Tissue engineering: *In vitro* embryonal nidation in a murine endometrial construct*

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Received 28 September 2002; revised 5 March 2003

The epithelial and mesothelial cellular components of organs can be obtained as dissociated cells using adequate procedures of enzymatic digestion followed by pycnotic separation on density gradients. Using a specially developed procedure for tissue dissociation, the epithelial and connective tissue components of endometria from pseudopregnant mice were grown in culture using a combination of three dimensional culture of connective tissue components in collagen gel, with the superimposition of epithelial components in liquid medium on the surface of the gels. After a few days of growth, when the cultures became dense, murine blastocysts obtained on postcoital day 4.5 by fallopian flushing of hormonally primed and mated mice, were transferred onto the imitated endometria. The blastocysts hatched and grew on the endometrial epithelium as spherical coherent conglomerates of cells quite different from hatched blastocysts grown on the surface of a petri dish, in which the presumptive trophoblasts spread around the central mass. Light and electron microscopy of resin embedded sections (2 days after nidation on the simulated endometria) revealed that at least two populations of cell types were recognisable as layers. This is interpreted as an early sign of morphogenesis and the first visible steps of differentiation. The presence of mitotic figures indicates viability and continuing growth. Electronmicroscopy of cell types grown under conditions simulating *in vivo* tissue architectonics showed overtly less cytopathology and better cell function. Simulated endometria may, therefore, serve as an attractive model for studying early mammalian embryogenesis and the effects of toxic agents.

**Keywords:** Endometrium construct, Nidation *in vitro*, Tissue engineering

The early events in mammalian embryogenesis from the time of nidation until organogenesis have remained elusive because of the lack of an adequate *in vitro* system to simulate nidation¹. An attempt to achieve this requires, in the first place, the production of a culture system composed of the essential cellular elements of the endometrium, organised in appropriate histological architectonic relation to one another. With this provision, blastocysts may be introduced into the system to allow hatching and attachment. Successful invasion by trophoblast precursors accompanied by appropriate cellular interactions between the embryonal and endometrial cells²,³ leads to cellular proliferation and early morphogenetic events. Sengupta *et al*⁴ contributed to an essential improvement in the culture of endometrial cells by growing them of floating collagen gels. However, this system cannot be regarded as a break through.

The cellular elements of the endometrium consist essentially of the epithelium and the stroma. From a histological perspective, the epithelium is organised as a layer of cells growing on a two-dimensional plane, delimited by the basement membrane. In contrast, the stromal cells grow in a dense three-dimensional network of collagen fibres. Cells from the two tissue types interact with one another.

As an attempt to create a model, endometria from pregnant mice were dissociated enzymatically, the cells from the respective tissue types retrieved, and a construct simulating the endometrium was created. The ability of the construct to support the nidation of embryos was tested. For this purpose, blastocysts were allowed to hatch and implant in the construct. Besides confirming the histological simulation of relevant tissues, light and electron microscopic observations presented here also reveal the interaction between hatched blastocysts and the mimicked endometrium, which is none other than a reflection of the initial events in embryo nidation. Early morphogenetic events in the embryos were obvious. Further work using tissue engineering methods could

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*Oedicated to Priv.-Doz. Dr. med. Carstens Alsen-Hinrichs, an uncompromising environmental toxicologist, on the ocassion of his 60th birthday.*
contribute essential information on early mammalian embryology. The model could be used for improved in vitro assay systems to study embryonal regulatory factors and also for toxicological testing.

**Materials and Methods**

Since sufficient tissue could not be obtained from normal mouse uteri, pseudogravid uteri had to be taken. Female mice were made pseudopregnant by priming the mice with 5 IU of pregnant mare serum gonadotrophin followed by 5 IU of human chorionic gonadotrophin 48 hours later. The stimulated females were mated with vasectomised males. The pseudopregnant mice were sacrificed within a week and the uteri removed under aseptic conditions. The endometria were scraped off and placed in a 15 ml polypropylene tube containing 0.25% collagenase with 0.25% dispase (Boehringer, Mannheim) in 10 ml Ham's F 10 culture medium. The tube was left lying overnight at room temperature. On the following morning, the digestion mixture was repeatedly drawn lightly through a 10 ml pipette, the filled pipette held vertical for about a minute or two to allow for non-dispersed fragments of tissue to settle toward the tip. The fraction containing tissue fragments (mainly epithelial cells) and the remaining fraction containing predominantly dispersed cells (Stroma elements) were both layered separately over Percoll gradients at 45% and 25% in Dulbecco's PBS. Intact cells settled at the interphase between the Percoll layers. The cells were washed in culture medium before being put into culture. This procedure yielded cell harvests of >90% viability as per trypan blue exclusion.

Stroma cells were plated in 1 mm thick collagen gels. The gels constituted of 7 parts collagen solution, 1 part 10x concentrated Ham's F 10 medium and 2 parts NaHCO₃ (11.76 g/l). Nine parts of this mixture were added to 1 part foetal calf serum (FCS) containing the stromal cells. After plating, the temperature was raised from ice-bath temperature to 37°C upon which the gels solidified.

The epithelial cells were plated onto the gels in Ham's F 10 containing 10% FCS, 1 to 2 hr after the gels had formed. The liquid medium layer was also 1 mm thick. Incubation was at 37° C, 5% CO₂ in air and saturated water vapour. Dense cultures were obtained within 3 to 4 days and were considered ready for nidation.

To obtain blastocysts, mice were primed as mentioned above, with the only difference that the females were then mated with normal healthy males. The blastocysts were collected by flushing of the fallopian tubes on the fourth postcoital day, day 1 being the day when vaginal plugs were visible. These blastocysts were then introduced into the endometrial cultures.

Two days after implantation, cultures were fixed in Karnowski's fixative and embedded in araldite for sectioning. Cultures were monitored with a Olympus M2 inverted phase contrast photomicroscope. Semi-thin (0.5 to 1µm) sections were examined with a Zeiss Photomicroscope and for transmission electron microscopy a Zeiss EM 9 operated at 60 kV.

**Results**

Figures 1 and 2 are phase contrast documentations of the in vitro endometrial mimic after about a week in culture. The surface of the culture appears as in Fig. 1, which reveals epithelial cells. These epithelial cells are recognised from their "cobblestone" appearance. The epithelial nature of these cells was confirmed by transmission light and electron microscopic examinations of semi-thin and ultrathin sections, respectively, of resin embedded material. The light microscopic observations are given in Figs 3 (a and b) and 4, in which the cells can be seen as a monolayer. The layer seemed multilayered, but upon close inspection the cells are seen to be more columnar in form (Fig. 4), which raises the probability of individual cells maintaining direct contact with the growth surface. Should that be the case, then such spots are just pseudomultilayered. As the section is not exactly vertical but rather at a slant, it provides a good view of the the intimate association of the epithelium with the stromal cells at that spot. The stromal cells growing beneath the epithelial layer in the gel are shown in Fig. 2. The stromal cells show profuse filopodia which anchor the cells to the three-dimensional network of collagen fibrils. The close association between stromal and epithelial cells is clearly visible in Fig. 4.

Transmission electron microscope pictures of epithelial (Fig. 5) and stromal cells (Figs 6-8) unusually low level of cytopathological changes which are otherwise observed in cultured cells grown on plastic substrata. A tight junction and intercellular spaces between two adjacent epithelial cells are seen in Fig. 5. The upper surface of these cells (towards the liquid medium) bears microvilli, as seen from their bases. The nuclei are highly euchromatic which is a sign of vitality and high activity. Figures 6 and 7 are sections through stretched stromal cells, the former in
longitudinal and the latter in cross section through the respective nuclei. Here too all features of healthy cells are seen. The clear presence of rough endoplasmic reticulum indicates synthetic activity. Fine filopodia in cross section are visible around the main cytoplasmic bulk in Fig. 7. Figure 8 shows a part of a stromal cell through a aborescent cytoplasmic arm which bears contacts (gap junctions) with other filopodia (marked X in two vesicles).

Figure 9 shows murine blastocysts which were obtained after uterine flushing and put into Ham's F10 culture medium containing 10% foetal calf serum. After about 6 hours in culture, the blastocysts approached the hatching stage. The rupturing of a zona pellucida is visible, while the inset (a) shows initiated hatching. Figure 10 shows the appearance of a blastocyst attached to the surface of the culture dish one day later. In Fig. 11, hatching is shown in conjunction with the endometrial construct in vitro. The implanted embryos (in the endometrial construct) one day later is shown in Fig. 12. The differences between “implantation” on the culture dish surface and the same in the endometrial construct is obvious.

Implantation in the endometrial construct is certainly a closer simulation of nidation in vivo, with a retention of morphological integrity.

After 48 hr of embryos implantation (which is about 72 hr post uterine flushing), the cultures were fixed with Karnowski’s fixative and processed for embedding in araldite. Vertical sections (0.5-1 um) through embryos after Richardson’s staining (which is essentially a methylene blue stain) are presented in Figs 13-16. Figure 13 (a-c) give an overview of 3 sections photomicrographed at low magnification (x200) in which peripheral masses of migrating cells are seen; d-f show only the embryo cut in th median plane (e) or parallel to it (d and f). Figures 14-16 show the same sections at higher power. What is obvious, is that the cells have organised themselves into layers. The inner mass which develops into the embryo proper can be distinguished into two distinct areas or layers (Figs 14 and 16). In Fig. 15, at the base of the inner mass, large cells which do not belong to the inner mass are visible. These large cells are probably presumptive trophoblast cells which in vivo grow to form the placenta. The arrow heads in Fig 16

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Figs 1-4 — Phase contrast photomicrographs of an endometrial construct. Fig. 1 — Surface view of epithelial cells grown as a monolayer (in liquid medium) on the gel surface of the underlying culture—"cobblestone" appearance. Fig. 2 — Stromal cells anchored to the network of collagen fibres provided by the gel (semi-solid medium). Fig. 3 — Vertical section through epithelial monolayer (b) and for the purpose of comparison, Fig. 3a shows a section of epithelial cells grown on the plastic surface of a culture dish. Fig. 4 — Slanted section through a dense spot of the epithelium also showing the close association with stromal cells.
Figs 5-8 — Ultrastructure of epithelial and stromal cells. Fig. 5 — Two adjacent epithelial cells. Tight junctions with hemi-desmosomes can be seen. Fig. 6 — Elongated stromal cell longitudinally sectioned. Fig. 7 — Section through an abortescence stromal cell at the nucleus. Fig. 8 — Cross-section through a major filopodium. Intercytoplasmic contacts (marked X) with gap junctions can be seen.

Figs 9-12 — Hatching and implantation of murine blastocysts on plastic surface (culture dish) and in an endometrial construct. Fig. 9 — Murine blastocysts 4.5 days post-coitus. Inset (a) shows a hatching blastocyst. Fig. 10 — Blastocyst attached to plastic substratum (culture dish). Fig. 11 — Blastocysts in the process of hatching after introduction into the endometrial construct. Arrows point to zonae pellucidae. Fig. 12 — Implanted blastocysts. 48 hr after introduction into the endometrial construct.
point to mitotic figures, which must be taken as an indication of the viability of the embryo.

Figures 17 and 18 give further views of the large presumptive trophoblast cells. In Fig. 17 organisation into discrete areas can be seen. Figure 19 shows an area at the periphery of an embryo with physical intermingling of epithelial, trophoblast and stromal cells, which is essential for physiological interaction between these tissue types. As the section is only 0.5 to 1 μm thick, only the cytoplasm/cytoplasmic processes of the larger cells (trophoblast and stroma) can be seen. Figure 20 shows an area corresponding to the peripheral mass of migrating cell shown in Fig. 13 (a-c), containing unclear smaller cells with extensive cytoplasmic patches between them. These cells were not identifiable on the basis of morphology. The cytoplasmic borders were not clear.

Discussion

A central feature of efforts at simulating tissues is the creation of the appropriate extracellular matrix (ECM) for the tissue under consideration. As collagen is universally present in tissues and is easily available, it is the simplest choice, even if it may not be optimal for certain tissue types. Thus, a large scope for the development of ECM either from material of natural or of synthetic origin is given.

The method for enzymatic digestion which has been employed here has consistently proved to be reliable with respect to obtaining high yields (>90%) of viable cells. The procedure as given above was developed for processing clinical biopsy material. It is superior to the usual methods of trypsinisation. Trypsin causes extensive cell damage leading to significantly lower yields of viable cells. Besides the fact that the action of trypsin is unspecific, trypsinisation has to be done at 37°C in Ca²⁺ and Mg²⁺ free balanced salt solutions which is unfavourable for cell survival. A further disadvantage of trypsinisation as compared to the method used here, is that further processing of the material i.e. culture etc. must immediately follow. The advantage which trypsin offers is its low cost.

In addition to work convenience, the combination of collagenase with dispase is well suited for organ material in which epithelial and connective tissue (here the stroma) elements are simultaneously retrieved in dispersed form. In contrast to trypsin,
these two enzymes act with specificity. Collagenase disrupts collagen fibres while dispase disrupts the epithelial basement membrane, thus detaching the epithelial tissue. The epithelium is released in bits and pieces while the stroma cells are dispersed largely as single cells. This difference has been exploited for the purpose of separating the two fractions by sedimentation of the epithelial clumps. Of course, the epithelial fraction cannot be expected to be a homogenous population of cells. The glandular cell fraction is vastly separated with the epithelial fraction. This aspect could better be demonstrated with human endometrial material, which is not included in this communication. Since the epithelium is detached in aggregates of varying sizes, the re-grown epithelium after plating onto the stromal gel cultures often showed spots of apparent "piling", which upon careful scrutiny showed that the individual cells in such areas acquired columnar morphologies. The individual cells must therefore have polarity with cytoplasmic attachments to the basement.

The need for an in vitro system for the purpose of doing analytical studies on early mammalian embryos has been recognised\(^5\)\(^6\)\(^4\). The culture of blastocysts attached to plastic substrata (culture vessels), upon hatching, has been proposed as a model for such purposes, since cells of the inner mass are distinguishable from presumed trophoblast cells\(^4\(^7\). The shortcomings with this model are: (a) both the inner mass and presumed trophoblast cells generally degenerate; survivors which continue to proliferate transform into cell lines; (b) the constrains of a two-dimensional culture system do not allow for adequate cell-cell-interactions required for morphogenetic movements and cell differentiation. These severe limitations have been eliminated in the system presented here. The developing embryo implanted in an in vitro endometrial construct which is none other than an imitation of its natural microenvironment. The embryonal cells are able to interact among themselves and with the endometrial cells. This fosters cell proliferation and differentiation, as has been documented in this study by the obvious presence of different cell types at specific locations.

It was not clear from the sections which were examined as to whether the large presumed trophoblast cells were mononuclear or multinuclear, since they later develop into the syncytiotrophoblast. The mechanism of formation of the syncytiotrophoblast has been a matter of controversy with two competing concepts: (a) formation through cell fusion; and (b) formation through endomitosis i.e. nuclear division without cytokinesis.

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Figs 17-20—Light photomicrographs through growing presumptive trophoblasts. Figs 17, 18—Arrows point to growing presumptive trophoblasts in the periphery of the central mass. Fig. 19—A site of interaction between embryonal and epithelial cells. Fig. 20—Lightly stained cellular mass without clearly defined cell borders—initiation of syncytiotrophoblast?
proliferation without cytoplasmic division. It is at the moment not clear as to whether the cytoplasmic patches described in Fig. 20 may in fact be the initiation of the syncytiotrophoblast. Using this model, a careful analysis of serial sections could resolve this problem.

The development of the syncytiotrophoblast is important in vivo because it produces chorionic gonadotrophin and other hormones which are essential for maintaining conception. It is not known if it may produce other humoral factors for the local support and maintenance of embryonal growth. This and further aspects of humoral regulatory factors essential for early mammalian embryonal development could be studied using this model. It could also serve for the purpose of analysing specific effects toxic substances.

The model has its own limitations. It can only simulate the initial events of nidation. The natural architectonic relationship between endometrial microenvironment and the embryo is by far more complexer and cannot be mimicked, especially in further development when it is perfused by circulatory blood. A further important constituent of the placental microenvironment are the decidual cells which are lacking in the endometrial construct, since these cells have been supposed to be of haemopoietic origin. Nonetheless, the essential conditions for mammalian embryonal nidation and the very early stages of development can certainly be successfully provided in vitro and exploited for analytical studies on embryogenesis. The model can also be applied for specific studies in the area of reproductive toxicology.

References