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IMMUNOHISTOCHEMICAL ANALYSIS OF BASEMENT MEMBRANE FORMATION IN EMBRYOID BODIES DERIVED FROM EMBRYONIC STEM CELLS

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SUMMARY

The central role of basement membranes in organ and cell biology has made them an important subject for researchers who are investigating their structure, organisation and regulatory functions. Our study employed indirect immunohistochemistry to investigate the formation of basement membranes in embryonic bodies (EB) derived from embryonic stem (ES) cells. Basement membranes initially appeared in embryonic bodies at the end of the first day. Depositions of basement membrane components - laminin, collagen IV, nidogen and perlecan - appeared in cultures as linear staining. Thick basement membrane depositions were also observed in embryonic bodies at the end of the first week using anti-laminin, anti-collagen IV, anti-nidogen and anti-perlecan antibodies. Using TEM, the ultrastructure and thickness of the basement membrane was observed to be similar to Reichert's membrane, a membrane consisting of parietal endoderm cells derived from primitive endoderm cells, secrete basement membrane components and the trophectoderm basement membrane. However, ES cells, which are derived from inner cell mass cells, cannot be differentiated from trophoectoderm cells; therefore, basement membranes in EBs may form mechanisms other than Reichert's membrane. It is likely that different basement membrane types are present in EBs but cannot be distinguished by immunohistochemistry.

Key Words: *Basement Membrane, Embryonic Stem Cells, Immunohistochemistry*

ÖZET

EMBRİYONİK KÖK HÜCRELERİNDEN FARKLILANAN EMBRİYOBLASTLARDA GELİŞEN BASAL MEMBRANIN İMMUNOHİSTOKİMYASAL OLARAK İNCELENMESİ

Organ ve dokulardaki hücrenin temel biyolojik fonksiyonlarında önemli rol oynayan basal membranın, yapısı, organizasyonu ve regülasyonu günümüzdeki araştırma konularındandır. Bu nedenle, embriyonik kök hücrelerinden gelişen embriyoblastlardan oluşan basal membranın yapısı elektron mikroskobik ve indirekt immunohistokimya teknikleri ile incelenmiştir. Basal membranın formasyonu ilk olarak 1 günlük kültür yapılmış embriyoblastlarda gözlendi. Basal membran proteinleri olan laminin, kollagen IV, nidogen ve perlekanın immunoreaktivitelerinin pozitifliği bir günlük kültürde bir çizgi şeklinde saptandı. Embriyoblastların 1 hafta sonraki kültür dönemlerinin analizlerin de ise, basal membranın kalın bir tabaka olarak laminin, kollagen IV, nidogen ve perlekan antikorları ile immunohistokimya tekniği ile boyandığı gözlendi. Yapısal ve kalınlık açısından embriyoblastlarda farklılanan basal membranın TEM yöntemi ile Reichert'ın membranına benzediği gözlendi. Reichert'ın membranı primitif endoderm hücrelerinden farklılanan ve basal membran proteinlerini salgılayan parietal hücreler (Smith and Strickland, 1981; Hogan et al., 1981; Dziadek et al., 1985; Paulsson et al., 1985) ve trofoektoderm hücrelerinin basal membranı (Hogan et al., 1984; Salamat et al., 1995) tarafından oluşturulur. Bununla beraber, embriyonun iç hücre kitlesinden faklılanan embriyonik kök hücreleri trofoektoderm hücrelerine faklılanamaz. Bu nedenle, embriyoblastlarda gelişen basal membranın Reichert' ın membranının gelişiminden farklı bir mekanizma ile geliştiği düşünülmektedir. Tabiki, immunohistokimyasal olarak embriyoblastlardan gelişen basal membranın tipi ayırt edilemez.

Anahtar Kelimeler: *Basal Membran, Embriyoblast, İmmunohistokimya.*

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Basement membranes, which are secreted during early embryonic development, are found in all vertebrates and invertebrates except for sponges (1). For this reason, the characterisation of the biological properties of basement membranes has become an important theme in cell and developmental biology. Basement membranes are abundant in proteinaceous sheets underlying epithelial and endothelial cells and surrounding muscle fibres, fat cells and peripheral nerves. Only a few tissues (bone and cartilage) completely lack basement membranes (1). When analysed using standard electron microscope fixation methods, basement membranes appear to be divided into three layers. These are identified with respect to their staining behaviour and localisation: the lamina rara, the lamina densa and the lamina fibroreticularis.

Basement membranes exhibit structural and functional differences at different stages of development and in different tissues. For example, in the renal glomerulus, the basement membrane is sandwiched between a fenestrated endothelium and epithelial cells, providing a barrier between blood and urine. In the mouse embryo, Reichert's membrane, a multi-layered basement membrane, acts as a filter allowing free access of nutrients between the embryo and mother (2). In addition, basement membranes may play a central role both in the initial stages of development and in the maintenance of polarity of epithelial and endothelial cells (3).

The molecular organisation of the basement membrane depends on the inter-molecular interaction between its different components (1). The major mass of the basement membrane is built up of only a few of these components, including laminin, nidogen (entactin), collagen IV, perlecan, fibulin (BM-90) and SPARC (BM-40) (1). It is generally assumed that the formation of basement membranes requires specific interactions between the various constituents. Interaction of components may contribute to the structural and functional heterogeneity of basement membranes. Furthermore, the interaction of one basement membrane component with cells may modulate the deposition of other components within the basement membrane. The specific roles played by the individual components during basement membrane assembly remains a major question. They may include self-assembly processes involving identical components as well as the formation of the basement membrane containing different components.

In this study, embryonic stem (ES) cells were derived from the inner cell mass of 3.5 day blastocysts, which retained their pluripotency when allowed to grow under undifferentiated conditions (4,5). ES cells can be maintained in culture for many passages in an undifferentiated state by culturing on STO cells or primary cultures of mouse embryo fibroblast-feeder layers and/or with a differentiation inhibitor of ES cells, namely the leukemia inhibitor factor (LIF). In their absence, ES cells spontaneously differentiate in vitro into embryonic bodies (EBs) (6,7). EBs were found to be divided into two distinct parts - the outer endodermal cell and inner ectodermal cell layers, bordered by a basement membrane, which is similar in appearance to the egg-cylinder stage of the five-day embryo (Figure 1).

In using EBs derived from ES cells, it is significant that the analysis of basement membrane formation provides a very convenient model for the study of the biosynthesis and assembly of the basement membrane components. Thus, in order to understand the ultrastructure of the basement membrane in EBs, we used an electron microscope and indirect immunoflourescence staining to examine basement membrane formation and component deposition (laminin, nidogen, collagen IV and perlecan) in EBs derived from differentiating ES cells.

MATERIALS AND METHODS

Embryoid Bodies

Stock frozen cultures of undifferentiated mouse ES cells were allowed to differentiate in DMEM 10% FBS using the hanging drop method (8). Drops of 10µl (5x103 cells per drop) were placed on the lower surface of plastic petri dish lids containing PBS. Cell aggregates were plated

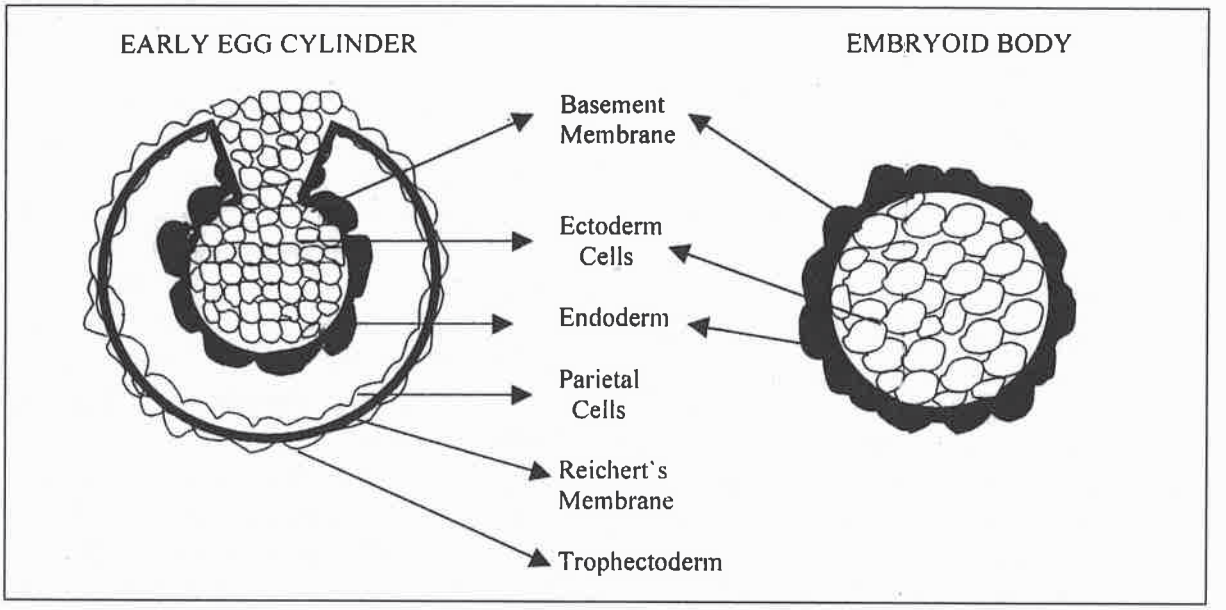

Figure 1: Analogy between structures in mouse egg cylinder and embryonic body. (Not to scale)

into plastic petri dishes after two hours in culture conditions, and the EBs were collected at the first day, second day and first week of culture. Specimens were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature prior to sectioning and immunostaining as above. EBs were washed in PBS prior to embedding in gelatin and freezing in Tissue-Tek (Sakura Finetek Europe).

Immunostaining of Frozen Sections

Rabbit polyclonal primary antibodies anti-EHS laminin (LA5 antiserum), which recognises all three subunits of laminin (9), anti-perlecan raised against recombinant domain III3 (10) and anti-collagen IV (Chemicon AB756) were used in this study. Rat monoclonal antibody against nidogen purified from the mouse EHS tumour was also used (15). Cryostat sections (7 µm) were washed in PBS for 30 minutes at 38°C, followed by a 10 minute wash in PBS at room temperature for the removal of gelatine. These sections were then incubated with a blocking solution consisting of 10% (v/v) normal goat serum (NGS Sigma G-9023). The primary antibodies (rabbit anti-laminin, rat anti-nidogen, rabbit anti-collagen IV and rabbit anti-perlecan) were then added. After washing with PBS, they were incubated with secondary antibodies [anti-rabbit IgG rhodamine-conjugated (Dako R-1569) for anti-laminin, anti-collagen IV and anti-perlecan antibodies; biotinylated anti-rat IgG (Dako E-0468) and streptavidin conjugated fluorescein isothiocyanate (FITC) (Amersham RPN 1323) for anti-nidogen antibody]. All immunostaining slides were mounted in a fluorescent mounting medium (DAKO). Slides were examined using a Leitz DMR photomicroscope with epifluorescence and phase-contrast microscopy and then photographed with HP5 film (Ilford, ISO 400).

Transmission Electron Microscopy (TEM)

EBs were taken from culture after a week and fixed in 2.5% (w/v) glutaraldehyde, 2% (w/v) paraformaldyde in 1 M sodium cacodylate buffer (pH 7.2) for two hours and postfixed with 2% (w/v) osmium tetraoxide in the same buffer. EBs were rinsed twice for five minutes with 0.15 M sodium acetate buffer, stained with 2% (w/v) uranyl acetate in 0.15 M sodium acetate buffer (pH 6.0) for 45 minutes, dehydrated in graded concentrations of alcohol and transferred into an embedding component (L. R. White). Sections

(70 nm) were taken with a Huxley microtome and mounted on 3000 mesh grids for examination by electron microscope (Philips 300 operating at 80 Kv). Sections were stained with 2% (w/v) uranyl acetate solution in 0.15 M sodium acetate buffer and lead citrate to increase contrast.

RESULTS

Using the hanging drop method, ES cell clumps appeared within 24-36 hours as simple EBs. Each clump had an outer layer of endodermal cells and inner ectodermal cells. There was an apparent increase in the size of the clump after 24 hours, probably due to both clump aggregation and cell multiplication. A basement membrane between the outer endodermal cell layer and the inner ectodermal cells was clearly visible under the dissecting microscope after a week in culture (Figure 2).

EB ultrastructure was examined by TEM. The thickness of the basement membrane measured $5x10^{-6}m$ between the outer layer and inner cells (Figure 3).

Immunohistochemical Analysis of Laminin in Ebs

EBs were stained with an anti-laminin antibody after a week in culture. Immunoreactivity of laminin was located underneath the outer layer of EB cells (Figure 4a). In addition, some punctuate staining was found in the inner layer cells. Figure 4-b shows the same EBs under phase microscopy. No staining was observed in the EB control sections for immunohistochemistry (Figure 4-c).

Time Course of Deposition of Basement Membrane in EBs

In order to analyse the induction of the basement membrane deposition, EBs were collected from culture at different times. After two hours in culture, single ES cells began to aggregate and form EBs (Figure 5-a). Under phase-contrast microscopy, some crescents of endodermal-like cells were seen on EB surfaces; however, no complete layers of endodermal cells encircling the EBs were seen (Figure 5-a). Immunoreactivity of laminin was seen both extracellularly (cell surfaces) and intracellularly (Figure 5-b). After one

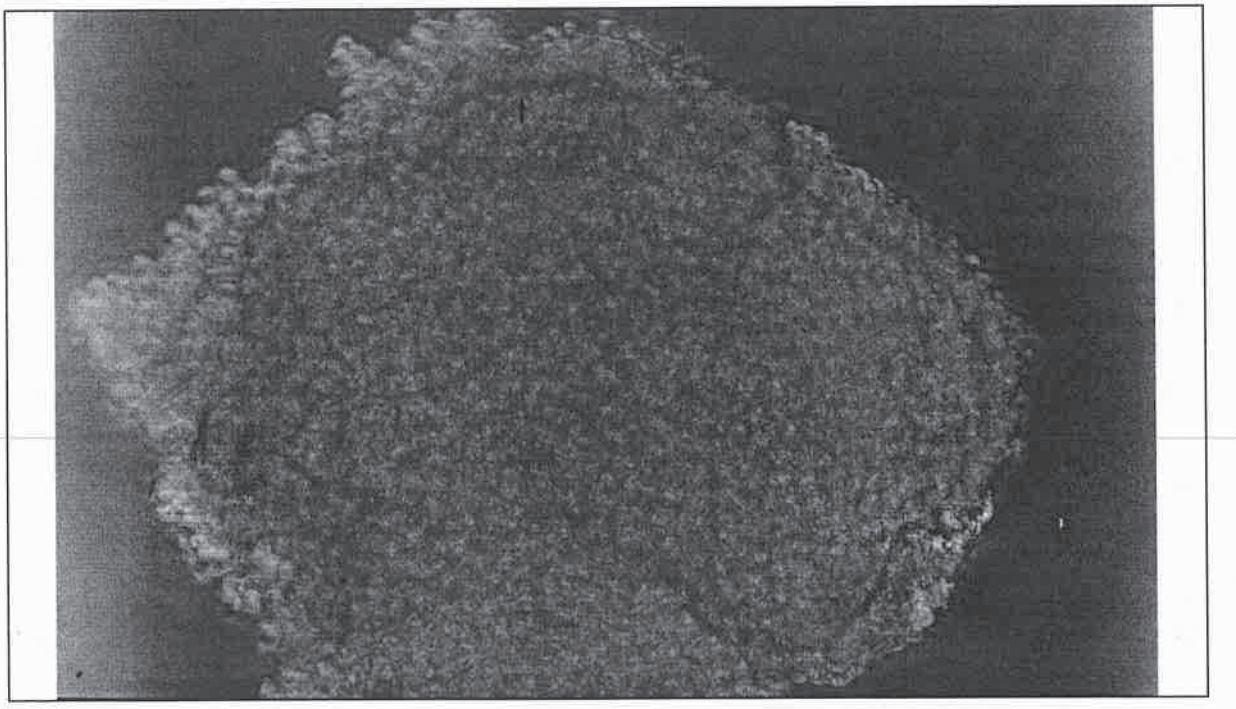

Figure 2: Phase micrographs of EBs produced using the hanging drop technique. After a week in culture, the basement membrane can be identified between the outer layer and inner cells (arrow). (Scale bar= 40 μm)

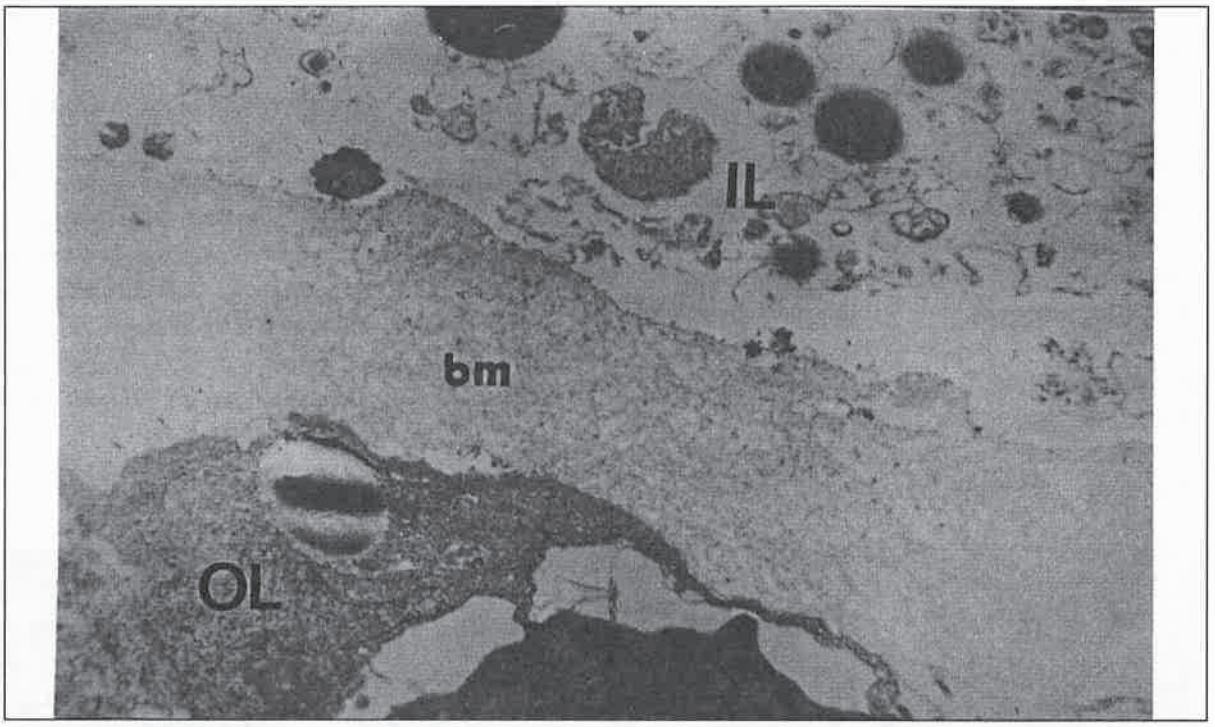

Figure 3: Electron microscopy of EBs after one week in culture. A sheet-like structure (basement membrane) seen beneath the outer layer. (BM:basement membrane; OL:Outer endodermal cell layer; IL:Inner ectodermal cell layer. Scale bar= 2 μm)

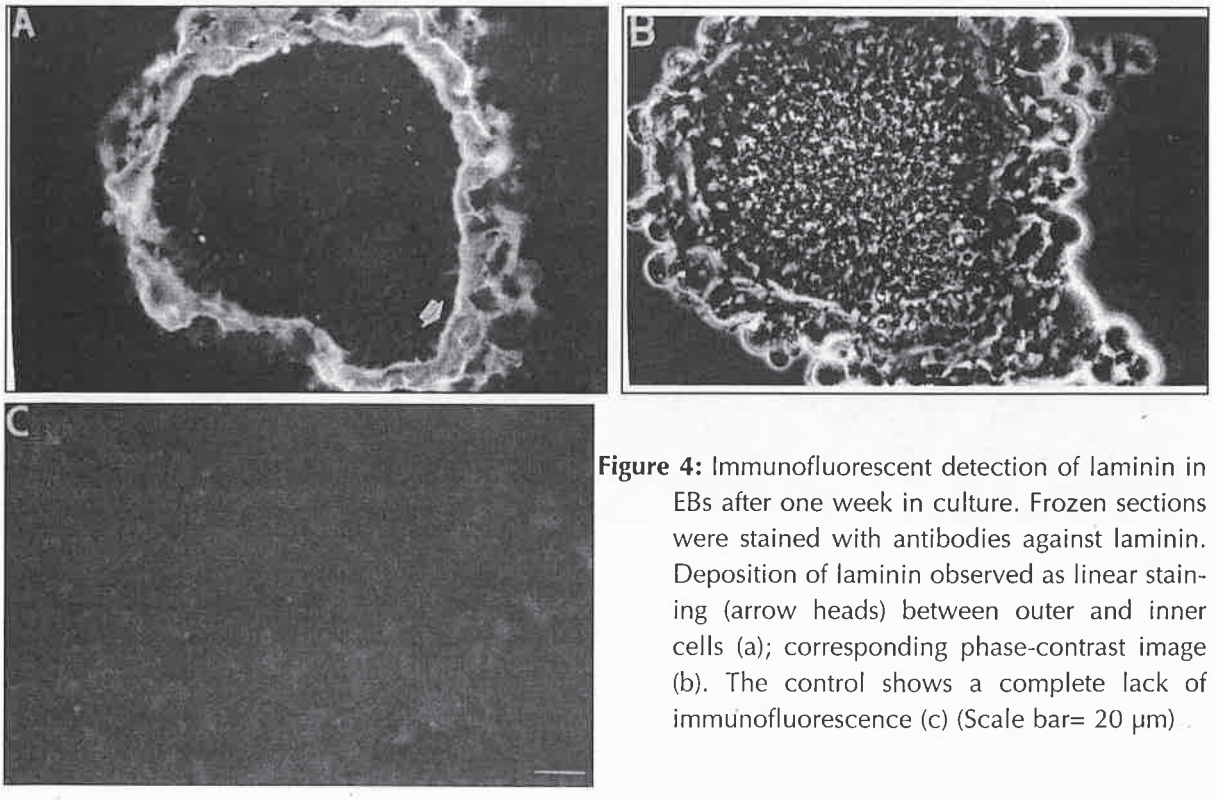

Figure 4: Immunofluorescent detection of laminin in EBs after one week in culture. Frozen sections were stained with antibodies against laminin. Deposition of laminin observed as linear staining (arrow heads) between outer and inner cells (a); corresponding phase-contrast image (b). The control shows a complete lack of immunofluorescence (c) (Scale bar= 20 μm)

day in culture, ES cell clumps became simple EBs (Figure 5-c). A comparison of Figures 5-a and 5-c shows the apparent increase in the size of EBs (four times greater than that of two-hour cultures) due to clump aggregation and cell multiplication. The outer cells of EBs appear as a layer that can be identified by phase contrast microscopy (Figure 5-c). Continuous linear staining with LA5 antiserum was seen between the outer layer and the inner cells in the periphery of EBs (Figure 5-d). Following two days in culture, the basement membrane was observed between outer and inner cells as a thick layer stained with laminin (Figure 5-e). Some immunoreactivity of laminin was seen to be associated with a few inner cells as well (Figure 5-e).

Deposition of Other Basement Membrane Components

EBs were stained with antibodies against collagen IV, nidogen and perlecan after two days and one week in culture (Figures 6-a, 6-c and 6-e, and Figures 6-b, 6-d and 6-f, respectively). Strong immunoreactivities of collagen IV (Figures 6-a, 6-b) nidogen (Figures 6-c, 6-d) and perlecan (Figures 6-e, 6-f) were seen as a continuous linear layer under the outer EB cells.

DISCUSSION

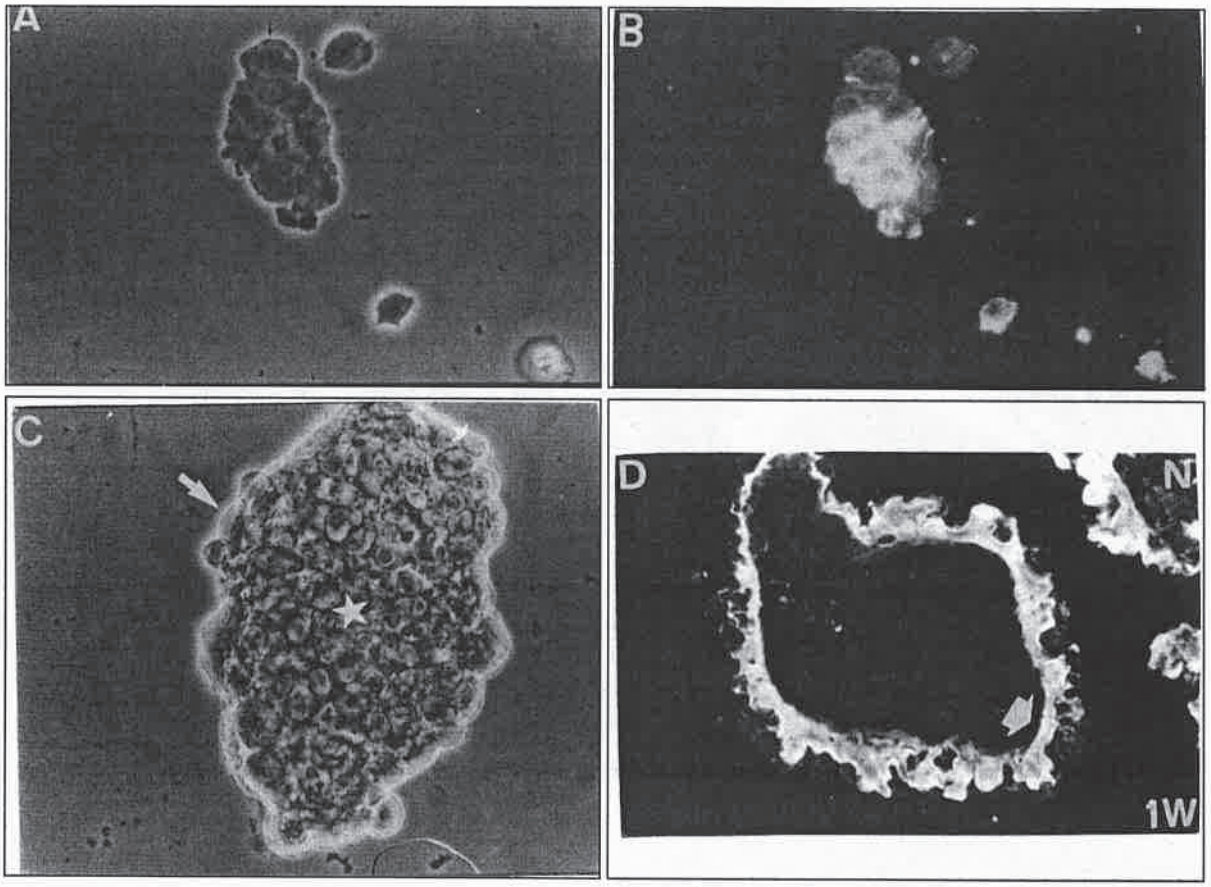

Figure 5: Immunofluorescent staining of EBs after two hours, one day and two days in culture. EBs were stained with LA5 antiserum against laminin. After two hours, primitive endoderm-like cells in outer layer (black arrow) were observed in phase-contrast image, and laminin immunoreactivity was shown as extracellular staining (b). After one day, EB growth and outer (white arrow) and inner cell layers (star) are clearly detectable in phase-contrast image (c). Immunoreactivity of laminin is also clearly detected between outer layer and inner cells (arrow heads) after one day (d) and two days (e) in culture. (Scale bar= 20 μm)

ES cells that are allowed to aggregate in the absence of LIF differentiate in vitro into EBs (6,7). Cell differentiation is promoted via cell-to-cell interactions similar to a compaction of the preimplantation mouse embryo (11). The use of the hanging drop technique is ideal for the rapid differentiation of defined EBs because it allows ES cells to aggregate spontaneously and quickly and permits EB size to be controlled by cell density (8). After cell differentiation, the EBs consisted of two parts, the outer layer and inner cells, which were separated by a basement membrane. The outer layer cells have the characteristics of both visceral and parietal endoderm cells (8). After a week in culture, a thick basement membrane was seen between the outer layer and inner cells by using TEM for EBs (Figure 3). The thickness of this basement membrane was estimated to be about $5x10^{-6}m$, giving it a resemblance to Reichert's membrane, a multilayer basement membrane seen at the blastocyst stage of mouse and rat development (12,13). Reichert's membrane is built up by both parietal endoderm cells, which are derived from primitive endoderm cells and produce basement membrane components (14-16), and the trophectoderm basement membrane (13,17). However, ES cells, which are derived from ICM cells, cannot differentiate into trophoectoderm cells. Therefore, EB basement membranes may form by a mechanism different from that of Reichert's membrane. Thus, it is likely that different basement membrane types are present in EBs but cannot be distinguished by immunohistochemistry. However, this basement membrane was deposited with major basement membrane formation.

Deposition of laminin was seen in our experiments as linear staining between the outer layer and inner cells, with some punctuate staining on or in the inner EB cells. Immunoreactivity of laminin was detected mainly in association with the outer layer cells (see below). Immunoreactivity of laminin was first demonstrated by immunohistochemistry using LA5 antiserum. The antibodies for laminin recognise all three subunits of type-1 laminin (9) and therefore do not distinguish individual subunits. When ES cells were allowed to differentiate after the hanging drop, they started to aggregate after two hours. However, basement membrane formation in EBs began on Day 1.

Indirect immunohistochemistry was used to detect basement membrane components including collagen IV, nidogen and perlecan to determine if any other basement membrane formation occurred. After 2 days in culture, immunoreactivities of collagen IV, nidogen and perlecan were extracellular, and their deposition was in the basement membrane between the EB outer and inner layers (Figure 6).

In recent years, the complexity of basement membrane deposition and components collagen IV and laminin has been revealed. The distribution of the two proteins vary in different basement membranes (18-21). In addition, recent studies have demonstrated that the deposition of the basement membrane is disrupted without laminin (22). The diversity of laminin and collagen IV may be important in the development and remodelling process and in providing tissue-specific variability of basement membrane structure and function. Also, interactions between laminin and other components, especially nidogen, play a role in basement membrane assembly (23). However, further research is needed to produce a clearer picture of basement membrane formation and assembly in the adult and during embryo development.

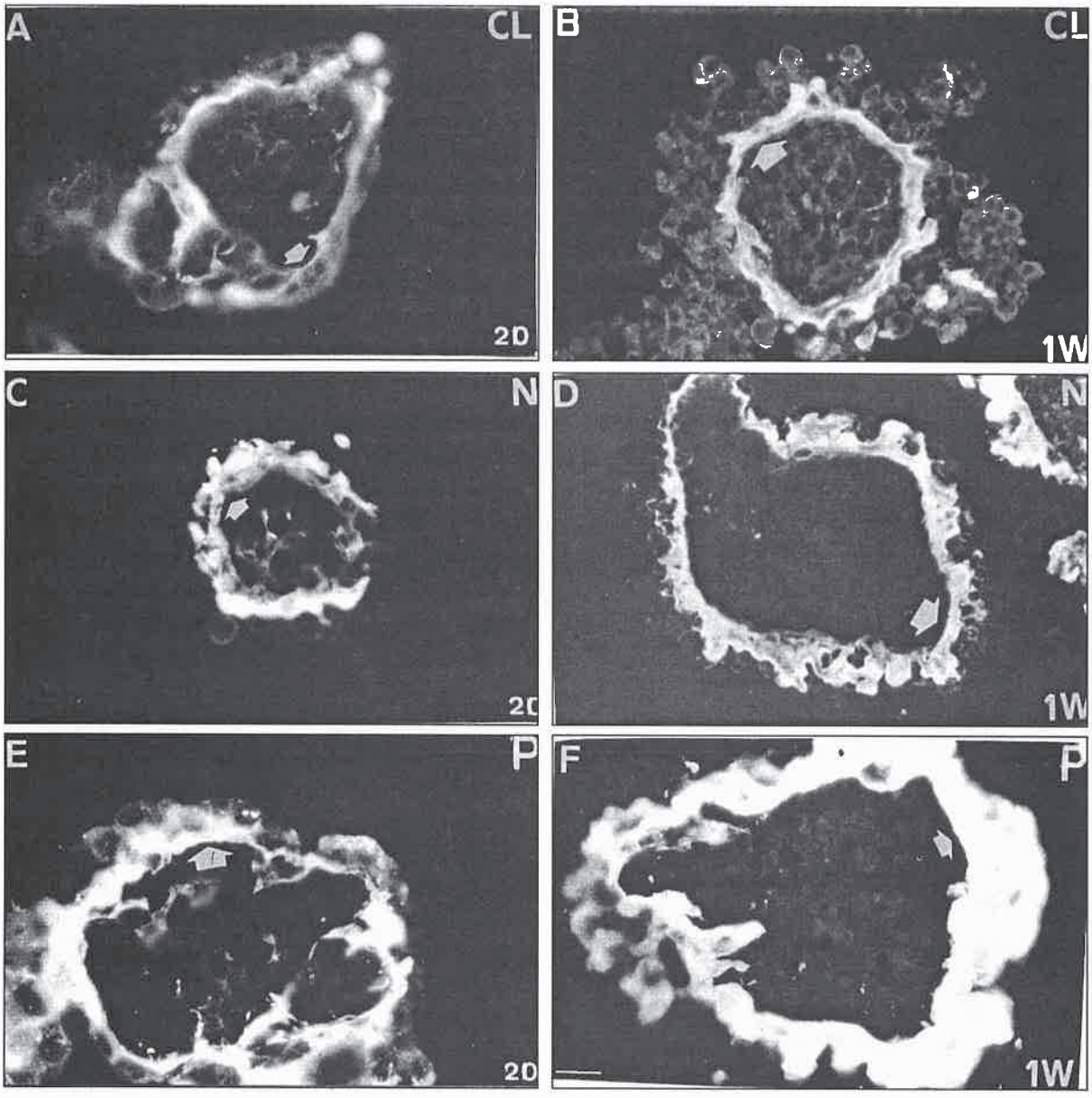

Figure 6: Immunofluorescent detection of collagen IV, nidogen and perlecan in EBs after two days and one week in culture. Immunoreactivity of collagen(CL) IV (a,b), nidogen (N) (c,d) and perlecan (P) (e,f) were seen between outer and inner layers of EBs (arrow heads) at two days (2D) and one week (1W) in culture. (Scale bar= 20 μm)

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