The functional regeneration of syncytiotrophoblast in cultured explants of term placenta

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Simán, C. M., C. P. Sibley, C. J. P. Jones, M. A. Turner, and S. L. Greenwood. The functional regeneration of syncytiotrophoblast in cultured explants of term placenta. Am J Physiol Regulatory Integrative Comp Physiol 280: R1116–R1122, 2001.—We have investigated the functional characteristics of term human placental villous explants kept in long-term (7–11 days) culture. Fragments of placental villous tissue (3–5 g wet wt) were cultured in supplemented CMRL-1066 culture medium for up to 11 days. After the first day of culture, the syncytiotrophoblast appeared vacuolated and eventually degenerated. However, a new syncytiotrophoblast developed before day 4, being indistinguishable from that of a fresh placenta by 11 days. Release of human chorionic gonadotrophin increased and activity of lactate dehydrogenase in culture medium decreased with culture time. Transport variables were measured over the first 7 days of culture. Basal 86Rb efflux was reduced with time in culture and was inhibited by Ba2+, suggesting the efflux was mediated by K+ channels. At all stages of culture, 86Rb efflux was stimulated by ATP, hyposmotic medium, and ANG II. A complex pattern of efflux changes with culture time and type of stimulator was observed, suggesting that several components of the tissue contributed to stimulated efflux. This culture system provides opportunities for studies of chronic regulation of placental function.

explant culture; potassium transport; angiotensin II; adenine 5’-triphosphate; osmolality

THE CYTOTROPHOBLAST AND SYNCYTIOTROPHOBLAST layers of the placenta are dynamic over the course of pregnancy. This is seen, for example, in changes in morphology (2), in human chorionic gonadotrophin (hCG) secretion (28), and in the expression and activity of transport proteins (3, 5). This capacity for change indicates that the trophoblast has mechanisms that enable it to respond appropriately to its environment. Furthermore, the interaction between cytotrophoblast and syncytiotrophoblast apparently allows repair mechanisms in response to damage (16).

Acute (over hours) regulation of transport mechanisms in the trophoblast is known to occur, for example, for ions (27) and amino acids (8, 10, 11), but it is not well characterized. Chronic (over days) regulation is even more poorly understood but may be particularly important in view of the changes in transport activity that occur over the whole course of pregnancy (21). A preparation of isolated cytotrophoblast cells in primary culture could provide a useful system for studying such chronic regulation. However, it has been argued that such cells suffer from the disadvantage of not achieving terminal differentiation (19) and of being divorced from potentially important interactions with other components of the normal villus. A second possibility is to place fragments of villous tissue in explant short-term culture. However, a recent study suggests that the viability of such explants of term placenta rapidly decreases after ~4 h in culture (24). On the other hand, it has been reported that, although the syncytiotrophoblast of first-trimester explants degenerates initially, it then regenerates over a period of days in culture (17, 31).

The purpose of the present study was to characterize both morphologically and functionally villous explants of term placenta over the course of up to 11 days in culture. We investigated morphology at both the light and electron microscope level. Lactate dehydrogenase (LDH) was assayed in the culture medium as a measure of cellular integrity, and hCG secretion was measured to assess endocrine function. To determine the transport activity of the explants, we measured 86Rb efflux as a tracer of K+ transport (29).

MATERIALS AND METHODS

Tissue culture. Placentas were obtained at term from the delivery unit of St. Mary’s Hospital (Manchester, UK) following normal pregnancies, delivered vaginally or by caesarean section. The culture system was developed from procedures of Trowell (26) and Watson et al. (30). Within 30 min of delivery, chorionic villi were dissected out and carefully rinsed in sterile Dulbecco’s phosphate-buffered saline with calcium chloride and magnesium chloride (37°C; Sigma) to remove maternal blood. The placental tissue was cut into pieces weighing ~5 mg. Three such pieces were cultured in individual Costar Netwell (15-mm diameter, 74 μm mesh; Corning, Corning, NY) supports in 1.5 ml of culture medium (CMRL-1066, 5% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml insulin, 0.1 μg/ml hydrocortisone, and 0.1 μg/ml retinyl acetate; Sigma).
The tissue was supported on the mesh in the liquid-gas interface. Cultures were maintained at 37°C in a humidified gas mixture of 5% CO₂ and 95% air, and medium was changed every 24 h. Supernatants were collected and analyzed freshly for LDH or stored at −20°C for hCG analysis. The cultures were maintained for up to 7 or 11 days for functional and morphological evaluation, respectively. Cultures with apparent bacterial contamination were interrupted and excluded.

**Morphological evaluation.** For inspection of morphology, placental explants were harvested and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer with pH 7.3 for 3 h, washed in buffer containing 3 mM calcium chloride, and stored in this buffer at 4°C until further processing. The specimens were then cut into two or three pieces and post-fixed in 1% osmium tetroxide in 0.05 M sodium cacodylate buffer with pH 7.3 for 1 h at 4°C. After being rinsed in buffer, the pieces were dehydrated in an ascending alcohol series, incubated in two 15-min changes of propylene oxide, and infiltrated with Taab embedding resin (Taab Laboratories Equipment, Aldermaston, UK) before polymerization in gelatin capsules at 60°C for 72 h. Sections, 0.5 μm thick, were cut, mounted on glass slides, and stained with 1% toluidine blue 0 in 1% borax. Suitable areas were selected, and ultrathin sections were cut and mounted on copper grids, contrasted with uranyl acetate and lead citrate before examination in a Philips EM 301 electron microscope at an accelerating voltage of 60 kV. Electron micrographs were obtained of the areas of trophoblast.

**Analysis of collected culture medium.** The concentration of hCG in culture medium was assessed by quantitative immunoradiometric determination using a commercially available kit (hCG solid phase component system, ICN Pharmaceuticals, Costa Mesa, CA). The hCG assay uses the “sandwich technique” where the solid phase binds the alpha subunit of hCG in culture medium was assessed by quantitative immunoassay. The hCG assay uses the “sandwich technique” where the solid phase binds the alpha subunit of hCG and a radiolabeled antibody in the liquid phase binds to the beta subunit.

LDH activity in fresh culture medium was measured daily.

Conversion rate of NAD to NADH at 37°C was measured as the absorbance at 340 nm over 2 min (LDH/LD reagent kit, Sigma).

**Protein measurement.** After lysis of the cultured placental explants in deionized water, membrane-bound protein content was measured by dissolving the tissue in 4 ml 0.3 M NaOH. Two times 80-μl samples from each dissolved explant were mixed with 320 μl samples from each dissolved explant were mixed with 320 μl 0.3 M NaOH, 400 μl 0.3 M HCl, and 200 μl Bio-Rad reagent (Bio-Rad Laboratories, Hemel Hempstead, UK) and vigorously mixed. Optical density was measured at 595 nm.

65Rb efflux. 65Rb efflux has been widely used as a tracer of K⁺ fluxes (23, 29). Efflux of 65Rb from the explants was measured using a technique modified from Shennan (20). All efflux procedures were performed with the placental tissue in the Netwells where they were cultured. At 0, 2, 4, and 7 days of culture, the placental tissues were incubated at 37°C in 4 ml of buffer [135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5.6 mM glucose, 10 mM HEPES (acid), pH 7.4, osmolality 290 mosmol/kg H₂O] with the addition of 65Rb (45 μM, 5 μCi/ml; Amersham Pharmacia Biotech) for 90 min. After incubation, the explants were washed in 2 ml buffer (with no added isotope) for 2 × 10 min. Subsequently, 4 ml of buffer were changed and collected every 2 min for 30 min at 37°C. In some experiments, efflux was inhibited by addition of Ba²⁺ (BaCl₂, 5 mM) or stimulated by ATP (0.1 μM Na₃ATP; Sigma), hypotonic buffer (55 mM NaCl, 138 mosmol/kg H₂O), or ANG II (10 μM; Sigma) after 10 min of basal efflux collection and throughout the experimental period. Preliminary experiments with fresh placenta fragments showed that maximal 65Rb efflux response was achieved with the agonist concentration used here (22). Effused 65Rb was measured in a beta-counter (Packard 2000 CA). The tissue was lysed in deionized water overnight, and nonmembrane-bound 65Rb was measured in the supernatant. This count represents the effluxable fraction of 65Rb taken up by the tissue during the incubation phase minus that which effluxed during washing and experimentation. Percent 65Rb efflux was calculated as 65Rb counts in the efflux samples per the calculated effluxable counts at each time point. For analysis of 65Rb data, it was assumed that efflux would reflect a predominant pathway from a single compartment at steady state. Thus efflux in control situations (and in the presence of Ba²⁺, which is likely to have a predominant effect on a single pathway) was measured by a first-order rate constant. Under this assumption of a single compartment, the rate constants for 65Rb efflux were calculated for the period of 30–50 min as the slope of a regression line for the log of effluxable counts per initial effluxable counts at each time point.

After stimulation of efflux in response to ATP, ANG II, and hypotonic buffer, the assumption of a simple first-order process could not be maintained. Accordingly, alternative methods were used: 1) visual inspection of the time course of efflux, 2) area under the curve of the peak, calculated by subtracting the corresponding control from the stimulated explants for the period of 30–50 min after loading, and 3) repeated-measurement ANOVA (full factorial model).

All data are given as means ± SE. Statistics were calculated using the SPSS software package.

**RESULTS**

**Morphology.** Light microscopic examination of semithin sections of the explants revealed progressive degeneration of the original syncytiotrophoblast layer and its replacement by a newly formed layer. This layer gradually thickened as new syncytiotrophoblast was regenerated, whereas the original syncytium was sloughed off and lost. This regrowth was first evident by 3–4 days of culture.

At the ultrastructural level, it could be seen that, even after 1 day in culture, the syncytiotrophoblast had become vacuolated and was tending to lift away from the underlying cytotrophoblast and/or basement membrane (Fig. 1A). Syncytial nuclei appeared apoptotic, whereas the microvilli on the surface were either lost or showed severe blebbing. In contrast, the cytotrophoblast cells appeared relatively normal. By 4 days in culture, the original syncytium was highly degenerated, whereas the area of new growth was composed of newly divided cytotrophoblast cells, as shown by the presence of cells with large, pale nuclei, often overlaid by more basophilic mononuclear cells that exhibited surface microvilli and many of the cytoplasmic features of syncytiotrophoblast (Fig. 1B, day 4). After 7 days in culture, there was evidence of syncytiotrophoblast development, indistinguishable from the original tissue, with well-developed microvilli covering areas that were clearly syncytial in nature, overlying intermediate cells and a population of cytotrophoblast cells. Remnants of the original syncytium were still present in places (Fig. 1C). By 11 days, there was a thick, healthy-looking layer of newly grown syncytiotrophoblast, with
a dense coat of microvilli and many cytoplasmic secretory droplets. The ultrastructural characteristics of the tissue appeared to be identical to those found in the intact placenta (9). Much of the original tissue was now lost, although apoptotic nuclei and shreds of vacuolated cytoplasm could still be found in places, sometimes in close contact with the underlying regenerating layer (Fig. 1D). The cells of the mesenchymal core maintained a surprisingly high level of preservation during the period of study. In general, there was some thickening of the trophoblastic basement membrane and increased villous fibrosis over time in culture, with some breakdown and congestion of intravascular erythrocytes, but, otherwise, the standard of ultrastructural integrity was high.

**hCG and LDH release from cultured placental explants.** During the first day of culture, the release of hCG into the medium dropped quickly (data not shown). From the second day of culture, release of hCG

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**Fig. 1.** A: explant culture after 1 day in culture, showing degeneration and detachment of the syncytiotrophoblast (S) that has apoptotic nuclei (N) and loss of surface microvilli. The underlying cytotrophoblast (C), where present, appears to be relatively normal. B: after 4 days in culture, the C, which is now multilayered, shows differentiation with the development of new microvilli (arrowheads) and increasing amounts of rough endoplasmatic reticulum. C: after 7 days in culture, there is evidence of new S development with a well-developed microvillous surface; C and intermediate cells (I) can also be seen. Remnants of the old detached S are present overlying the new growth. D: by 11 days, the S is well developed with many secretory droplets (arrowheads) and a dense covering of microvilli. An I is also present. Part of the old S still adheres to the new growth. Scale bars represent 5 μm.

**Fig. 2.** Release of human chorionic gonadotrophin to the culture medium per 24 h of culture. Data are means ± SE of 7 placentas.
different from control on day 7.

...remainder of buffer osmolality all stimulated $86\text{Rb}$ efflux (Fig. 5).

Rubidium efflux. The basal percentage efflux of $86\text{Rb}$ remained fairly constant from 30 min onward in control explants (Fig. 4). When the rate constants were calculated for each experimental run, in every case the correlation coefficient exceeded 0.99. The rate constant of efflux in control conditions showed a decreased $86\text{Rb}$ efflux with the number of days in culture (Table 1; repeated-measurement ANOVA for 3 placenta giving data for all 4 time points, $F = 8.516, P < 0.02$). After the addition of Ba$^{2+}$ at 30 min, the mean rate constant for efflux was $-0.0077 \pm 0.00059$ on day 0 and $-0.0061 \pm 0.00012$ on day 7. This was significantly different from control on day 7 (1-way ANOVA, $F = 20.5, P < 0.001$). On day 0, Ba$^{2+}$ reduced the mean efflux compared with control, but this did not reach statistical significance due to the high variability on day 0 (1-way ANOVA, $F = 3.671, P < 0.1$).

Addition of ATP or ANG II to the buffer or reduction of buffer osmolality all stimulated $86\text{Rb}$ efflux (Fig. 5).

The ATP-stimulated efflux increased with the number of days the explants had been in culture, when expressed either as a peak response over nonstimulated efflux (Fig. 5A; 1-way ANOVA, $P < 0.02$) or area under the curve in the experimental period (Fig. 6), although the latter was not statistically significant due to the high variability on day 0. The shape of the stimulated efflux was similar at all time points of culture.

The efflux peak response to hyposmotic buffer increased (1-way ANOVA, $P < 0.02$) and occurred faster with the number of days of explant culture (Fig. 5B). However, the recovery after the peak was faster with older explants, resulting in no change in total efflux (area under the curve) between fresh and 7-day cultured explants (Fig. 6).

By contrast, the peak efflux following administration of ANG II increased from day 0 to days 2 and 4, but, then at day 7, it returned to almost day 0 response (Fig. 5C). Total efflux in the presence of ANG II was at a maximum after 2 days in culture (Fig. 6).

**DISCUSSION**

When placental tissues are kept in culture, the syncytiotrophoblast tends to degenerate over the first few days. Sooranna et al. (24) found intracellular vacuoles in the syncytiotrophoblast of first- and third-trimester placental explants after 8 h in culture at the ultrastructural level. Similar degeneration in first-trimester explants was noted after 24 and 48 h of culture by Palmer et al. (17), but, by 48 h, a newly formed trophoblast layer was found that was further developed after 5 days of culture. Histological examinations of cultured placental explants have reported a “prominence” of the cytotrophoblast as seen in the light microscope (7, 25). The current report supports the previous findings and expands them by showing that the trophoblast degeneration/regeneration does occur in explants from term placenta and that the newly formed syncytiotrophoblast layer continues to develop and remain viable for at least 11 days in culture.

Our data suggest that the degeneration of the old syncytiotrophoblast may occur by apoptosis; typical features of this process including chromatin condensation and membrane blebbing were observed (Fig. 1). Furthermore, the appearance of the nuclei in the new syncytiotrophoblast suggests that they are derived from division and fusion of cytotrophoblast cells. The improved integrity of the explants with time in culture is further indicated by reduction of LDH release into the medium. In addition, the development of the new syncytiotrophoblast over time was paralleled by in-
creasing hCG release from the explants. This is in contrast to the observation of a direct correlation between LDH and hCG release in first-trimester placental explant cultures as reported by Watson et al. (30).

To further characterize the functional integrity of the cultured explant, we measured 86Rb efflux because of the importance of K\(^+\) in cellular homeostasis. There was a decrease in control, basal 86Rb efflux with number of days in culture. This reduction in basal 86Rb efflux with time in explant culture may be explained by initial damage to the original syncytiotrophoblast and its degeneration, followed by the formation of the new intact syncytiotrophoblast and consequent reduction of nonspecific leak. Alternative explanations could be that driving forces or channel activities alter with time in culture. We have previously shown that Ba\(^{2+}\) depolarizes the microvillus membrane potential difference in fresh villous fragments (1). Ba\(^{2+}\) inhibited basal 86Rb efflux from cultured explants in the present study, indicating that a component of the efflux is conducted via K\(^+\) channels and that this component remains the same over the time of culture. The Ba\(^{2+}\)-insensitive efflux is likely to result from other transport routes as well as “leak” across the membranes. A disadvantage of using villous explants for efflux experiments is that the tracer may not be coming solely from the syncytiotrophoblast. However, the high correlation coefficient for rate constants over 30–50 min suggests that 86Rb efflux originated predominantly from one compartment under control conditions during this time period.

We showed previously that ATP, ANG II, and hypotonic medium stimulate 86Rb efflux from villous fragments in short-term culture (<4 h) (22). All of these agents act via intracellular signaling involving Ca\(^{2+}\) (12, 18) and were studied to verify functional integrity of the cultured explants in response to a regulatory challenge.

In trophoblast cells, ATP is most likely acting on purinoceptors with its effect mediated via inositol trisphosphate and Ca\(^{2+}\) to open Ca\(^{2+}\)-activated K\(^+\) channels in the plasma membrane (12). In primary cultures of cytotrophoblast cells, ATP-stimulated K\(^+\) efflux is charybdotoxin sensitive but apamin insensitive in response to ATP (4). In the current study, responsiveness to ATP (whether measured as peak response or total efflux) increased with time in culture, following the course of regeneration of the syncytiotrophoblast, indicating either improved ATP sensitivity of the cells or presence of more ATP-sensitive cells with time in culture.

The hypotonic medium used for stimulating 86Rb efflux was designed to mimic cell swelling secondary to the rise in intracellular osmolality, which occurs during nutrient uptake (6). Hypotonic solutions provoke an increase in cell volume, activation of transport

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**Fig. 5.** Efflux of 86Rb from explants after 0 (○), 2 (△), 4 (□), and 7 days (×) of culture. Efflux was stimulated by addition of ATP (A), hypotonic buffer (B), or addition of ANG II (C) from 30 min onward. Data are means ± SE of 3 placentas.

**Fig. 6.** Area under the peak of stimulated 86Rb efflux from cultured explants (day 0, black bars; day 2, cross-hatched bars; day 4, hatched bars; and day 7, white bars). The peak area was calculated by subtracting efflux of the corresponding control explants from the stimulated explants. ANOVA did not reveal any significant changes with time in culture. Data are means ± SE of 3 placentas.
mechanisms extruding ions (particularly K\(^+\) and Cl\(^-\)), and subsequent return of cell volume to normal (a regulatory volume decrease). Volume-activated K\(^+\) efflux appears to be at least partially mediated by a rise in intracellular Ca\(^{2+}\) concentration in cytotrophoblast cells (28). The peak response to hyposmotic buffer increased with time in culture, again approximating the time course of regeneration of the syncytiotrophoblast. However, as the efflux returned to baseline faster with time in culture, the total \(^{86}\)Rb efflux over the experimental period did not change significantly with the time in culture. This may suggest that the combination of cell types contributing to the hyposmotic stimulated \(^{86}\)Rb efflux changes with time in culture or that time in culture influences the nature and/or regulation of pathways in volume regulation.

In contrast to ATP and hyposmotic stimulated efflux, ANG II-stimulated efflux was highest on the second day of culture and then diminished. Furthermore, total \(^{86}\)Rb efflux during exposure to ANG II tended to decrease with days in culture. Specific binding sites for ANG II (AT\(_1\) receptor) are present on both syncytiotrophoblast (14) and placental vascular muscle homogenates (13, 15). Karl et al. (12) found that only 8% of isolated trophoblast cells responded to ANG II in terms of a rise in intracellular Ca\(^{2+}\), whereas 100% so responded to ATP. Thus it may be that the effect of ANG II on \(^{86}\)Rb efflux from the villous explant is of mixed origin, with the predominant response from non-trophoblast cells. The reduced ANG II stimulation with time could be explained by regeneration of a new syncytiotrophoblast layer that prevents ANG II from reaching the mesenchyme and mediating its predominant effect. Another potential mechanism affecting the shape of the ANG II-induced efflux may be tachyphylaxis in response to the high dose used in this experiment. In fresh placental fragments, doses of 1 and 10 \(\mu\)M ANG II induced a similar response (with a peak rapidly tailing off), whereas a dose of 10 nM ANG II initiated a steady but small increase in efflux throughout the experimental phase (unpublished observation).

Perspectives

Taken together, the \(^{86}\)Rb efflux data suggest improved integrity and responsiveness of the explants with time in culture over the 7 days studied. This improvement coincides with restoration of normal morphology and increasing hCG release from the explants, suggesting that the placental tissue has been able to “repair” the severe trauma caused by preparation in terms of receptor expression, second messenger activation, volume homeostasis, and endocrine function. It is, however, impossible at this stage to estimate to what extent the new trophoblast layer mimics the in vivo placenta, although, by morphological criteria, the two are indistinguishable. We suggest that this preparation might be valuable for studying death and repair of the syncytiotrophoblast and for investigating chronic regulation of function, including transport physiology. The explants have the advantage of retaining the normal spatial relationships between the various cell types of the villus but the disadvantage for transport studies that several components might contribute to the observed fluxes. However, this might be overcome by following transport activity in relation to degeneration and regeneration of the syncytiotrophoblast.

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