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Morphology of Mouse Embryo Cultured in a Newly Established Culture System Using Collagen Gel Layer

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UEHARA, S. and YAJIMA, A. *Morphology of Mouse Embryo Cultured in a Newly Established Culture System Using Collagen Gel Layer.* Tohoku J. exp. Med., 1987, **153** (3), 245-258 —— A new in vitro culture system of embryo using collagen gel as the substrate was developed, in which mouse blastocysts were cultured. Morphological analyses including electron microscopic observation disclosed that after hatchig, the mouse blastocysts were attached to the collagen gel layer and were satisfactorily developed and differentiated. In the embryo observed on the 3rd or 4th day after culture, cellular processes and villi had penetrated the collagen gel from the mural trophoblast. While there were many vacuoles in the cytoplasm and a large number of destroyed cells in the mural trophoblast mass, some steroid producing cells were also observed. The polar trophoblasts, though having a small number of vacuoles, partially exhibited characteristic activities of steroid production. Between the mural and the polar trophoblasts, a sort of desmosome or intermediate junction was observed. There were no morphological differences among the cells derived from the inner cell mass. From these results, it was concluded that this new embryo culture system using collagen gel layer as the substrate can be of value in studies on development and differentiation of the embryo; moreover, this system can be used to replicate several phenomena similar to those appearing in in utero implantation. ———— mouse embryo; in vitro culture; collagen gel; mural trophoblast; polar trophoblast

Hsu (1971) described attachment of the hatched blastocyst to the dish surface and its differentiation in in vitro culture. Since then, several studies have ascertained that the embryo is able to sufficiently differentiate to the stage displaying hematopoietic activity and cardio-pulmonary function even in in vitro cultures (Hsu 1972). However, since in such an in vitro culture system, the embryo is attached to the dish either directly or after coating with the collagen, the embryo in vitro culture differs from those observed in the early stage of in vivo implantation in that the mural trophoblast does not sufficiently develop. In order to improve growth of the fetus in an in vitro culture system so that it more closely approximates growth in in vivo implantation, it is necessary to find

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substrates as adequate as the endometrium. So, with reference to previous studies of in vitro culture in which the dish surface was coated with collagen, we used collagen gel layer as the substrate so as to establish this type of culture system and observed the morphological features of the embryo obtained in this culture system.

Materials and Methods

Collection of the mouse blastocyst

Eight- to sixteen-week-old female mice of ICR strain were randomly mated with a male mouse of the same strain. The first day of pregnancy was confirmed by the formation of vaginal plug the next morning. In the afternoon of the 4th day of pregnancy, these mice were killed under ether anesthesia. After the uterus was aseptically extirpated and divided, blastocysts were obtained by flushing each uterine horn with Hank's solution (containing 1 g/liter of glucose).

Production of the collagen gel layer

The collagen used in our study as substrate was that for tissue culture classified as acid soluble type I group (Cell-Matrix type I-A, Nitta Gelatin Co., Ltd., Osaka). We prepared a solution of the collagen containing a suitable dose of antibiotics (penicillin G), CMRL 1066 ($\times 10$ solution, Gibco), and an aseptically filtered mixture which consisted of 100 ml of 0.05 N NaOH, 2.2 g of $NaHCO_3$ and 4.77 g of HEPES, the proportions being 8 : 1 : 1. Then, after adding fetal bovine serum (20%) and checking the pH color by phenol red, 2 ml of this solution was placed in an aseptic plastic dish, 3.5 cm in diameter. This procedure was carried out in iced water to prevent gelation of the collagen. Gelation of the collagen was performed by leaving the dish under temperature conditions of 37°C for 30 min. Thereafter, 1.5 ml of CMRL 1066 containing fetal bovine serum (20%) was added to the gelated surface, and CO_2 saturation was completed by placement in an incubator containing 5% CO_2 in air at 37°C for 2 hr. After adding the blastocyst, the resulting collagen mixture was used for the static culture. Each of the culture media on the gelated surface was renewed every 3 days.

Observation of the cultured embryo in vitro

In addition to continuous observation of the cultured embryo with an inverted microscope, its ultrastructure was examined with Hitachi SEM-450 scanning electron microscope and Hitachi H-600 transmission electron microscope. For electron microscopic observation, a part of the collagen gel connected with the embryo was separated from the surroundings with a syringe needle. For purposes of pre-fixation, this collagen gel specimen was immersed for an hour in 2.5% glutaldehyde (0.1 M cacodyl acetaldehyde buffer) and then irrigated with 0.1 M cacodyl acetaldehyde buffer. For post-fixation, the collagen gel specimen was immersed for an hour in 1% osmium tetroxide (0.1 M cacodyl acetaldehyde buffer). The above washing procedure was repeated and the gel specimen was dried with ethanol. For obseration by scanning electron microscope, critical-point drying and gold vacuum evaporation were conducted on the material which had been briefly immersed in isoamylhydrochloride. For observation by transmission electron microscope, after displacement with a mixture of propylene oxide and epoxide, first in a proportion of 1 : 1 and then in a proportion of 1 : 3, the material was embedded in epoxy resin and cut into ultra-thin sections. The sections were then electronically stained with lead citrate and uranium acetate. At the same time, specimens for light microscopic examination were prepared by staining with toluidine blue.

Results

Morphological changes of embryo cultured in vitro

Among the blastocysts obtained on the 4th day of pregnancy, only the ones exhibiting mild or high expansion before hatching were used for the culture. On the afternoon of the next day of culture (2nd day of culture), most of the blastocysts were observed in the middle of or after completion of hatching. On the 3rd day of culture, the embryo began to attach to the collagen gel layer, but since its area was still so small, it appeared to be only placed on that layer. The mural trophoblast of the embryo observed on the 4th day of culture spread toward the periphery, demonstrating single layer proliferation on the collagen gel layer. At this stage, the part derived from the inner cell mass, which had become considerably enlarged, was also clearly recognized on the mural trophoblast layer. After that, the mural trophoblast was observed to grow and proliferate, and stratification was noted near the center. The growth of the part derived from the inner cell mass became more obvious, the cell mass increased, and even the egg cylinder was formed (Fig. 1).

Observation of the embryo cultured on the collagen gel layer by scanning electron microscope

Early on the 3rd day of culture, there was no indication of mural trophoblast growth, and the oval shaped blastocyst was observed to be merely attached to the collagen gel layer (Fig. 2). However, high-power magnification of the area of

Fig. 1. Photomicrography of the embryo attached on the collagen gel layer. Seventh culture day, inverted microscope, ×50.
The embryo grew to the egg cylinder stage. The mural trophoblast (arrow head) around the cell mass is observed to proliferate and to spread.

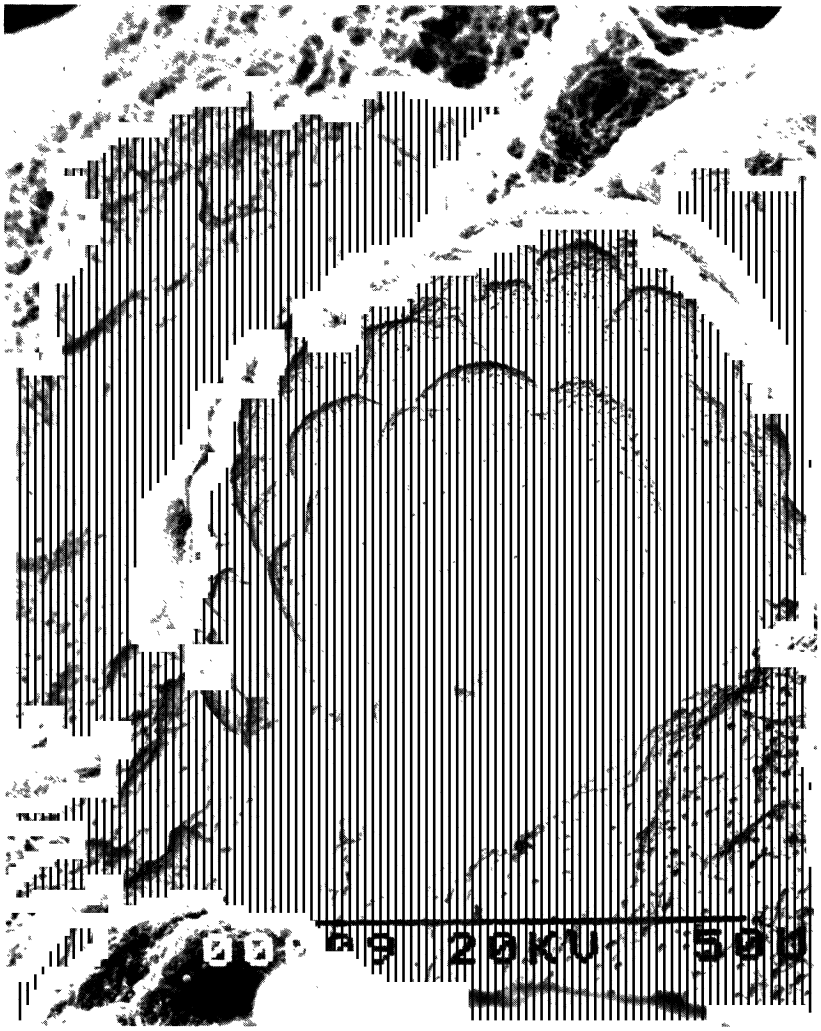


Fig. 2. Photomicrography of the embryo attached on the collagen gel layer. Third culture day, scanning electron microscope.

The embryo just attached on the collagen gel layer is oval and still showing the shape of blastocyst.

contact between the mural trophoblast and the collagen gel layer showed the existence of a large number of villi and cellular processes, somewhat thicker than villi, which extended into the collagen gel layer (Fig. 3). Halfway through the 3rd day of culture, the mural trophoblast was observed to expand, accompanied by distension of villi and cellular processes. At this stage, the part derived from the inner cell mass covered by the polar trophoblast with short villi assumed a hemispheric shape, indicating that the blastocoele had already disappeared (Fig. 4).

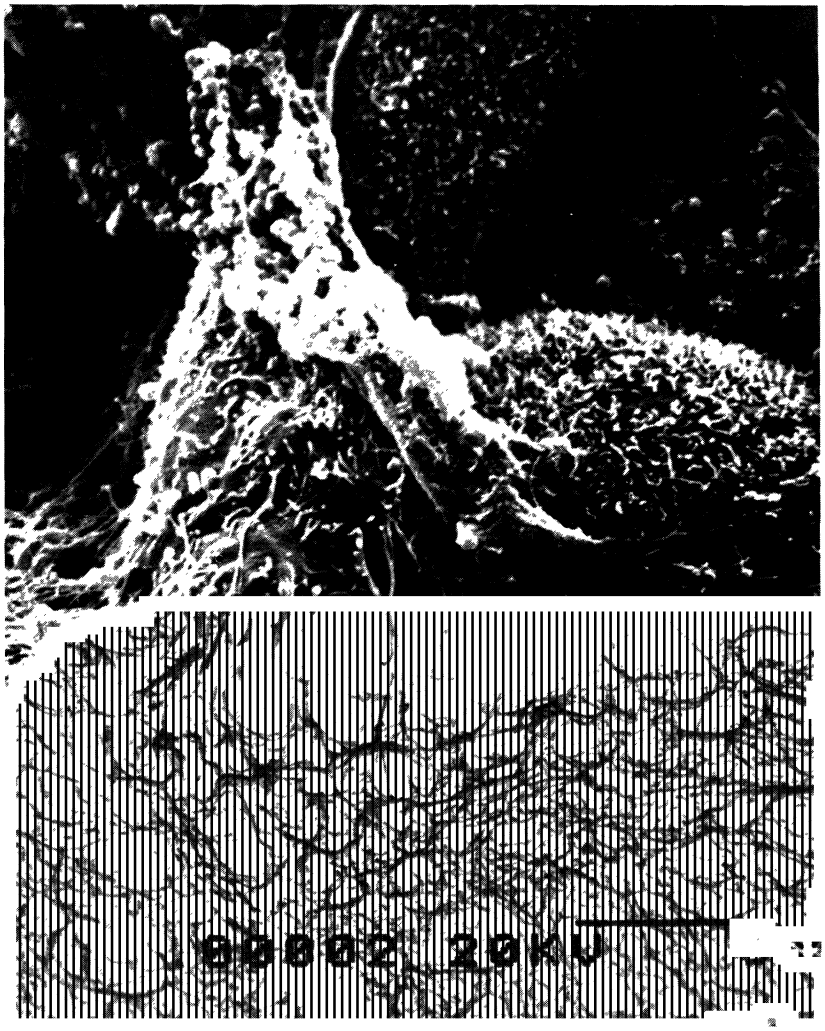


Fig. 3. Photomicrography of the mural trophoblast of the embryo attached on the collagen gel layer. Third culture day, scanning electron microscope.
On observing an area of contact of the mural trophoblast with the collagen gel layer, there are recognized a large number of villi and cellular processes which stick into the collagen gel layer.

Observation by transmission electron microscope of the embryo cultured on the collagen gel layer

Figs. 5 and 6 illustrate the morphological character of the embryo on the specimen for light microscopic examination on the 4th day of culture. Growth of the mural trophoblast was seen on the surface of the collagen gel layer, and the part derived from the inner cell mass surrounded by polar trophoblast was

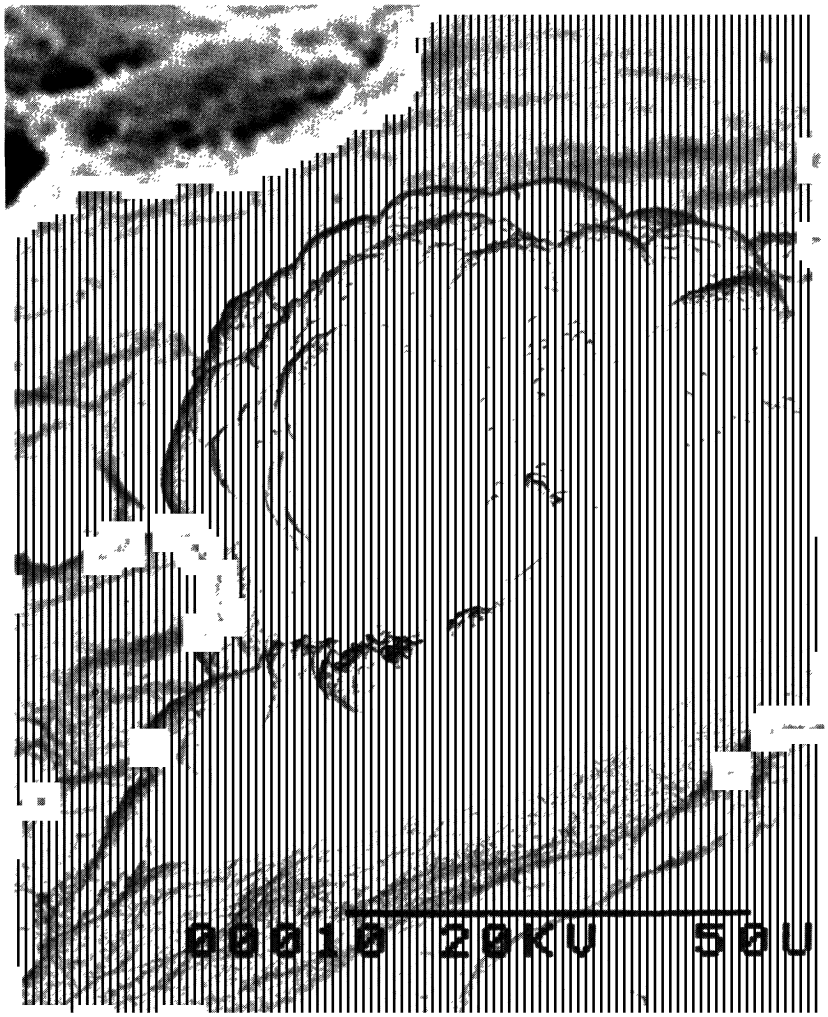


Fig. 4. Photomicrography of the embryo attached on the collagen gel layer. Fourth culture day, scanning electron microscope.

Comparing with the embryo of the 3rd culture day, the mural trophoblast is observed to expand. The part derived from inner cell mass is clearly visible on the mural trophoblast layer.

observed on the mural trophoblast. Inside the collagen gel layer, several cellular processes, which were thought to extend from the mural trophoblast, were noted. Every mural trophoblast contained vacuoles of various sizes. While a small number of vacuoles in the polar trophoblast enclosing the part derived from the inner cell mass was seen, some of the cells possessed secretion granules.

Among the mural trophoblasts observed by transmission electron microscope, many destroyed cells were noted, and vacuoles of various sizes were evident even in the normal cells. Furthermore, a number of these normal cells contained fat

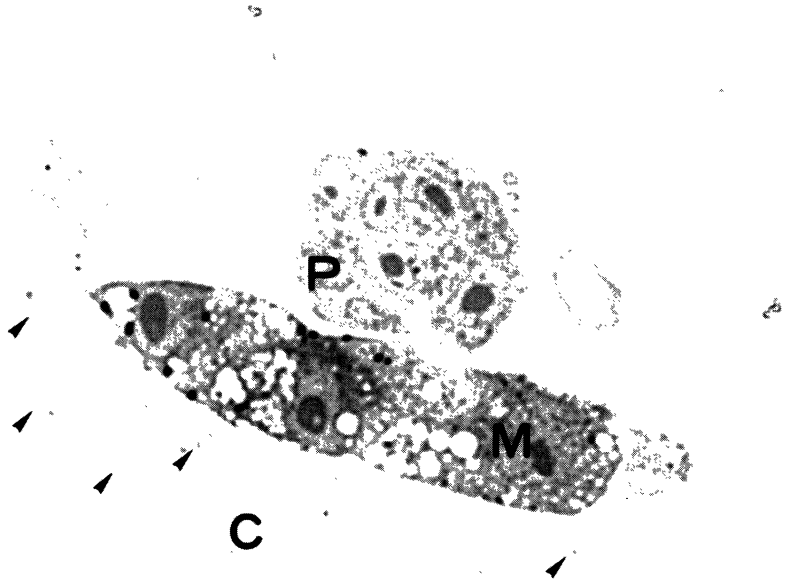


Fig. 5. Photomicrography of the embryo attached on the collagen gel layer. Fourth culture day, light microscope, toluidine blue staining, ×360.

The mural trophoblast (M) containing vacuoles and secreting granules is observed to spread and to form single layer on the collagen layer (C). The part derived from inner cell mass which is surrounded by the polar trophoblast (P) is clearly visible on the mural trophoblast. In the collagen gel layer, there are observed processes (arrow head) extending from the mural trophoblast.

droplets, granular endoplasmic reticulums, Golgi complexes and mitochondria. Inside the collagen gel layer, transverse sections of numerous villi were observed ; these were thought to originate in the mural trophoblast. In addition, there were several other processes of transverse sections which were thicker than those of villi. Collagen fibers which surrounded the villi and the processes were found to be dispersed (Fig. 7a-c).

Between the mural and the polar trophoblasts, there were some connecting apparatuses similar to desmosomes or intermediate junctions (Fig. 8).

Generally, the polar trophoblast appeared to be normal without vacuoles such as those observed in the mural trophoblast. A certain number of polar trophoblasts held an abundance of fat droplets, granular endoplasmic reticulums, Golgi apparatuses and mitochondria. Such inclusions were not so frequent, however, as those seen in the mural trophoblast (Fig. 9).

In the part derived from the inner cell mass, there were some granular endoplasmic reticulums and mitochondria ; however, they were not so abundant.

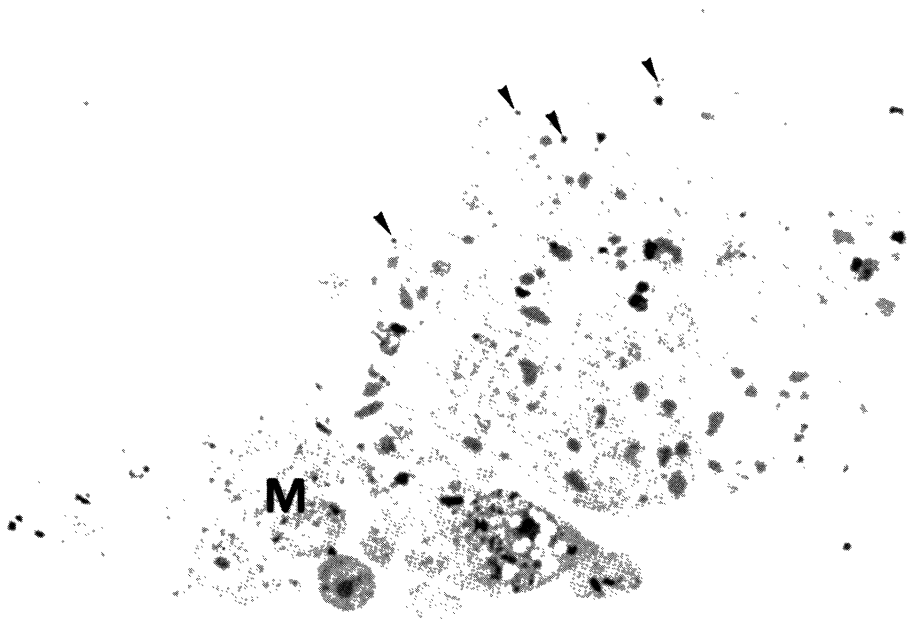


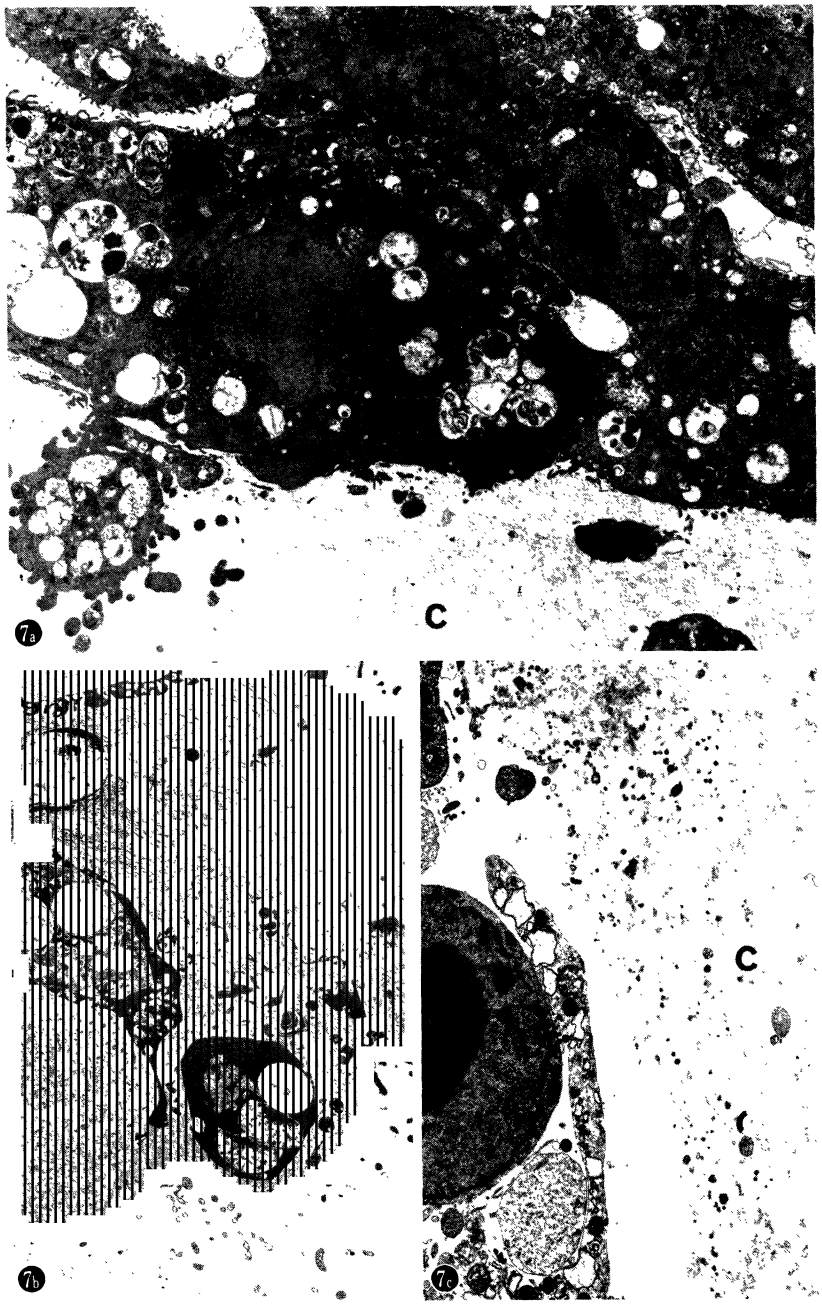
Fig. 6. Photomicrography of the embryo attached on the collagen gel layer. Fourth culture day, light microscope, toluidine blue staining, ×180.
The part derived from inner cell mass is visible on the mural trophoblast group (M). In the mural trophoblast, vacuoles and/or secreting granules are observed. Some of the polar trophoblast also contain secreting granules (arrow head).

In some intercellular spaces, a few desmosomes were noted However, there were no other obvious characteristics suggestive of morphological or functional differentiation in each cell (Fig. 10).

Discussion

There have been various attempts to culture the embryo in vitro and to

Fig. 7. Photomicrography of the mural trophoblast of the embryo attached on the collagen gel layer. Fourth culture day, transmission electron microscope.
a: Vacuoles and secreting granules are observed in the mural trophoblast. There are processes sticking into the collagen gel layer (C). ×1,600
b: In the mural trophoblast containing secreting granules, there are observed endoplasmic reticulums, Golgi complexes and mitochondria. There is a destroyed cell beside the intact cell. ×2,000
c: The collagen fibers around the processes or the villi seemed to be loose. C, collagen gel layer. ×2,800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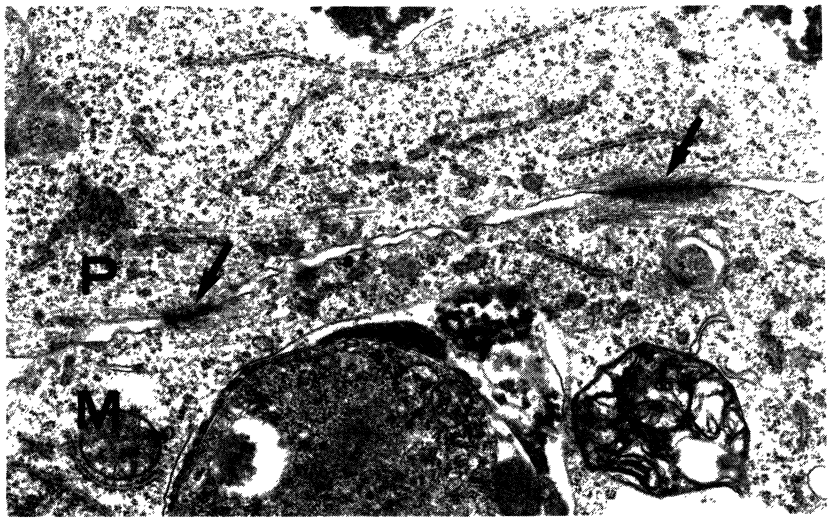

Fig. 8. Photomicrography of the cell connection between the mural and the polar trophoblasts of the embryo attached on the collagen gel layer. Fourth culture day, transmission electron microscope, ×10,000.

There are observed junctional complexes (arrow) which resemble the desmosome or the intermediate junction. M, mural trophoblast; P, polar trophoblast.

observe its development and differentiation. Cole et al. (1966) were the first to report a culture of rabbit embryo on the dish surface coated with the collagen obtained from rat tail tendon. Hsu (1971) also created a series of in vitro cultures of the mouse embryo on collagen film. The culture method using collagen gel originated with that of Cleator and Beswick (1972) who used cultured cells. Since then,various materials have been utilized for this type of culture. Recently, methods of suspending collagen gel, to which cells are attached, in the culture medium (Michalopoulos and Pitot 1975) or methods of embedding cells in collagen gel (Yang and Nandi 1983) have been reported as modified techniques and have made it possible to accelerate cell differentiation and to obtain three dimentional cell proliferation.

In this study, the blastocyst became connected with the collagen gel layer, and then differentiated to the degree of egg cylinder formation. However, the level of progression in development and differentiation was almost the same as in the case of culture on the dish reported by Hsu (1971, 1972). Therefore, the speed of development and differentiation was considered to be unaffected by the use of collagen gel as substrate. The collagen gel used in this study was the same kind as the collagen obtained from rat tail tendon. When collagen is used as the gel layer, however, the embryo, especially the mural trophoblast, maintains the cell structure, proliferates and piles up. This phenomenon is thought to be the most advantageous from the viewpoint of studying the morphology and functions of the

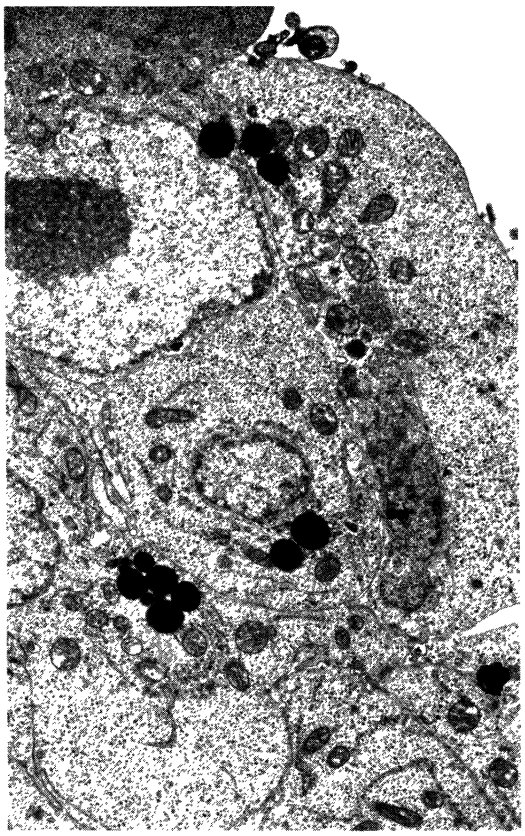

Fig. 9. Photomicrography of the polar trophoblast of the embryo attached on the collagen gel layer. Fourth culture day, transmission electron microscope, ×4,000.

In the polar trophoblast containing secreting granules, endoplasmic reticulums, Golgi complexes and mitochondria are observed. Comparing with the mural trophoblast, there is no vacuole in the cytoplasm.

trophoblast.

It seems that the process of the mural trophoblast seen on the electron microscopic picture in this study is identical to the lamella observed by Chavez and Van Blerkom (1981) and Sobel et al. (1981) who cultured the embryo on the dish surface as the substrate. But they did not refer to the presence of villi originating in the mural trophoblast in their observations. From this point of view, moreover, our method using collagen gel as substrate might be more appropriate for observation of mural trophoblasts than that using dish surface as substrate. These villi are thought to strengthen the connection of the embryo by penetrating the endometrium in utero in a way similar to that of processes.

Based on morphological observation of the human embryo, Hertig and Rock (1945) claimed that trophoblasts penetrate the endometrium in the course of

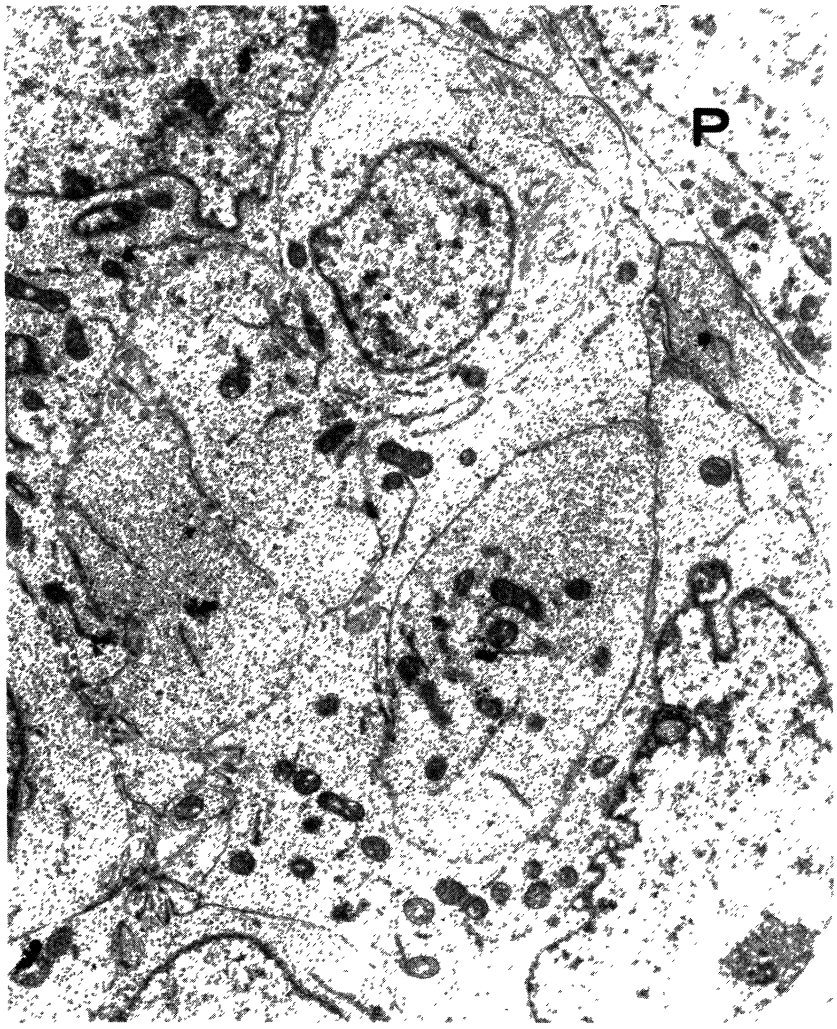

Fig. 10. Photomicrography of the part derive from inner cell mass of the embryo attached on the collagen gel layer. Fourth culture day, transmission electron microscope. ×4,000.

In the cell of the part derived from inner cell mass, there are contained endoplasmic reticulums and mitochondria, but not so abundantly and very few other organella. No prominent finding suggestive of morphologic or functional differentiation are observed in each cell. P, polar trophoblast.

implantation in utero. Therefore, we initially assumed that the trophoblast itself would penetrate the collagen gel layer, because the collagen gel used in this study was pure glycoprotein which was the ordinary substance existing in the endometrium. However, even after the 5th day of culture, the trophoblast itself remained on the surface of the gel layer, only showing penetration of the gel by processes and villi. As collagen fibers around the processes and villi disappeared

or became disarranged, it could be presumed that some kind of proteolytic enzyme was likely released from the trophoblast. For further analysis to clarify the role of the processes and villi, it would be necessary to employ a modified culture system for embedment of the embryo in the collagen gel.

On the 3rd or 4th day of culture, an abundance of fat droplets, granular endoplasmic reticulums, Golgi complexes and mitochondria were observed to be contained in the mural trophoblasts and some of the polar trophoblasts. Based on these morphological characteristics, it was assumed that these trophoblasts are capable of producing steroids (Bloom and Fawcett 1968). Furthermore, this activity is considered to be acquired after attachment of the embryo to the collagen gel, since these findings were not notable in the blastocyst before implantation (Uehara et al. 1986). It is presumed that this steroid might have a local activation effect on the endometrium in utero and would be valuable for facilitation of the progress of implantation.

In our study, vacuolization of many trophoblasts was observed. This phenomenon was the same as that reported by Chavez and Van Blerkom (1981) who used the dish surface as the substrate. In our study, it was further observed that vacuolization was less frequent in the polar trophoblast than in the mural trophoblast. This fact suggests that the polar trophoblast, though located in the trophectoderm together with the mural trophoblast, may present certain differences in form and function following implantation, depending on its exact position. Similar findings with regards to vacuolization in the human embryo were observed in the early stage of implantation in utero (Hertig and Rock 1945) and in the mouse blastocyst in the stage after hatching (Chida et al. 1987). In our study, many destroyed cells were also observed in the mural trophoblast group. From these findings, it is speculated that vacuolization and cell destruction are phenomena caused by the synthesis of the proteolytic enzyme which makes it easy for the embryo to penetrate the endometrium in the course of implantation.

Between the mural trophoblast and the polar trophoblast enclosing the part derived from the inner cell mass, connecting apparatuses such as desmosomes or intermediate junctions were observed. It is not obvious whether or not these connecting apparatuses are formed at the time of implantation in utero. However, based on results of our study, it is assumed that the intensity of their connection is fairly strong, because the area of contact between the mural trophoblast and the polar trophoblast was not so great.

The part derived from the inner cell mass gradually forms the fetal organ as differentiation progresses (Hsu 1972). Because there were no morphological changes in the cells of this part on the 4th day of culture, it is hypothesized that differentiation occurs at first in the trophoblast and that differentiation of the fetal organ begins later, some time after implantation.

As mentioned above, the form of the embryo on the collagen gel layer is not completely the same as that in utero. In spite of such differences in overall

appearance of the embryo, each constituent sufficiently retains its essential qualities. If this were not so, development and differentiation up to the stage of egg cylinder might not be accomplished. In conclusion, it can be hypothesized that our culture system using collagen gel layer as the substrate adequately reproduces the phenomenon which takes place in utero.

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