Cultured human trophoblast cells reproduce the initial events of placental biology

S. GARBISA - M. ONISTO - L. MAZZANTI (*) - A.L. TRANQUILLI (**)
A. PUGNALONI (***) - G. BIAGINI (***) - N. CESTER (**) - C. ROMANINI (**)

Summary: OBJECTIVE. The objective of this study was to determine whether cultured trophoblast cells shared the same morphological and biological properties observed in trophoblast, *in vivo*. STUDY DESIGN. Trophoblast cells from human term placenta were cultured, morphologically, biochemically and immunochemically monitored for as long as 30 days. RESULTS. Single cells progressively aggregated and fused into a syncytio, the Ca^{2+} and the Ca^{2+}-ATPase activity drooped, and the 72 kDa collagenase (MMP-2) was consistently expressed. CONCLUSIONS. Term placenta trophoblast cultures can be viewed and used as a model system mimicking morphological and biochemical events of placenta biology and differentiation.

Key words: Placenta; Trophoblast; Ca^{2+}-ATPase; MMP-2.

INTRODUCTION

The human syncytiotrophoblast plays numerous roles, including the regulation of ion, nutrient and immunoglobulin transport from the maternal to the fetal circulation ([1-5]), and the secretion of steroids and hormonal proteins ([6]). However the structural stages by which this syncytiotrophoblast coating is formed await further elucidation. While some Authors believe that the mononucleated cytotrophoblast leads to the syncytio through a process of cellular differentiation and fusion, other Authors have forwarded the hypothesis that the syncytial trophoblast is organized by means of the endomitosis of the trophoblast cells ([7-11]).

To tackle the problem of the genesis of the syncytiotrophoblast and of the control of its functions, investigators have set up reproducible *in vitro* systems by which these events may be studied and followed as a sequence of defined sequential steps, then focussing on one step at the time. Placenta explants have been maintained *in vitro* and cultures have been obtained from trophoblast cell suspensions ([12-14]). Systems of isolation are currently being standardized which use density gradients to concentrate the cytotrophoblast cell population. The aim of our study was to identify and define same key-role biochemical and morphological characteristics of trophoblast cells, isolated

Institute of Histology and General Embriology, University of Padova
(*) Institute of Biochemistry
(**) Department of Obstetrics and Gynecology and (***) Department of Morphology, University of Ancona
Padova and Ancona, Italy

from term placentas. It has been observed in other cellular models that cell fusion is preceded by modification in intracellular Ca^{2+} content, which in turn has been proposed to regulate the expression of the gene of the Ca^{2+} pump of the sarcoplasmatic reticulum ([15]). It has also been reported that cultured cytotrophoblast expresses gelatinase that seems critical for the invasion of basement membrane substrates ([16]). At the time of isolation, term placenta trophoblast cells were then morphologically identified, their stuctural modulation investigated, and type IV collagenase (MMP-2) expression, intracellular Ca^{2+} content and Ca^{2+}-ATPase activity determined.

MATERIALS AND METHODS

Normal term placentas were obtained from the Obstetric Departments of Ancona and Padova Medical Schools. Trophoblast cells were purified according to Morgan and Kliman's method, e.g. digestion of placental tissue and cell separation on Percoll concentration gradients ([17, 18]). The trophoblast cells concentrated in the 55% Percoll isolation band were either immediately studied ([19]), or cultured for 24 hr, 48 hr, 4, 5 days, and 30 days in a culture medium (Ham's F12 medium supplemented with 30% fetal calf serum, 2 mM glutamine, and 400 mg/ml each of penicillin and streptomycin) ([20]). By this technique, we isolated trophoblast cells free from blood elements, macrophages and mesenchymal cells contamination by trypsin digestion followed by a discontinuous Percoll gradient. Blood cells were found to prevail particularly in the 75% concentration band. In the 55% density band concentrated cells with trophoblast characteristic structure: cytotrophoblastic cells, cytotrophoblastic aggregates and occasional polynucleated trophoblastic elements. Cellular debris and mesenchymal cells were observed prevalently in the Percoll density bands below 5%.

Isolated trophoblastic cells underwent morphological investigation immunohistochemical and biochemical analyses as follows.

Morphological investigations

Morphological investigations, using transmission (TEM) ([19]) and scanning electron microscope (SEM). For SEM preparation, specimens fixed in 2% glutaraldehyde in 0,1 M cacodylate buffer (pH 7,4) and post fixed in 1% O_sO_4 in cacodylathe buffer, were then dehydrated in alcohols, critical-point dried in liquid CO_2, mounted on metal stubs and coated with gold. The samples were observed with a Philips 505 scanning electron microscope.

Immunohistochemical analysis

Immnohistochemical analysis for the expression of the 72 kDa type IV collagenase (MMP2), by the use of an affinity purified rabbit polyclonal antibody (1:200), raised against a synthetic amino terminal polypeptide, as already described ([21]), and a second peroxidase-conjugated goat-anti rabbit antibody (1:1000).

Biochemical analyses

– Ca^{2+}-ATPase pump activity, which was assayed according to the method of Davis *et al.* ([22]), by measuring Pi hydrolyzed from 1 mM Na_2ATP at 37 °C in the presence or absence of 0,15 mM Ca^{2+}. The ATPase activity assayed in the absence of Ca^{2+} was subtracted from the total ATPase activity to calculate the activity of Ca^{2+}-ATPase. The Pi was measured according to Fiske and Subbarow ([23]), and the protein concentration determined by the Lowry method ([24]) using albumin as standard. The results were expressed as mmoles Pi (mg membrane protein/h).

– Cytosolic free Ca^{2+} concentration, which was measured by fura-2-acetoxymethylester: the fluorescence intensity was read at a constant emission wavelength (490 nm) with changes in the excitation wavelength (340 and 380 nm) according to the method of Grynkiewicz ([25]). The results were expressed as mmol/l.

RESULTS

The cultured trophoblast cells already showed an evident change in their morphological characteristics within the first 48 hours of culture. On the fourth day of culture, mononucleated cellular elements were present. They tended to a spindle like shape, with cytoplasmic layers adhesive to the substrate. At the same time, however, it was possible to observe cellular aggregates composed of flattened cells adhering tightly to one another, yet still having adhering tightly structured individuality, with scattered nuclei and cell border mainly defined by cytoplasmic membrane. The signs of initial fusion among the single cell elements were only focal, even though they could be seen

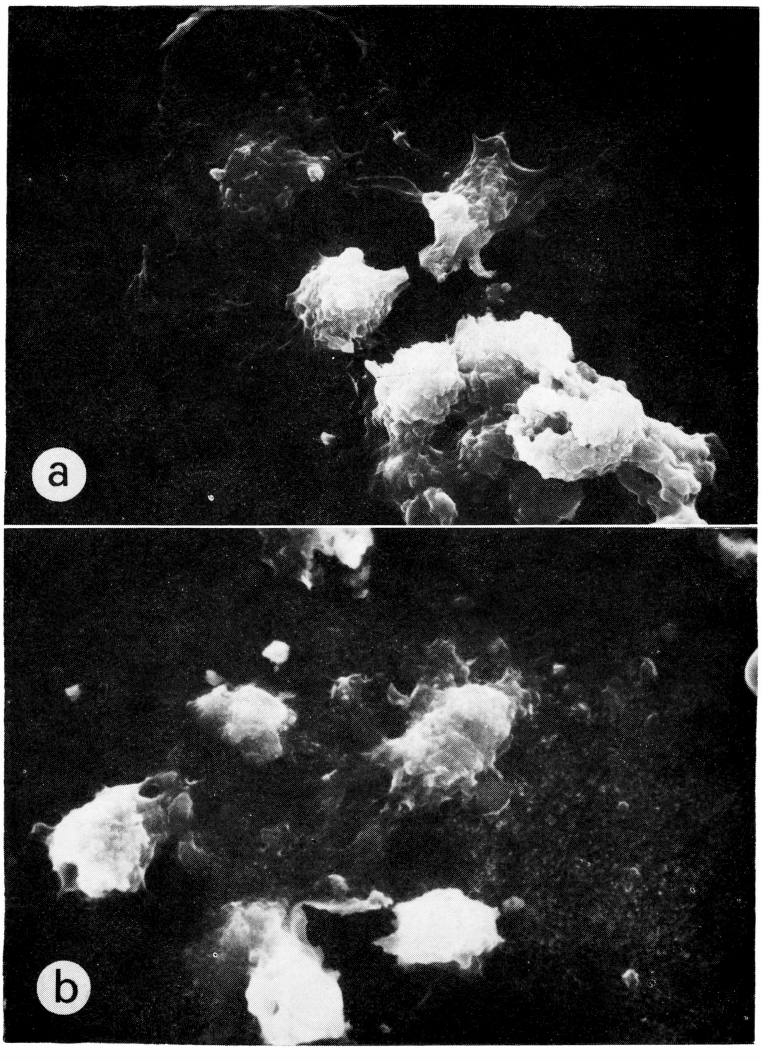

Fig. 1. — Scanning EM of trophoblastic elements at the fusion stage. Prominent nuclei are tending to join the cell body centrally (2,500×/a, 2,200 ×/b).

fairly frequently. For some groups of these cells, however, more evolved processes of plasmalemma fusion were noted, with the presence of prominent nuclei tending to join the ample cell body at its center. Finally, large sized cellular elements could be seen as the result of the aggregation process and successive fusion of the mono- or pauci-nucleated cells, possibly expressing a syncytial state (Figs. 1a, 1b, 2a, 2b, 3).

After 5-10 days of culture, such syncytial elements proved to be the most frequent cellular forms observed, while still retaining their ability to join to mono- or pauci-nucleated cells. However, such cul-

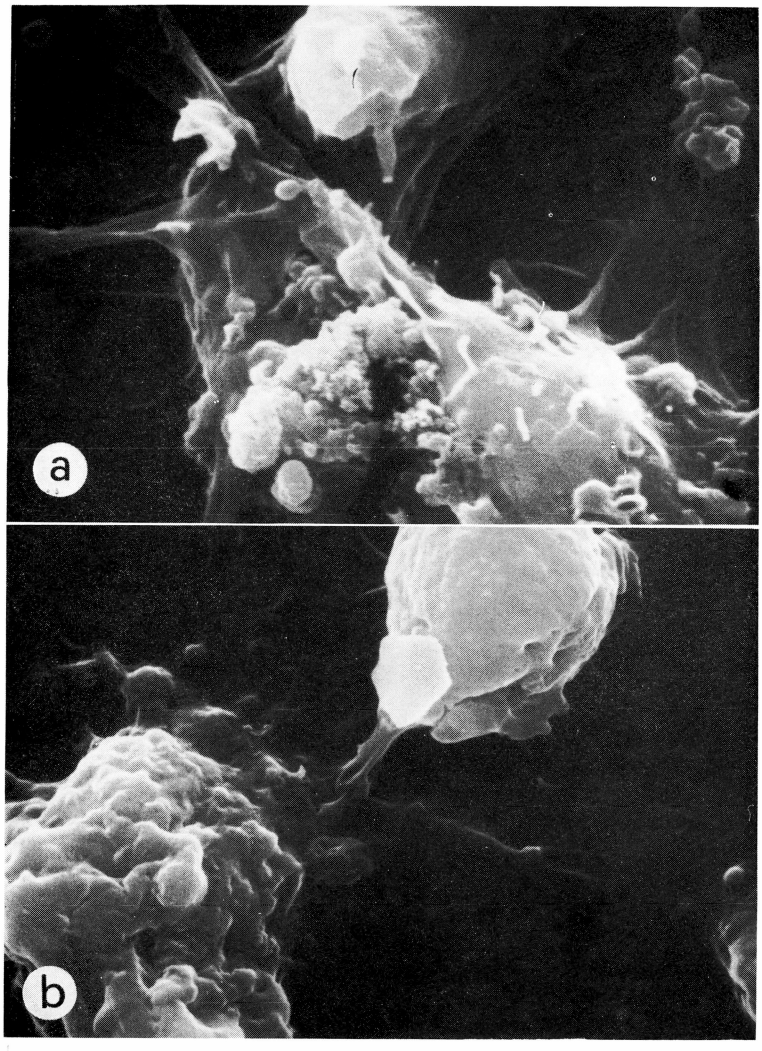

Fig. 2. — Scanning EM showing, at higher magnification than Fig. 1, some details of cell fusion (11,000 ×/a, 9,200 ×/b).

ture did not always seem able to lead the cells to conditions of full syncytiotrophoblastic maturity, and after 10-15 days it was still possible to detect flattened cells that, although structurally adjacent, were incompletely fused and without centralized nuclei (Fig. 4). This evidence either demonstrates the difficulty of reproducing *in vitro* what naturally happens *in vivo*, or highlights phases of partial or incomplete fusion that are not clearly identified *in vivo* (Fig. 5). After longer term culture (20-30 days) a further peculiar structural feature could be seen, which the phase-contrast microscope revealed as optically dense areas with rounded borders, perhaps reproducing the formation of villi-like elements.

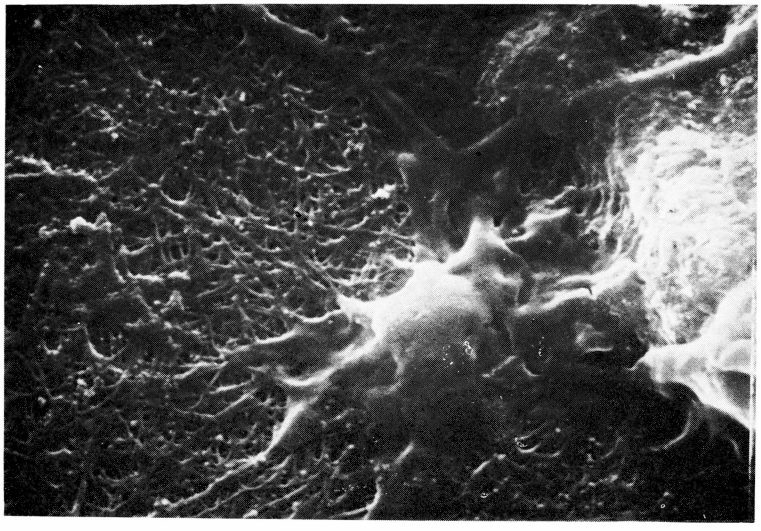

Fig. 3. — Scanning EM of trophoblastic elements. Enlarged cellular elements after fusion: details of cytoplasm (15,400 ×).

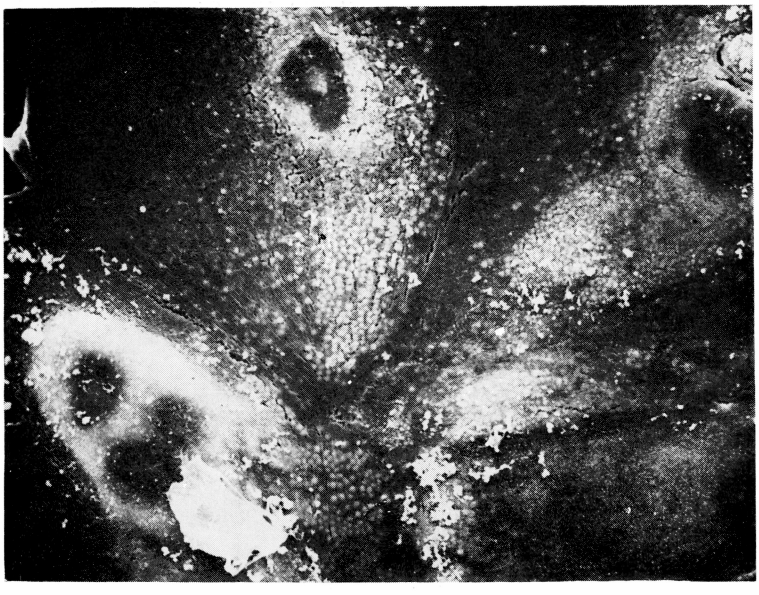

Fig. 4. — Trophoblastic cells strictly adjacent, with no centralized nuclei (800 ×).

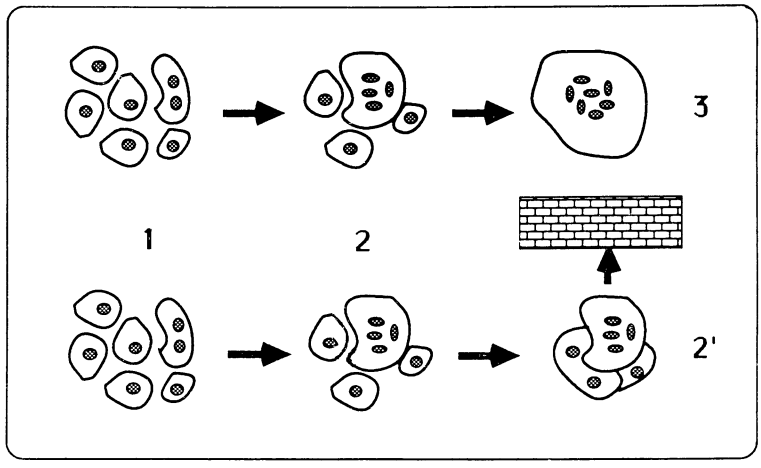

Fig. 5. — Scheme of syncytiotrophoblast formation as hypothesized by other Authors and ourselves: after initial aggregation, the mono- and bi-nucleated trophoblast cells (1) evolve into the form of a multinuclear aggregate (2) with focal signs of fusion. These aggregates will later originate syncytial cells (3), which are predominant after 48 hours in culture, though *in vitro* such aggregates could not complete the fusion (2'), and cease their structural evolution.

Immunostaining of the cultured term placenta trophoblast cells now reveals the expression of the 72 kDa type IV collagenase (MMP-2), either cytoplasmic or pericellular, which became evident by the end of the first week and remained consistent all along the culture period (Fig. 6).

Ca^{2+}-ATPase and intracellular Ca^{2+} contents were assessed in the cultured trophoblast for a time span during which cell fusion takes place. Both Ca^{2+}-ATPase activity and intracellular free Ca^{2+} content increased in the first 24 hours of culture, and dropped after the second day (Fig. 7).

DISCUSSION

Our results demonstrate that properly isolated and cultured trophoblast cells may reproduce trophoblast maturation *in vitro*. Morphological changes begin fairly early (48 hours) and progress through different stages up to 30 days. At this time, it is possible to observe structures that resemble the villi.

Concerning enzyme expression, our results show the production of the MMP-2 by the end of the first week of culture onwards. It has demonstrated that tissue remodelling such as the implantation of mouse blastocysts in the uterine wall ([26]), or at many other stages of embryogenesis, may frequently require secreted proteases. Recently it has been reported that in culture only early-gestation human cytotrophoblast invades a basement membrane-like substrate ([16]), and the proteolytic actions include a 92 kDa type IV collagenase (MMP-9) which seems critical for such invasion. The elaboration, by term placenta derived trophoblast cells, of MMP-2 which is a member of the metalloproteinase gene family ([27]), may be a phenotypic expression functional to mature placenta: its active degradative interaction which breaks the main structural component of basament membranes (collagen type IV) is probably functional to the delivery processes. It will be worth while investigating whether MMP-2 is as well expressed, concomitantly with MMP-9, in

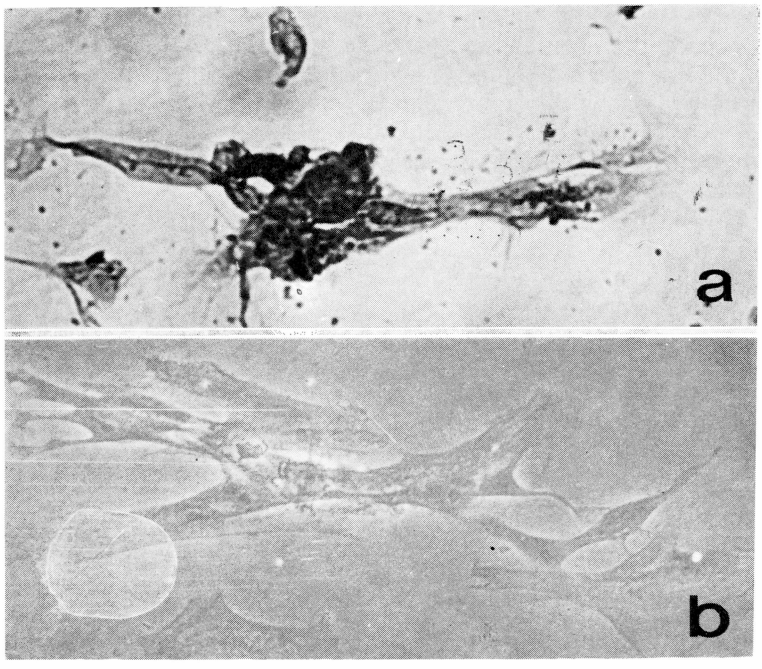

Fig. 6. — Immunostaining of trophoblast cells, after 14 days in culture, using anti-MMP-2 (72 kDa collagenase) antibody (a), and pre-immune serum (b).

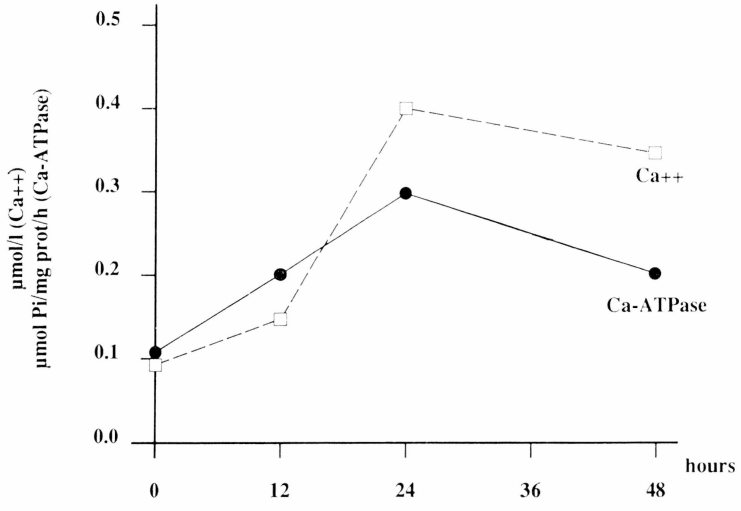

Fig. 7. — Ca^{2+}-ATPase activity and intracellular Ca^{++} concentration of human trophoblast cells at the time of isolation, and after 12, 24, and 48 hours in culture. The plot represents the mean value of three separate experiments, and for all values after 24 hours $p < 0{,}001$.

the early gestational stages, playing an active role also in the adhesion to and invasion of the maternal uterine epithelium, which takes place by the end or the first week of development. If this is the case, the proposed *in vitro* approach will stand even closer to the first steps that charcterize the trophoblast-microenvironmental-interactions.

Besides the biochemical events which mediate such interactions, other cellular mechanisms, involved in the cell-cell interactions, may act in mediating trophoblast structural modifications. Fusion of chicken myoblasts is preceded by an increased Ca^{2+} influx, and prevented in low-Ca^{2+} medium ([15]). The expression of the protein thought to be Ca^{2+}-ATPase pump of the sarcoplasmic reticulum is retarded in low-Ca^{2+} medium and accelerated by some, but not all, treatments suspected of elevating the cytoplasmic-free Ca^{2+} concentration. We demonstrated an increase in Ca^{2+}-ATPase activity and intracellular Ca^{2+}. Ca^{2+}-dependent ATPase is usually thought to be associated with transport since it is found on plasma membranes of transporting epithelial cells, such as renal, intestinal cells, and placental brush borders ([3]). The reduction in the activity of the Ca^{2+} pump after 48 hr of culture could express the creation of a metabolic condition ([28]) in which intra- and extracellular exchanges appeared modified in favor of important morphogenetic changes, characteristic of the very early phase of culture.

The conclusion that seems to emerge is that the trophoblast culture could be viewed as a valid model for studying the placenta biology and differentiation. Such cultures, imitating the *in vivo* trophoblast progression, may help in following either morphologically or biochemically the development of the cytotrophoblast, which, through aggregation and fusion progressively changes into a syncytio, and may contribute to the clarifying and monitoring of crucial events that take place during normal or pathological development of the placenta *in vivo*.

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PRECIS

Term-placenta-derived single trophoblast cells in culture fuse into a syncytio, increase Ca^{2+}-ATPase activity, and consistently express the enzyme MMP-2, and are proposed as a model mimicking the initial events of placenta biology.

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Address reprints requests to:
Prof. S. GARBISA
c/o Istituto di Istologia
Via Trieste, 75
35121 Padova (Italy)