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Technical Note

Double Immuno-labelling of Proliferating Villous Cytotrophoblasts in Thick Paraffin Sections: Integrating Immuno-histochemistry and Stereology in the Human Placenta

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ABSTRACT

In order to understand the pathological basis of abnormal villous trophoblast development in diseased placentas, the organ must be sampled by non-biased methods and subject to analysis by stereological tools. This approach permits quantification of cytotrophoblast density and syncytiotrophoblast structure including evidence of apoptotic shedding via syncytial knots. The stereological quantification of cells (or their) nuclei requires that each should be unambiguously identified and counted within a defined volume of tissue. A major limitation of such studies at present is the inability to accurately identify and phenotype subsets of villous cytotrophoblasts that either proliferate or are destined to fuse into the overlying syncytiotrophoblast.

We describe the development of a novel double immuno-labelling protocol to selectively identify proliferating villous cytotrophoblast cells in human placental villi using thick (25 μm) paraffin sections suitable for stereological quantification. Cytotrophoblast cells were selectively stained using a monoclonal anti-cytokeratin 7 (CK 7) antibody without antigen retrieval, followed by nuclear Ki-67 co-localisation. Both antibodies displayed full depth penetration with sharp, clearly defined staining precipitates and no cross-reactivity. This double immuno-labelling protocol is reproducible, cost effective and time efficient (8 h). Use of a variety of antibodies following antigen retrieval will be a significant advancement in the ability to accurately quantify sub-populations of villous cytotrophoblast in normal and pathological placentas.

1. Introduction

The regulation of villous trophoblast turnover in human pregnancy is of increasing importance especially in pathological pregnancies [1–3]. Considerable molecular knowledge in normal trophoblast development and pathologically mediated dysregulation is available [4]. Yet to date few studies have been able to integrate specific changes occurring at a molecular level with predicted structural changes in the syncytiotrophoblast layer responsible for fetal growth and maternal well-being. This dual approach of integrating molecular techniques with stereological quantification within a single placenta therefore provides a physiologically integrated model to study trophoblast development and dysregulation. The numerical correlation between proliferation, fusion and apoptotic shedding within the trophoblast compartment is paramount and can be achieved using stereological methods.

In order to investigate trophoblast kinetics using stereological techniques two factors need to be met 1) each cell or nucleus to be counted must be unambiguously identified and 2) cells or nuclei must be counted within a defined volume of tissue (i.e. the area of the counting frame multiplied by the disector height, see equation (1)) within which they reside. Counting within a defined volume of tissue at this level provides a numerical density estimate (N_v), which then multiplied by total placental volume, provides an estimate for the total number of cells within the entire placenta (N_{tot}).

The ability to accurately identify and phenotype the villous cytotrophoblast population from the overlying syncytiotrophoblast histologically has been attempted using morphological features or various antibody markers. Reliable identification based on

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morphology alone is difficult, especially as villous cytotrophoblast changes from a cuboidal-like continuous layer of cells to a discontinuous layer at term. Identification based on antibody markers, for example, E-cadherin [5], hepatocyte growth factor inhibitor (HAI-1) [6] and cytokeratin 7 [7] have all been used in a variety of applications. Despite their specificity for cytotrophoblasts, these markers are not exclusive displaying some cross-reactivity with the syncytium and stromal cells. Since no generic marker with the ability to consistently and accurately label cytotrophoblasts exists, attempts were made to modify the staining procedure using a monoclonal cytokeratin 7 antibody [8].

Stereological techniques have previously been employed to quantify the number of cytotrophoblast cells and syncytiotrophoblast nuclei based on their morphology and topography. Mayhew and colleagues used a Gomori trichrome tinctorial stain to identify different trophoblast populations using thin histological sections [9]. By using the physical disector technique [10,11], which employs two thin sections a known distance apart, these authors were able to quantify the different population of nuclei within a defined volume of tissue. Although unbiased, this method is time consuming since each pair of sections requires registration (see Sterio 1984 [11] for a detailed explanation). A more efficient method for generating a defined volume is the optical brick/disector technique [12,13], which employs a single thick (25 μm) section. This can be achieved using resin sections; however, full depth antibody penetration in thick resin sections is not possible.

To overcome these limitations, a method for the reliable and consistent identification of double immuno-histochemically labelled proliferating cytotrophoblast cells in a thick (25 μm) paraffin section, which fulfilled the criteria for stereological quantification was conceived. The ability to accurately and consistently identify subsets of trophoblast cells in a defined volume of placental villous tissue is an important technical advance for future quantitative analysis and will provide invaluable insight into villous trophoblast kinetics in pathological pregnancies.

2. Materials and methods

2.1. Tissue collection and preparation

Placental tissues from normal third trimester pregnancies were obtained from Mount Sinai Hospital, Toronto, Canada, following local ethical approval and informed patient consent. Uniform/systematic random placental samples were taken as previously described [1,14], fixed in 10% neutral buffered formalin for 48 h processed and embedded into wax blocks. Paraffin sections were cut at 5 μm and 25 μm thickness and mounted onto 3-aminopropyltriethoxysilane (APTS) coated slides (Sigma, UK) and dried at 60 °C overnight.

2.2. Antibodies

Cytotrophoblast cells were immuno-localised using an anti-human mouse monoclonal cytokeratin 7 [1:300] (clone OV-TL) antibody. Proliferating cytotrophoblast cells were visualised using a mouse monoclonal antibody to Ki-67 [1:50] (clone MIB-1). Both antibodies were obtained from Dako, Cambridge, UK.

2.3. Double-labelling of cytokeratin 7 and Ki-67

All incubations were carried out at room temperature (RT) and all reagents were obtained from Vector labs, Peterborough, UK unless otherwise stated. A high salt TBS-T wash buffer (100 mM Tris, 300 mM NaCl, 0.05% Tween, pH 7.6) was used for all washes during the double-labelling procedure to ensure minimal background staining.

Localisation of cytokeratin 7 *exclusively* to the cytotrophoblast required *omission* of antigen retrieval, necessitating it as the first antibody in the double-labelling procedure. Sections were dewaxed in xylene for 15 min and rehydrated through descending alcohol concentrations following routine procedure. Endogenous peroxide activity was quenched by incubation with 10% hydrogen peroxide (H_2O_2) in methanol for 30 min, followed by washing with TBS-T (Sigma, UK) for 3 × 3 min. Non-specific protein binding sites were blocked using 2.5% ready-to-use normal horse serum for 20 min, immediately followed by a 20 min incubation with mAb CK 7. Sections were then incubated with Vector ImmPRESS anti-mouse polymer detection reagent for 20 min, followed by extensive washing in TBS for 3 × 5 min. Cytokeratin 7 was visualised using ImmPACT DAB (diaminobenzidine) incubated for 2 min, producing a brown end product; sections were then washed in deionised water for 5 min.

Immediately after staining with CK 7, antigen retrieval was carried out on the same sections to allow for labelling of the second antibody Ki-67. Slides were placed in a coplin jar containing 20 mM citrate buffer, 2 mM EDTA, 0.05% Tween (Sigma, UK), pH 6.2, heated to 90 °C for 15 min, followed by cooling at RT, for a further 20 min. After washing in TBS-T for 3 × 3 min, non-specific proteins were blocked using 2.5% ready-to-use normal horse serum for 30 min, followed by incubation with mAb Ki-67 diluted in TBS for 2 h, and subsequently a 30 min incubation with Vector ImmPRESS anti-mouse Ig polymer reagent. Sections were thoroughly washed with TBS-T for 3 × 5 min to remove unwanted polymer. Ki-67 was visualised using a contrasting purple chromagen, Vector VIP, incubated for 2 min, followed by immersion in deionised water for 10 min. Sections were then counterstained using methyl green for 2 min at RT, differentiated in 0.05% acetic acid/acetone for 10 s, rinsed in deionised water for 10 s, dehydrated in 95% and 100% ethanol (2 min each), and finally cleared in xylene and mounted in DPX; excess DPX was used to avoid sections 'drying out' due to their increased thickness.

2.4. Estimating numerical density using the optical brick technique

The double-labelled sections were used to obtain the numerical density of CK 7 and Ki-67 positive cytotrophoblast cells. Numerical density estimation was performed with the aid of a BH2 Olympus light microscope (magnification ×100 NA 1.25), a Heidenhain microcator (ND 281A) attached to the z-axis of the microscope stage, and Kinetic Digital Stereology 5.0 software. An unbiased counting frame (UCF) was applied under software control to each uniformly–randomly selected image (field of view) and the microcator set to zero. Each section was focused through in the z-axis in a continuous motion; cells in the first (0–5 μm) and last (20–25 μm) 5 μm of the 25 μm sections were not counted to avoid the lost cap effect [13], generating a disector height of 15 μm within which cells were counted (verified by the microcator). Cells positive for CK 7 (non-proliferating), and CK 7 with Ki-67 (proliferating), were counted provided they obeyed the rules of the UCF and were within maximum focus within the 15 μm disector height (see Fig. 2). Approximately 10 fields of view per slide were sampled using a uniform random sampling approach resulting in an average 50 fields of view per placenta.

Numerical density estimates were calculated using the following formula (equation (1)):

$$N_v = \frac{\sum Q}{\sum P\left(A_f h\right)} \tag{1}$$

where (N_v) is numerical density, $\sum Q$ is the total number of cells counted divided by the total number of disectors $\sum P$ multiplied by the volume of each disector i.e. area of the unbiased counting (A_f) and the disector height (h).

The total number of cells (N_{tot}) was estimated by multiplying numerical density with total placenta volume (equation (2)):

$$N_{tot} = N_v \times V_{ref} \tag{2}$$

where (V_{ref}) is the volume of the placenta estimated by fluid displacement.

3. Results and discussion

Cytokeratin 7 is an intermediate filament protein expressed in epithelial cells. Although this monoclonal antibody stains both villous cytotrophoblast cells and the overlying syncytiotrophoblast in antigen-retrieved sections [15] we demonstrate the specific localisation of CK 7 exclusively to the cytotrophoblast cytoplasm when the antigen retrieval step is omitted (Fig. 1). Ki-67 was co-localised to the nucleus of labelled villous cytotrophoblast cells following antigen retrieval in this double-labelling protocol in 25 μm thick paraffin sections (Fig. 1). Both antibodies displayed full depth penetration of the tissue sections permitting accurate identification and future quantification of proliferating cytotrophoblast cells from other proliferating cell populations in the stromal compartment of chorionic villi.

Each antibody in the double-labelling protocol was first optimised as a single stain on thin 5 μm paraffin sections and then tested for full penetration and cytotrophoblast specificity in 25 μm thick sections; once individually optimized, they were combined in a sequential double-labelling protocol. This approach ensured complete penetration of each antibody, minimal background