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Collagenase-IV in human trophoblast invasion and differentiation

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Trophoblast cells are unique with respect to their functions and responsibilities. These cells demonstrate three sequential phenotypes, proliferation and invasion into the endometrium, differentiation to form syncytia and endocrine secretions. Equipped with these properties placental trophoblasts are endowed with a variety of functions, like implantation of the blastocyst to the endometrium, providing nutrition to the developing embryo and also transmitting extraordinary array of signals for the embryonic development. Experimental evidences and logical extrapolation suggest that these functions are precisely controlled by growth factors, cytokines and hormones produced either by the trophoblast themselves or by the utero-placental unit. Any error in this control mechanism has extremely adverse consequences. The cells also synthesize a large number of enzymes, amongst which collagenase type IV secretion is involved in digestion of underlying basement membrane necessary for the process of invasion. Our results implicate the enzyme in the functional differentiation of the trophoblast as well. Inhibitors to this enzyme inhibit trophoblast differentiation as monitored by secretion of hCG and progesterone, the two markers of trophoblastic differentiation. In contrast, BeWo cells,a choriocarcinoma cell line which does not differentiate spontaneously, undergo increased proliferation when challenged with EGF. The results indicate the possibility of invasive and differentiative phenotypes to be coupled. Exact molecular involvements in this coupling process are looked into.

Human implantation and embryogenesis are associated with endocrine and metabolic readjustments as well as extensive tissue remodelling and cellular migration. Trophoblast cells of the embryo play an important role in these activities during the process of implantation and subsequent placentation. The cells form the ultimate interface between fetal and maternal tissue and may therefore hold the key to the tissue remodelling. The trophoblast cells have remarkable growth and invasive properties *in vivo* so that they come to resemble neoplastic characteristics. Human cytotrophoblast cells exhibit their invasive properties as early as the first week of gestation[1], but this invasive property later subsides by yet unknown mechanism(s).

Successful human placentation requires that fetal trophoblast cells rapidly invade genetically dissimilar maternal tissues during the early stages of pregnancy. Many elements of trophoblast invasion are similar to the events that occur during tumor cell invasion. The phenotypic change from carcinoma *in situ* to invasive carcinoma occurs when tumor cells acquire the ability to penetrate the epithelial basement membrane and invade the underlying stroma[2,3,4]. Likewise, after a brief adherent stage of the blastocyst to the endometrium, cytotrophoblast cells penetrate the basement membrane of the uterine epithelial cells and invade the stroma and its associated arterioles[5]. The apparent similarity in these systems suggest a possible common mechanism between the two invasive processes. The degradation of the basement membrane by the invading trophoblast cells is carried out mostly by a metalloprotease, the type-IV collagenase[6] and a serine protease, urokinase type plasminogen activator[7]. A schematic representation of the proteolytic mechanism of basement membrane and extracellular matrix dissolution by these metalloproteases has been depicted in Fig.1. Unlike tumor invasion and metastasis, trophoblast invasion is precisely regulated, confined spatially to the endometrium of the uterus and temporally to the early pregnancy. Any error in this regulation can have extremely adverse consequences, like shallow invasion is associated with preeclampsia[8] and over

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Abbreviations used:
hCG , human chorionic gonadotrophin; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium, CMF-HBSS, calcium-magnesium free Hank's balanced salt solution; EDTA, ethylene diamine tetra acetic acid; FCS, fetal calf serum; RIA, radioimmuno assay, SDS sodium dodecyl sulphate

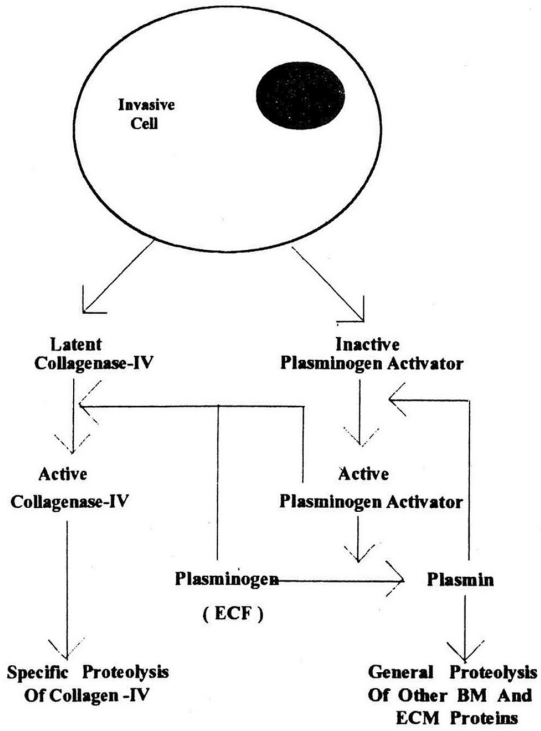

Fig.1— Schematic representation of the proteolytic mechanism of basement membrane and extracellular matrix dissolution.

aggressive invasion is associated with placental site tumors, choriocarcinoma and placental accreta.

Trophoblast cells display another unique property. Along with the process of invasion and placentation, many of the mononucleated cytotrophoblast cells aggregate together and then get coalesced to form multinucleated syncytiotrophoblast cells[9]. Once differentiated, syncytiotrophoblast cells acquire the ability to secrete many hormones, growth factors and cytokines necessary for fetal development and maintenance of pregnancy[9]. All these *in vivo* functions of human trophoblast cells could be reproduced under *in vitro* culture conditions thus providing a system particularly useful for studying trophoblast invasion and differentiation.

Trophoblast invasion and differentiation occurs sequentially i.e. invasion precedes differentiation. Hence, it is logical to assume that differentiation is the end point of invasion. Differentiation could be one of the controlling point for invasion. It is also possible that differentiation is an aftermath of trophoblast invasion and proliferation where latter two events are tightly coupled processes.

Materials and Methods

Trypsin (Type III) and DNase (Type I), antibiotic/antimycotic solution and 1:10 phenanthroline were obtained from Sigma Chemical Co., USA. DMEM, Ham's Nutrient Mixture F12 and fetal calf serum were obtained from Life Technologies, USA. Percoll was purchased from Pharmacia, Sweden. All components for electrophoresis were purchased from Biorad,USA. [^{3}H]Thymidine (sp. activity 18000mCi/mmole) was obtained from Isotope Division, Bhaba Atomic Research Center. All other chemicals (analytical grade) were obtained from local sources.

Sampling of tissue

Term placentae were obtained from normal deliveries at AIIMS or Safdarjung Hospital. They were collected in normal saline and were processed aseptically within half an hour of delivery. Portions of villous tissue were sampled from several cotyledons and washed thoroughly in normal saline to remove traces of blood. All connective tissue elements and blood vessels were removed. Finally, 20-30 g of tissue were processed for further isolation of trophoblast cells.

Isolation and purification of trophoblast cells

To isolate and purify trophoblast cells previously established method of Das and Catt[10] was used with minor modifications. Briefly, cells were isolated by differential trypsinization (0.125g % of 0.01g % DNase, for 3×30 min) in calcium, magnesium free Hank's Balanced Salt solution (CMF-HBSS) containing penicillin (100 IU/ml), streptomycin (100μg/ml) and fungizone (2.5μg/ml). Homogeneous population of trophoblast cells were obtained from the enzymatically isolated heterogenous population of cells by subjecting them to discontinuous percoll gradient (5-70%) centrifugatio The band corresponding to the density of 1.048 was 95 % pure trophoblast cells[9]. These cells were plated onto 16 mm, 24-well tissue culture plates (Costar) at a density of 0.3×10^6 cells/well/ml in DMEM containing 10% fetal bovine serum and antibiotic. The plates were maintained at 37°C in sterile humidified atmosphere

for 5 days with daily change and collection of media.

BeWo Cell line

BeWo cell line was obtained from National Facility for Animal Tissue and Cell Culture, Pune, India. Initially cells were expanded in tissue culture flask in Ham's Nutrient Mixture F12 with 15% FCS and antibiotic/ antimycotic solution. Cells were harvested by trypsinization (0.25g % trypsin, 0.03g % EDTA in phosphate buffer saline), counted and subsequently plated in 16 mm, 24-well tissue culture plates (Costar) at an initial density of 5×10^4 cells /ml/well.

Thymidine incorporation study

0.7-0.8 μCi of [^{3}H]-thymidine was added to each well at 0, 12, 24 hrs after plating the BeWo cells in the 24 well tissue culture plates. Spent media were discarded at 12 hourly intervals and the cells were harvested and filtered through 0.22μ membrane filters. Filters were washed with normal saline three times and then transferred to scintillation vials followed by overnight drying at room temperature. Finally, 5 ml of scintillation fluid was added to each vial and the radioactivity was counted in a Wallac scintillation counter (Pharmacia).

Challenge of cells

After 24 hr of plating, the trophoblast cells isolated and purified from term placenta, were challenged with increasing concentrations of 1:10 phenanthroline dissolved in DMEM in respective wells. In control wells plain media was added.

The BeWo cells were challenged with Epidermal Growth Factor (EGF) dissolved in F12 media at a concentration of 10 ng /ml. Equal volume of plain media was added to control wells.

Radioimmunoassay of hCG and progesterone

The collected spent media were assayed for progesterone and hCG by RIA following the WHO protocol and using the National Matched Assay Reagents of the Indian Council of Medical Research, New Delhi. Results of the assay were calculated by a special software 'RiaCalc RM, version 2.69 by Wallac Inc.' obtained from Pharmacia, Sweden.

Concentration of secreted hCG and progesterone were expressed in terms of total cellular protein level. In each experiment 6 replicates (6×2) were taken for analysis. The data were expressed in terms of mean ± S.D.

Determination of total cellular protein

After the collection of the spent media at the end of each experiment, the remaining cells were washed twice with normal saline. NaOH (0.1*N*) in a volume of 0.5ml was added to each well and the plates were shaken for 15 min. Dissolved cellular protein was collected from each well and stored at -20° C until assay. Finally, total cellular protein was determined by Lowry's method[11].

Substrate gel zymography for Collagenase-IV

To determine the molecular species of collagenase IV secreted by the cultured trophoblast cells, substrate gel zymography for collagenase IV was carried out by the method of Sawaya *et. al.*[12]. Spent media was concentrated by lyophilization and 100μg of media was subjected to electrophoresis in 5% SDS-polyacrilamide gel containing 1% gelatin. At the end of the electrophoresis the gels were rinsed twice with 2.5% Triton-X for two hrs. They were subsequently incubated at 37°C for 15 hr in 50 m*M* Tris-HCl buffer containing 15 m*M* $CaCl_2$ and 0.05% NaN_3. The gels were stained with 0.05% Coomassie blue and destained in a mixture of ethanol, acetic acid and water (4.5:1:4.5). Enzyme activity was detected as transparent bands on the blue background of Comassie blue stained slab gel.

Results

Normal secretion profile of hCG and progesterone by cultured trophoblast cells

The secretion of both hCG and progesterone followed a temporal pattern during the period of culture. The cells secreted negligible amounts of hCG (Fig.2) at 24 hrs after plating. However, a considerable amount of hCG was secreted by them at 72 hrs of culture. Microscopic observation at this time point showed differentiating cells i.e. many mononucleated cytotrophoblast cells fusing together to form multinucleated syncytiotrophoblasts. At 120 hrs the cells were fully differentiated and secreted highest amounts of hCG.

The secretion of progesterone by the trophoblast cells in culture followed almost the same pattern as

that of hCG secretion (Fig.3). The increased secretion of the steroid was observed at 72 hrs which was maintained till 120 hrs of culture. At the initial periods of culture however, rise in progesterone occured later than the rise in hCG.

Effect of 1:10 phenanthroline on hCG and progesterone secretion by cultured trophoblast cells

1:10 phenanthroline, a specific inhibitor of collagenase-IV, when added to the media inhibited secretion of both hCG (Fig. 4) and progesterone

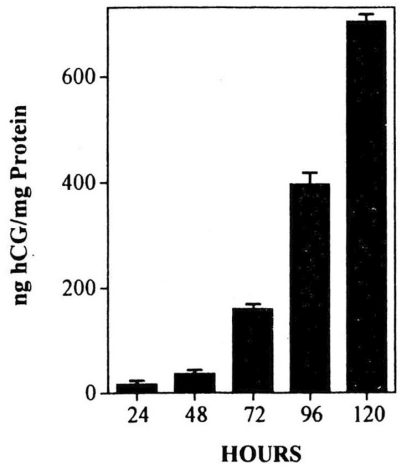

Fig.2— Secretion profile of hCG by normal term placental trophoblast cells maintained under culture conditions.

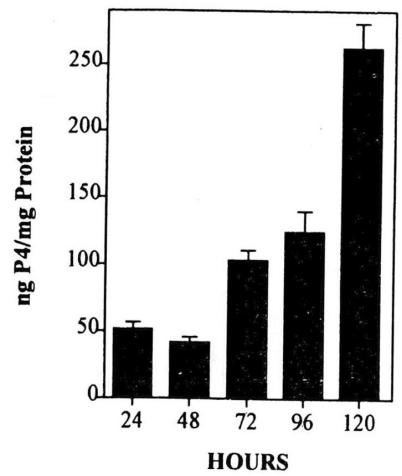

Fig.3— Secretion profile of progesterone by normal term placental trophoblast cells maintained in culture.

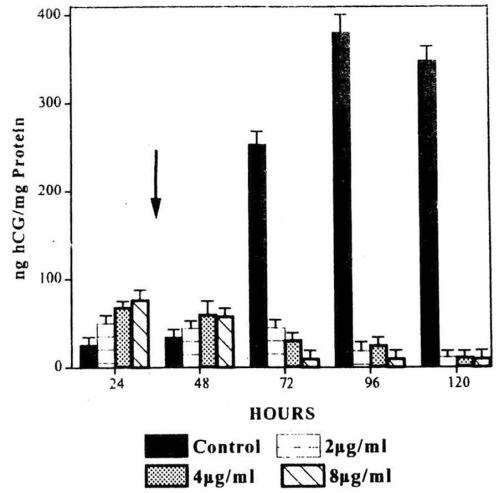

Fig.4— Effect of 1:10 phenanthroline on hCG secretion by the trophoblast cells in culture [Challenge was started after 24 hrs of plating the cells]

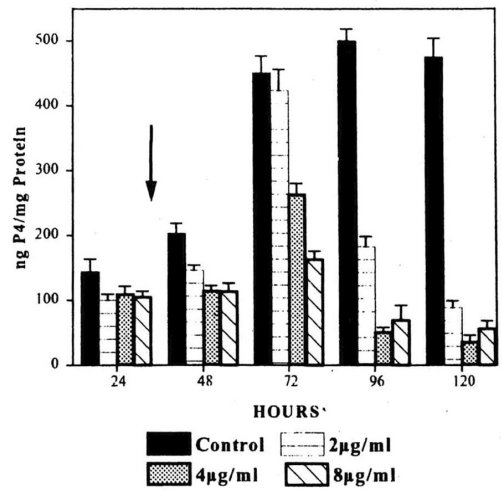

Fig.5—Effect of 1:10 phenanthroline on progesterone secretion by the trophoblast cells in culture. [Challenge was started after 24 hrs of plating the cells]

(Fig. 5) in a dose dependent manner.

Effect of EGF on [H^3]-thymidine incorporation by BeWo cell line

BeWo cell line when challenged with EGF at a dose of 10 ng/ml significantly ($p \leq 0.01$) increased [^{3}H]thymidine incorporation (Fig. 6).

Identification of isoforms of collagenase secreted by cultured trophoblast cells

Substrate-gel zymography of collagenase-IV

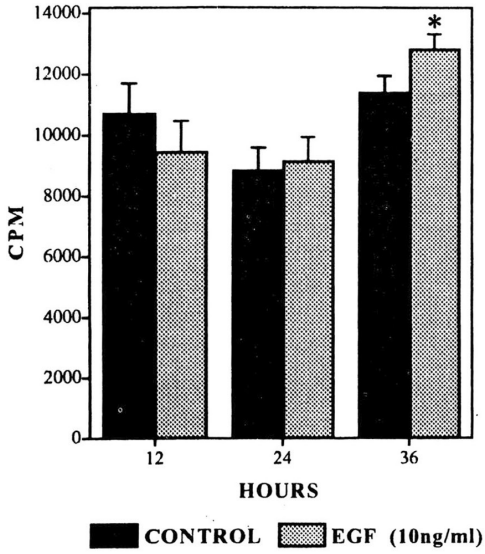

Fig.6— Effect of EGF on incorporation of [^{3}H]thymidine by BeWo cells. [At 36 hrs of culture EGF increased [^{3}H]-thymidine incorporation significantly* ($p \leq 0.01$)]

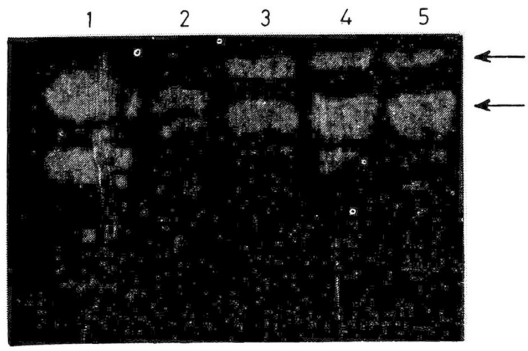

Fig.7—Substrate-gel zymography for collagenase-IV [Lanes1and 2, bacterial collagenase (1μg and 0.1 μg respectively), lanes 3-5, lyophilized media (100 μg)]

revealed the presence of two molecular forms of collagenase in the spent media of cultured trophoblast cells (Fig.7).

Discussion

Trophoblast cells isolated from term placenta by enzymatic dispersion, when maintained under culture conditions, differentiated to form multinucleated syncytiotrophoblasts. During the undifferentiated (cytotrophoblast) state (24-48 hrs), the cells secreted very low levels of hCG and a basal level of progesterone. Alongwith the cellular differentiation a spontaneous increase in the secretions of hCG (Fig.2) and progesterone (Fig.3) was observed at 72 hrs of culture. Interestingly, rise in hCG secretion always preceded progesterone secretion which is in agreement with our previously published report[13]. Considering the fact that increased levels of hCG and progesterone are secreted only when the cells have differentiated into multinuclear syncytia, the high concentrations of the two hormones could be used as an indicator of cellular differentiation under culture conditions.

Under *in vivo* situation, trophoblast cells are invasive before they differentiate to syncytiotrophoblast cells. The invasive process is accomplished by several enzymes amongst which collagenase-IV secreted by the trophoblast cells is very important. The enzyme is necessary for degradation of collagen-IV molecules which provide the major resilience strength to the basement membrane and act as the main hurdle for a cell to invade underlying stroma[2]. The activity of the secreted enzyme, when inhibited by exogenous addition of 1:10 phenanthroline, a specific inhibitor of collagenase-IV, blocks cellular invasion[14]. Interestingly, we observed that blocking of this enzyme blocked trophoblast cell differentiation as well. This conclusion is drawn from the fact that 1:10 phenanthroline inhibited secretion of hCG and progesterone, the two markers of differentiation (Fig. 4 & 5). It seems logical that apart from its contribution to invasiveness, trophoblast derived collagenase also contributes to differentiation and both the processes share collagenase-IV as common molecular tool.

As evident from substrate-gel zymography, trophoblast cells secreted two isoforms of collagenase (Fig 7). This may correspond to the previously known isoforms of collagenase-IV[15]. However, their exact molecular nature are yet to be worked out. The secretion of collagenase-IV in two isoforms raises several questions: for example, are both the isoforms involved in invasion and differentiation or each isoform is specific to a specific process?

BeWo cells are trophoblast derived choriocarcinoma cell line which proliferate but do not differentiate[16]. The cells also demonstrate the inherent potential to invade and metastasize. In these cells proliferation and invasion are tightly coupled phenomena. When the cells were

challenged with EGF, it resulted into proliferation as indicated by increased uptake of [H^3]-thymidine (Fig.6). It is thus logical to extrapolate that EGF increases invasion in BeWo cell line as proliferation and invasion are tightly coupled in these cells. This observation has important physiological significance in relation to *in vivo* invasiveness of trophoblast cells i.e., for implantation of the blastocyst to the endometrium and subsequent formation of the placenta. Since uterine environment is rich in different growth factors, one of them being EGF, our experiments indicate involvement of EGF in the trophoblast invasiveness .

Conclusion

When different pieces of observations and logical arguments are put together in a single frame it can be concluded that trophoblast derived collagenase-IV is utilized by both invasion and differentiation process as a common mechanistic tool.

Though indirect, it can also be concluded that EGF exerts influence over invasion and differentiation implicating it as a potential candidate for controlling both the processes.

Acknowledgement

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