phosphate medium[20], and in this medium glucose metabolism was impaired in rat heart[21]. SHAW and STADIE[22] proposed that in diaphragm muscle the Embden-Meyerhof pathway was inhibited at the phosphofructokinase step when the tissue was incubated in phosphate medium. If it could be assumed that the effect of pyruvate on halothane-depressed rat atria may be similar to that above, our investigation might be concerned with the manner in which halothane influences the myocardial metabolism. Either the uptake or utilization of glucose may be impaired by halothane. Using partition coefficient data of LARSON et al.[23], a saline concentration of 6 mg/100 ml halothane would be equivalent to a blood concentration of 19.8 mg/100 ml. Blood levels of 17.9–20.3 mg/100 ml were found necessary to anesthetize a dog to produce a loss in the pain reflex of foot pad[12]. Thus, the concentration of halothane employed in this study was similar to that assumed to produce in the dog.

Zusammenfassung. Die nach Halothan eingetretene Verminderung der Kontraktilität des Myokards kann durch Pyruvat verhindert werden. Dieses Ergebnis stützt die Hypothese, Halothan behindere die Aufnahme oder die Verwertung von Glycose.

KYE-CHANG KO[24,25] R. R. PARADISE and DAE-SUP HAN[24]

Pharmacology Department School of Medicine, Indiana University, Indianapolis (Indiana, USA); and Department of Pharmacology School of Medicine, Kyung-Hee University, Seoul (Korea), 30 May 1972.

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[24] Present address: Department of Pharmacology School of Medicine, Kyung-Hee University, Seoul (Korea).
[25] Reprint requests to: Dr. Kye-Chang Ko, Dept. of Pharmacology, School of Medicine, Kyung Hee University, Dongdaemoon-ku, Seoul, Korea.

Some New Aspects in Human Trophoblast Cultures

Studies concerning the immunological aspects of pregnancy strongly depend on maintaining trophoblast cells in vitro[1–6]. In the present work, a simple method is given for keeping human trophoblast cultures active for a longer period of time, and the morphological evolution of these cells, as well as their hormone secreting activity, during in vitro culture, is described.

The cultures were performed from sterile dissected chorion villi, washed in sterile 199M[7], and carefully cut with fine scissors and forceps. The fragments were transferred to a sterile tube with a rubber stopper, and 0.25% trypsine was added. The tubes were left for about 30 min in incubator at 37°C, shaken for 1 min every 5 min, and then centrifuged. The sediment, washed 2–3 times with phosphate buffer saline until supernatant remained clear, was resuspended with 199M containing 10% sterile calf serum, filtered through sterile gauze, and suspension adjusted to a proportion of 3×10^5 cells/ml[3]; an amount of 1 cm[3] was put into each sterile tube, and left at 37°C. Two types of culture tubes were used: regular ones and Leighton tubes[8], which allow the cells to grow on a removable glass cover slip. Parallel cultures were performed from same cell suspension, in sterile Petri dishes incubated in a CO^2 incubator. The tubes and the Petri dishes were examined every day in the inverted microscope. Cover slips from the Leighton tubes were removed at different intervals. Some of the cover slips were examined without staining in the phase contrast microscope, the others were stained by the May-Grunwald-Giemsa technique, and examined in the bright field microscope. Samples of growth media (199M/10% sera) were taken after 10 days of incubation and kept at 4°C. On about the 20th day, the growth media was removed and replaced by maintenance media (199M without sera). The maintenance media was changed at different intervals. All the removed media samples were concentrated by alcohol-ether extraction, and presence of humanchoriongonadotropin tested by the aid of the haemagglutination inhibition test (HIT)[9].

The development of the Petri dish cultures in CO^2 atmosphere was rapid but limited, and degenerated cells could be observed 2 to 3 weeks after incubation. In contrast to this, the cultures performed in tubes could be kept alive for 2 months and more. These cultures, performed in regular tubes as well as in Leighton, developed rather rapidly. Several aspects could be noted at different intervals.

During the 24–48 h of incubation, multinucleated giant cells and many epitheloid polygonal cells were observed, as well as few fibroblast cells.

The multinucleated giant cells contained about 10–30 nuclei in a pale stained protoplasma, and could be found until about the 8th day of culture (Figures 1–4). Serial examination of cover slip cultures, in the 2nd week, showed groups of cells congregating closely and similar to those included in the 'multinucleated giant cells', but without being delimited by a common external membrane. Their protoplasma was small as compared to the nucleus and increased later. These cells seemed to be remainders of the prior multinucleated giant cells. (Figures 5 and 6).

The epitheloid polygonal cell had a relatively large nucleus with dense and strongly stained chromatine and sometimes with big nucleolus (Figures 7–9). These cells change their shape during the in vitro evolution. At 6–9 days, the protoplasmic edges were more elongated and sometimes curved (Figures 10 and 11). After the 2nd week of culture, these cells were larger, with large nuclei full of

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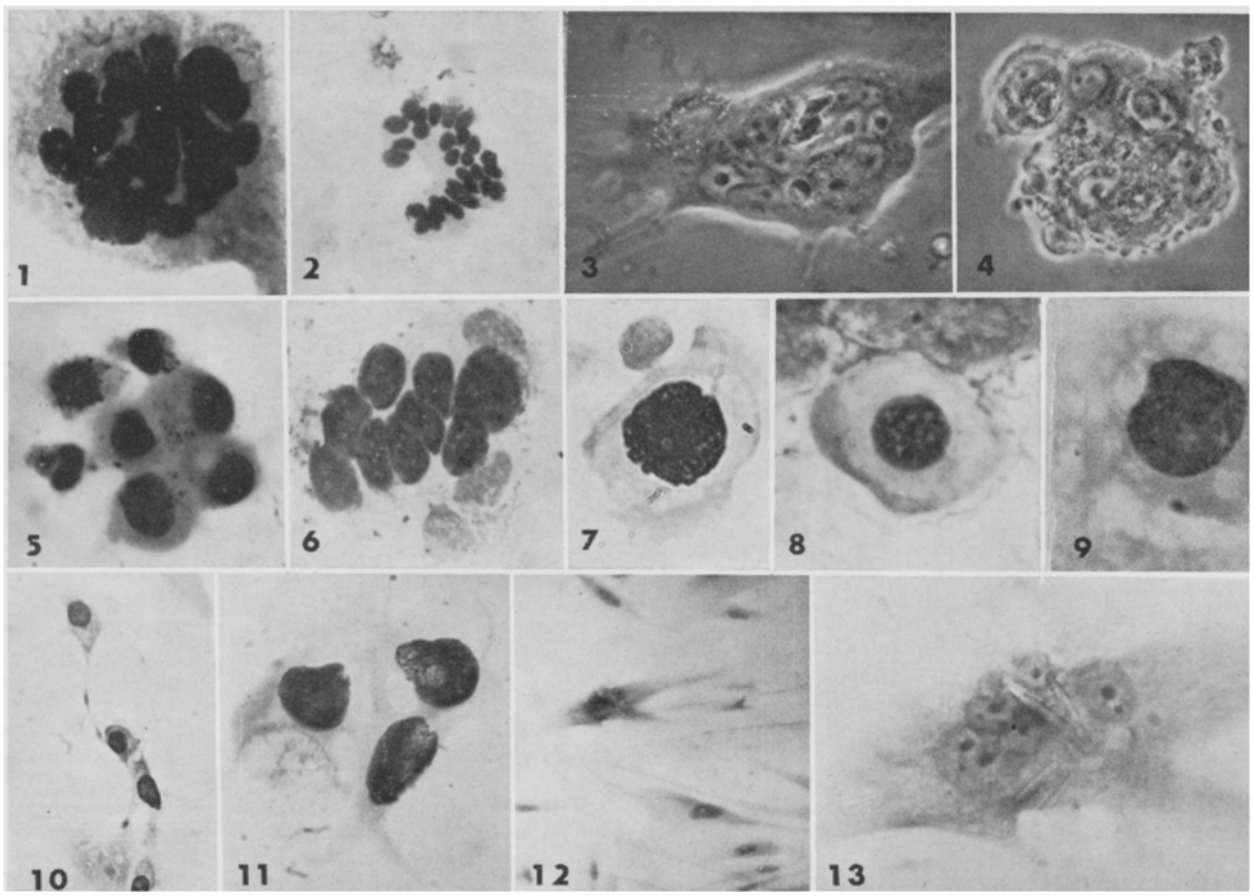

Fig. 1. 8-day-old culture, stained May-Grünwald Giemsa (MGG). Bright field microscope. Obj. 100.

Fig. 2. 8-day-old culture, stained MGG. Bright field microscope. Obj. 25.

Fig. 3 and 4. 5-day-old cultures, non-stained. Phase contrast microscope. Obj. 40.

Fig. 5 and 6. 2-week-old cultures, stained MGG. Bright field microscope. Obj. 100.

Fig. 7, 8 and 9. 5-day-old cultures, stained MGG. Bright field microscope. Obj. 100.

Fig. 10. 9-day-old cultures, stained MGG. Bright field microscope. Obj. 25.

Fig. 11. 9-day-old cultures, stained MGG. Bright field microscope. Obj. 100.

Fig. 12. 1-month-old culture, stained MGG. Bright field microscope. Obj. 10.

Fig. 13. 1-month-old culture, stained MGG. Bright field microscope. Obj. 40. The big fibroblast-like cell in the middle of Figure 12 appears in Figure 13 as a trophoblast cell with several nucleus and large spread protoplama. Cristals can be observed in the protoplasma.

chromatine and strongly elongated protoplasma. Their spindle-shaped aspect can lead at first examination to confusion between these trophoblast cells and similar elongated fibroblasts. Attentive serial examination showed that most of the elongated 'fibroblast-like cells' are actually true trophoblasts, as can be judged from the structure of the protoplasma, the shape of the nucleus and the aspect of the chromatin (Figures 12 and 13).

Isolated fibroblasts, or fibroblasts islets were usually observed after the 2nd week. The HIT reaction gave positive results in the freshly removed supernatant, as well as in 199M, which was added after 2 weeks, 1 month or 75 days, and incubated with the old cultures for about 10 days.

Our results are somewhat in contradiction to those of other authors. The multinucleated giant cells were present in our cultures for the first 8 days, and not for the first 16 h[1], or starting only after 3 days[2], as previously described. We believe that these formations are not giant multinucleated cells, but a transversal section of chorionic villi containing syncitial trophoblasts. These so-called 'giant cells' do not disappear; probably the external membrane gradually degenerates and the cells separate from each other forming groups of congregated cells, as we have herein described.

The trophoblast cells do not transform themselves into fibroblasts, but into fibroblast-like cells, which keep their hormone secretion activity. The number of true fibroblasts was small, probably because of a suitable dissection of the chorionic villi. Not only is the amount of fibroblasts low, but they seem to have a rather slow evolution. Whereas, in regular cultures, fibroblasts tend to form full monolayers rapidly, we could not find fibroblastic layers in our cultures, but only isolated cells or islets of cells, even after 20 days of incubation. The question arises whether the presence of the human chorion-gonadotropine or of some other hormones, secreted by the trophoblasts, might be responsible for inhibiting the fibroblasts development.

Studies are in progress in our laboratory to elucidate this problem[10].

Résumé. L'évolution morphologique des cellules trophoblastiques humaines, ainsi que leur potentiel de sécrétion hormonale, in vitro, ont été examinés pendant plus de 2 mois, à l'aide d'une technique simplifiée de culture de placenta. La signification des cellules dites «géantes», l'aspect fibroblastique des trophoblastes dans les cultures agées, et le développement des vrais fibroblastes dans ces cultures, sont discutés.

J. J. FOLDES and JEANNA SCHWARTZ[11]

Mitchell Medical Clinic, Department of Obstetrics and Gynecology, Ramat Aviv, of 'Kupat Holim' – Health Insurance Institution of the General Federation of Labour in Israel, and the Tel-Aviv University, Medical School, Department of Human Microbiology, Ramat Aviv (Israel), 23 May 1972.

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Effect of N^6O^2-Dibutyryl Cyclic 3', 5'-Adenosine Monophosphate on the Pinocytosis of Brain Capillaries of Mice

Pinocytotic activity in brain capillaries, except for those capillaries of brain areas not protected by the 'blood-brain' barrier, is known to operate at very low level under normal conditions[1]. The failure of penetration of certain substances from the blood circulation into brain substance has already been connected with the low rate of pinocytosis revealed in the endothelial cytoplasms of brain capillaries[2]. In contrast, in almost every study of pathological damage of the 'blood-brain' barrier, during which the capillary macromolecular transfer is usually enhanced, a considerable increase in the number of pinocytotic[3] and coated vesicles[4] has recently been reported.

The present investigation was designed to elucidate whether the pinocytosis of capillaries in the 'blood-brain' barrier-protected brain areas could be influenced experimentally by the lipid soluble substituted derivative of cyclic adenosine monophosphate (cAMP).

Mice, weighing about 20–25 g, were each given, by i.p. injection, a single dose (10 mg/kg) of N^6O^2-dibutyryl cyclic 3', 5'-adenosine monophosphate (dibutyryl cAMP, Sigma) dissolved in 0.1 ml saline. Control mice were given 0.1 ml saline solution only. The animals were killed by decapitation 5 or 20 min after injection. Small cubes of the parietal cortex and the cerebellar vermis were fixed in Karnovsky's aldehyde fixative and postfixed in Millonig's buffered osmic acid. Thin sections were examined in a Jeol 100B electron microscope. Plates of the non-nuclear areas of the endothelial cells to be measured were taken from randomly selected capillaries at a magnification of × 30,000. Portions of the endothelial cytoplasm were outlined and planimetrically measured on prints of final magnification × 90,000. The pinocytotic and coated vesicles, both attached to the luminal and abluminal surfaces and lying free in the cytoplasm, were counted for 1 μm^2 of endothelial cytoplasm on prints derived from the experimental and control groups. The counts of vesicles per unit area of endothelial cytoplasm in the experimental groups were compared statistically with those of the controls using the Student's *t*-test.

Evaluation of the results obtained is summarized in the Table. There was a significant increase in the numbers of pinocytotic vesicles lying free and attached to the basement membrane 5 and 20 min after the treatment and also in the counts of coated vesicles 5 min but not 20 min after the dibutyryl cAMP administration. A possible rate of induced pinocytosis, judged by comparing the total counts of vesicles obtained for brain capillaries after dibutyryl-cAMP treatment with those previously reported by BRUNS and PALADE[5] and CASLEY-SMITH[6] for qlood capillaries of diaphragm and endothelium of lymphatics, respectively, can be estimated. It appears that

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Effects of dibutyryl adenosine 3',5'-cyclic monophosphate on the counts of vesicles involved in the pinocytosis of brain capillaries (mean ± S.E.M.)

	pvp	pvi	pva	cp	ci	total count of vesicles
Control	0.03 ± 0.04	7.09 ± 0.10	0.41 ± 0.02	0.05 ± 0.06	0.55 ± 0.21	7·89 ± 0.11
5 min	0.41 ± 0.25	14.84 ± 0.18	4.88 ± 0.1	0.32 ± 0.01	1.51 ± 0.3	22.14 ± 0.24
P value	<0.02	<0.001	<0.001	<0.05	<0.001	<0.001
20 min	0	15.37 ± 0.48	4.15 ± 0.16	0.33 ± 0.03	1.02 ± 0.52	21.15 ± 0.52
P value	–	<0.001	<0.001	<0.05	<0.20	<0.001

Legend: pvp, pinocytotic vesicles pinching off from the luminal membrane; pvi, pinocytotic vesicles inside the endothelial cytoplasm; pva, pinocytotic vesicles attached to the basement membrane; cp, coated vesicles pinching off from the luminal membrane; ci, coated vesicles inside the endothelial cytoplasm; 0, evaluation was not made. *n* in control = 54; *n* in 5 min group = 53; *n* in 20 min group = 25.