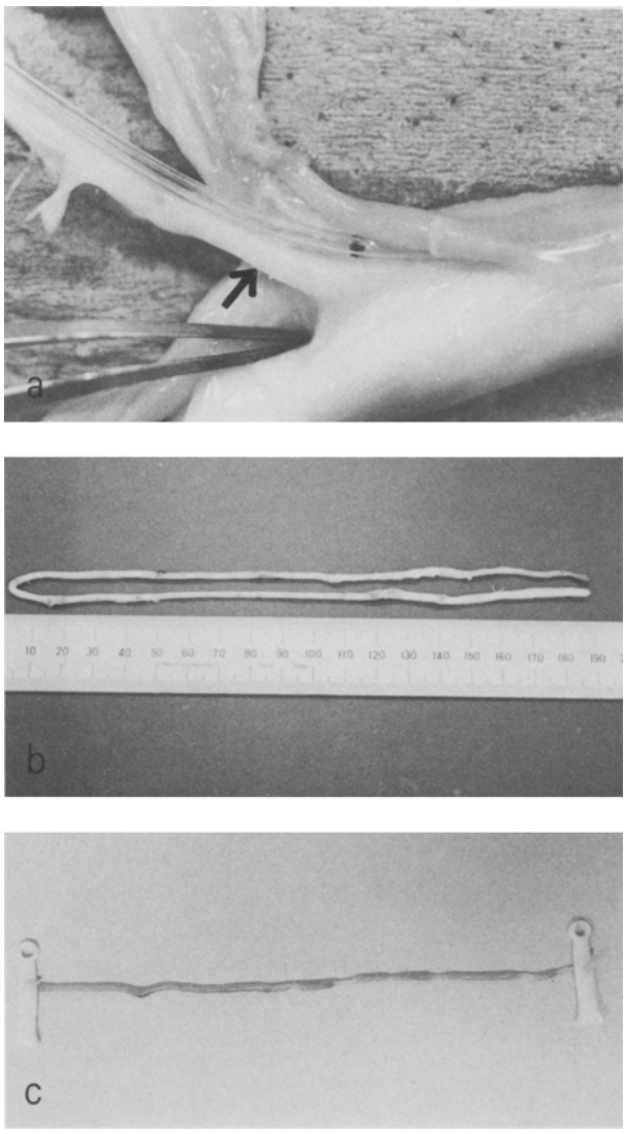

Figure 1. *a* The umbilical cord is torn to isolate the artery (arrow) using the catheter as a guide.
b The isolated, constricted artery with residual blood.
c The artery infused with dispase solution. By pretreatment with a vasodilator, nifedipine, dispase solution can be easily infused into the lumen of the artery.

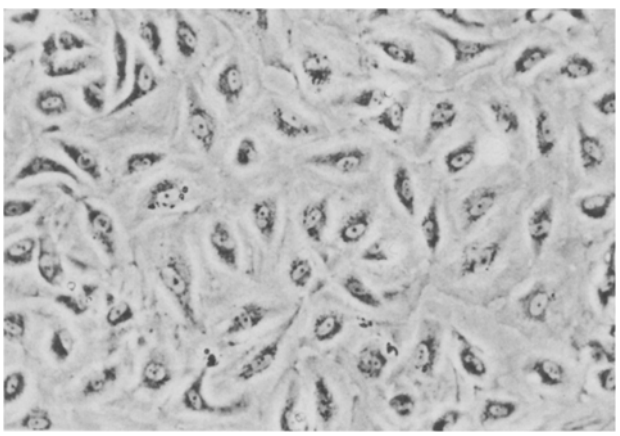

Figure 2. Phase photomicrograph of human arterial endothelial cells from umbilical cord (9-day-old). × 50.

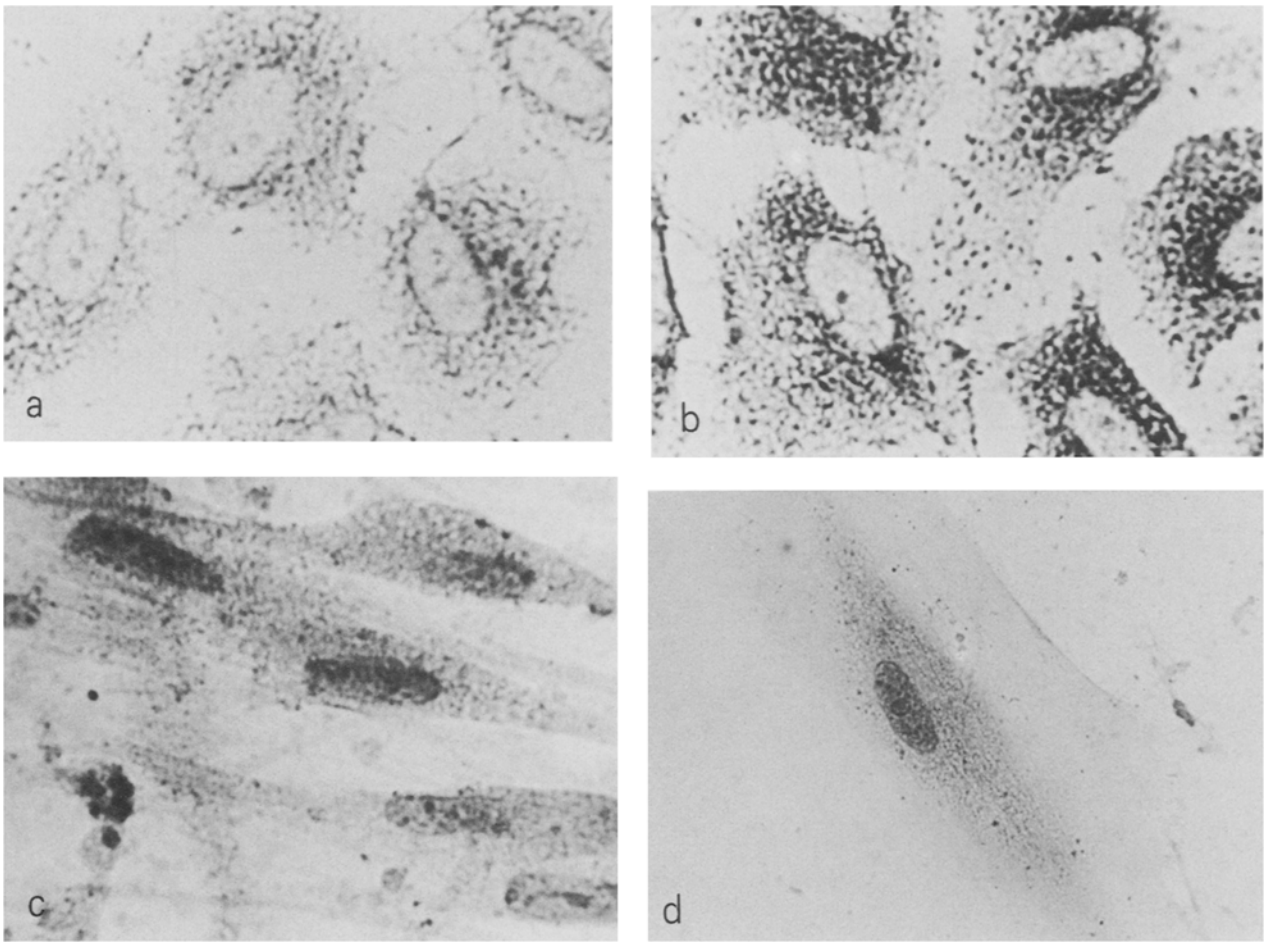

Figure 3. Antifactor VIII PAP staining of cultured cells. The cell sheet grown in a tissue culture chamber/slide (Lab-Tek, No. 4804) was fixed in methanol-3% hydrogen peroxide (4:1; v/v), followed by a PBS rinse and treatment with normal swine serum. The specimen was subsequently incubated with rabbit antihuman factor VIII antibody, rinsed in cold PBS and again incubated in excess amounts of swine antirabbit immunoglobulin. After a rinse in cold PBS, the material was processed with PAP complex (rabbit antiperoxidase antibody and peroxidase) followed by the addition of substrate solution containing hydrogen peroxide and aminoethylcarbazole. The specimen was counterstained with Carazzi's hematoxylin.
a Arterial endothelial cells (× 250);
b venous endothelial cells (× 250);
c human fetal fibroblasts (× 250);
d human vascular smooth muscle cells (× 125).

vessels in clumps, resulting in active cell proliferation. In this respect, dispase is also recommended as a useful dispersing agent for endothelium.

Immunoperoxidase techniques have been shown to be well suited for the study of the distribution of specific antigens in routinely processed paraffine-embedded sections under the light microscope. Both arterial and venous endothelial cells showed the intensely stained granular pigments – presence of factor VIII antigen – exclusively around the nucleus (fig. 3, a and b); fibroblasts and smooth muscle cells were negative (fig. 3, c and d). This method, therefore, proved superior to conventional immunofluorescent staining for the study of the localization of factor VIII antigen in endothelial cells. The cultures were not contaminated with other cell-types.

Progress in the development of in vitro systems for biomedical research has been hampered by the difficulties of obtaining suitable human materials. In our present work, a technique to isolate arterial endothelial cells from a ready human source, umbilical cord, has been substantially improved and refined. Recently, metabolic differences between arterial and venous vasculature have become a matter of interest[3–5], with special reference to vascular pathology. Cultured human arterial as well as venous endothelium from the same umbilical cord may offer a useful model for the comparative study of cell functions from various vascular origins.

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