Virchows Archiv A Pathol Anat (1991) 418:509–514

Virchows Archiv A
Pathological Anatomy and Histopathology

Laminin and fibronectin expression during in vivo growth of embryoid bodies derived from teratocarcinoma

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Received October 24, 1990 / Accepted December 19, 1990

Summary. Embryoid bodies (EB) derived from teratocarcinoma OTT6050 are a useful model for the study of early embryogenesis and tumorigenesis and are found in two forms: cystic and simple. After injection of cystic EB intraperitoneally into isogenic 129/Sv mice, their growth and the expression of laminin (LAM) and fibronectin (FN) were studied at 5, 7 and 9 days post-injection. LAM was highly expressed on the internal face of endodermal and on some internal cells in cystic type EB and in both endodermal cell faces in simple EB. Only slight FN expression was observed in cystic and simple EB. Extracellular matrix proteins can be studied in this model.

Key words: Teratocarcinoma – Embryoid bodies – Extracellular matrix – Embryonal carcinoma

Introduction

When teratocarcinoma (TC) tumour cells are injected intraperitoneally, an ascitic form of the tumour is obtained in which structures morphologically similar to young mouse embryos, embryoid bodies (EB) are isolated (Pierce and Dixon 1959; Stevens 1960, 1983; Damjanov et al. 1987). EB have an external endodermal layer surrounding embryonic carcinoma (EC) cells (Martin 1980). There are two EB types: cystic, which are similar to 3-day mouse embryos; and simple, which are morphologically similar to 5-day mouse embryos (Martin 1978).

TC are very useful for studying the early stages of embryogenesis (Martin 1980; Hogan et al. 1983; Leivo and Wartiovaara 1989; Lehtonen et al. 1989). Several authors have studied various aspects of extracellular matrix (ECM) proteins using EB or cellular lines derived from TC. Wartiovaara et al. (1978) observed fibronectin (FN) in EB derived from EC cells (OC15 S1 cell line) and Wolfe et al. (1979) reported its presence in TC cells. Laminin (LAM) and collagen IV were found in PYS-1 cells (Leivo and Wartiovaara 1989) and the same proteins were detected in F9 cells by Adamson and Grover (1983). Grabel and Watts (1987) observed the contribution of these proteins in the growth of PSA-1-derived EB. All these studies were carried out in vitro but little is known of ECM protein expression when EB grow in vivo.

The study of the behaviour of EC cells in vivo is more complex than in vitro, as it is difficult to control the evolution of these cells, and also because of the interactions established with the host animal. It has been shown that EC cells injected into different strains of mice can develop tumours, and their capacity for growth and differentiation is regulated by a set of genetic and epigenetic interactions between the injected cells and the host (Damjanov et al. 1983, 1987). Obviously, this kind of interaction does not occur in a system in vitro. Thus the aim of this paper was to show whether cystic EB become simple EB when injected intraperitoneally into isogenic mice and, if so, how EC cells grow inside the EB. Similarly, FN and LAM expression during in vivo growth was studied by immunofluorescence and immunoperoxidase (PAP).

Materials and methods

OTT6050 tumour was derived from a 6-day old embryo of a strain 129/Sv mouse grafted to a histocompatible host testis. It was converted to the ascitic form and maintained intraperitoneally by serial transfers of ascitic fluid which contained a large number of EB (Stevens 1970). The EB derived from TC OTT6050 and syngenic strain 129/Sv mice were kindly supplied by Prof. Jacob of the Pasteur Institute (Paris). EB have been maintained in our laboratory by intraperitoneal passage into isogenic 129/Sv mice twice a month since 1984.

EB were obtained from ascitic fluid of isogenic 129/Sv mice. After several washings in Dulbecco's modification of Eagle's medium (DMEM), they were centrifuged at 1000 rpm at 4° C for 15 min in a discontinuous Ficoll gradient at 6–10% in phosphate-buffered saline (PBS).

Cystic EB were isolated in the 6–10% interface; these EB were washed several times in DMEM and 10^4 EB in 0.5 ml PBS was injected intraperitoneally into isogenic 129/Sv mice. Mice were killed from day 5 to day 30 (D5–D30) post-injection. The EB obtained were counted and studied by light microscopy and immunohistochemistry. As mentioned in the Results, the most important

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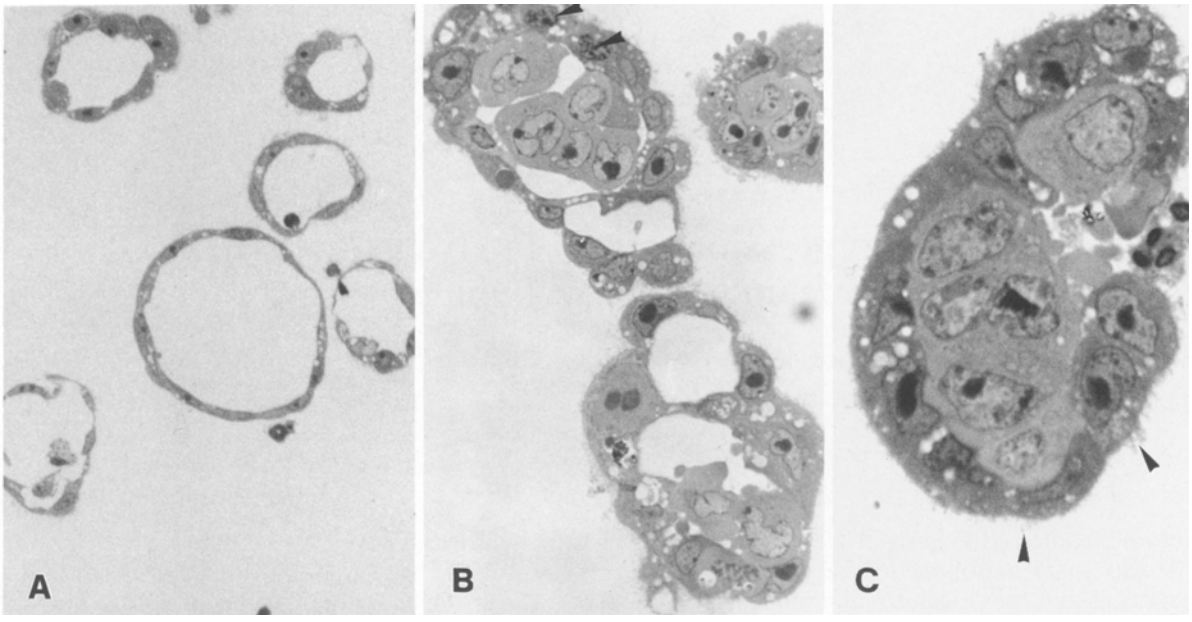

Fig. 1A–C. Embryoid body morphology. **A** Appearance of cystic embryoid bodies (EB) obtained from Ficoll gradients. They are formed by a layer of endodermic cells which surrounds a small number of embryonal carcinoma cells. **B** EB 5 days (D5) postintraperitoneal injection of cystic EB. The elongations from endodermic cells to the cavity of EB, and cells with a granulocytic aspect, can be seen (*arrows*). **C** EB 7 days (D7) post intraperitoneal injection of cystic EB. They are formed by endodermic layer cells with microvilli (*arrows*) and a large number of carcinoma cells. **A–C** Approximately ×1000

events occurred from D5 to D9 post-injection and this period was closely monitored. For light microscopy cystic EB and those obtained from D5 to D9 were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 for 2 h at room temperature, washed several times in PBS, dehydrated in a graded series of ethanol. EB were then embedded in Araldite, cut at 1 μm in an ultracutmicrotome and observed by optic microscope (Olympus AH-2). Immunofluorescence in EB was examined in cystic forms and those obtained from D5 to D10 post-injection. They were washed with 1:1 DMEM – PBS, pH 7.6; fixed in 3.5% paraformaldehyde – PBS for 45 min at 4° C and then washed and soaked with 0.5% Nonidet P-40 PBS for 30 min at 4° C. After several washings in PBS, EB were incubated in 1:50 PBS, 1% bovine serum albumin (BSA) rabbit anti-fibronectin (Biogenex Lab Dublin, CA, USA) or 1:200 PBS 1% BSA rabbit anti-laminin (Gibco Pasley, UK) at 4° C overnight. After this period they were washed in PBS and incubated in 1:40 PBS swine anti-rabbit Ig-FITC (Dako Glostrup, Denmark) for 2 h at 4° C, washed in PBS, mounted in Citifluor (Agar Essex, UK) and observed by Leitz fluorescence microscope.

For immunocytochemistry in paraffin-embedded material, cystic EB and those obtained from D5 to D10 post intraperitoneal injection were fixed by the Guy Saint-Marie method (1962), dehydrated in alcohol and xylene, embedded in paraffin at 54° C for 2 h and sectioned at 5 μm in a Reichert-Jung microtome. The slides were incubated with the same antisera and for the times described for immunofluorescence.

For the PAP technique 1:40 Tris-buffered saline (TBS), 1% BSA goat anti-rabbit IgG (Dako) was used as a second antibody for 1 h at room temperature and 1:100 TBS 1% BSA – PAP (Dako) for 1 h at room temperature as a third. After three TBS washings, diaminobenzidine was added to develop the slides. As a negative control fresh and paraffin-embedded EB were incubated with normal rabbit serum and swine anti-rabbit FITC or goat anti-rabbit IgG plus PAP.

Results

Cystic EB obtained in a discontinuous Ficoll gradient have an external endodermal cell layer with a central nucleus and some vacuolization in their cytoplasm. This

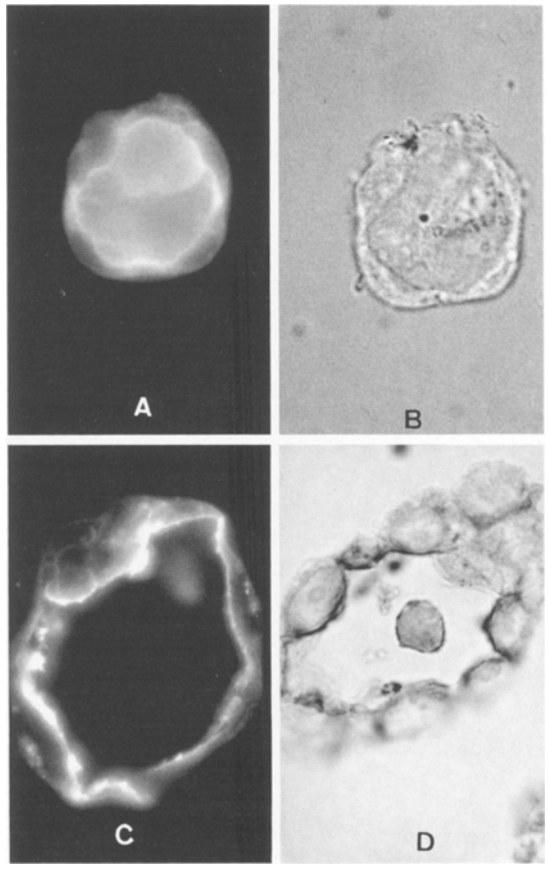

Fig. 2A–D. Immunofluorescence and immunoperoxidase staining for laminin in cystic EB. **A** Fresh and permeabilized cystic EB stained with anti-laminin antibodies. **B** Phase contrast of **A**. **C** Section of cystic in paraffin-embedded EB: fluorescence is seen largely on the basal face of the endodermal cells. **D** Immunoperoxidase staining shows that some internal cells are also positive for laminin. **A, B** ×350; **C, D** ×600

layer surrounds a cavity which contains some EC cells (Fig. 1A). EB obtained 5 days post cystic EB injection had increased in size and most of the endodermal cells presented some elongations within the EB cavity and surrounded an increasing number of EC cells. Cells with a granulocytic appearance were observed very occasionally next to endodermic cells (Fig. 2B). Seven days post cystic EB injection, the initial morphology of D5 EB had changed with more EC cells surrounded by endodermic cells. These had a large number of microvilli and a basal nucleus (Fig. 1C) and acquired some degree of polarization. Nine days post-injection morphologically different EB types were observed.

The number of EB obtained on D5 was lower than the number of EB injected and their morphology was

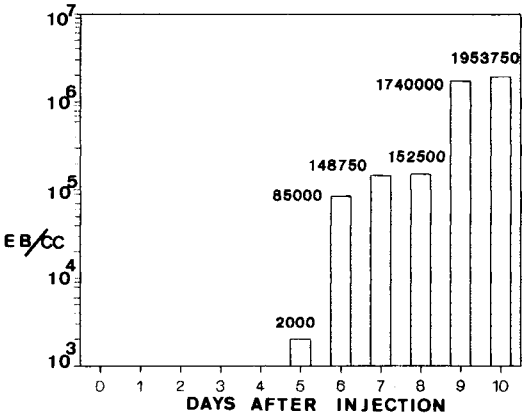

Fig. 3. EB growth after cystic EB intraperitoneal injection

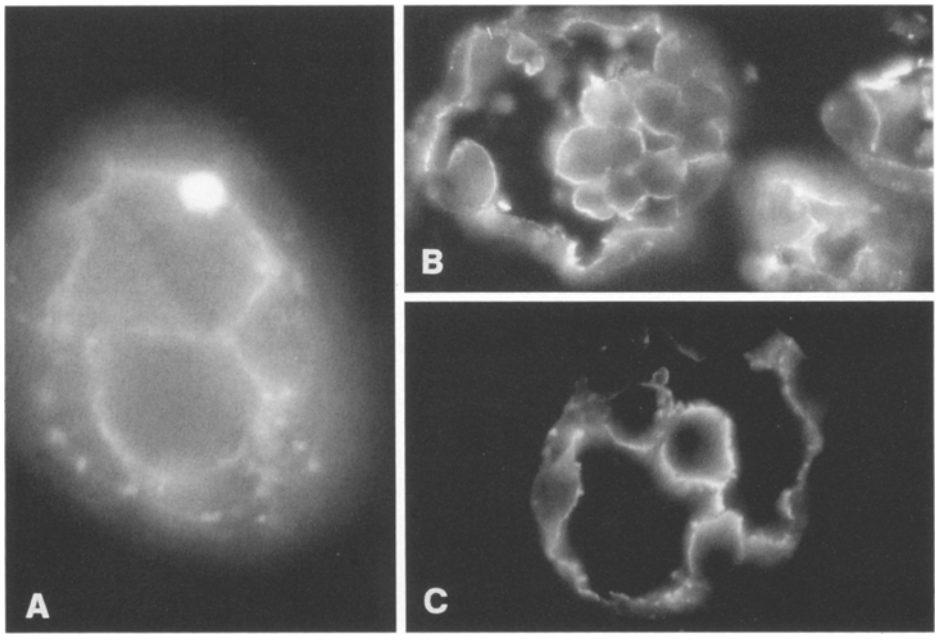

Fig. 4A–C. Immunofluorescence staining for laminin in D5 EB. **A** Immunofluorescence localization of laminin in fresh and permeabilized EB. **B, C** Laminin localization in paraffin-embedded EB sections. **A** ×850, **B, C** ×600

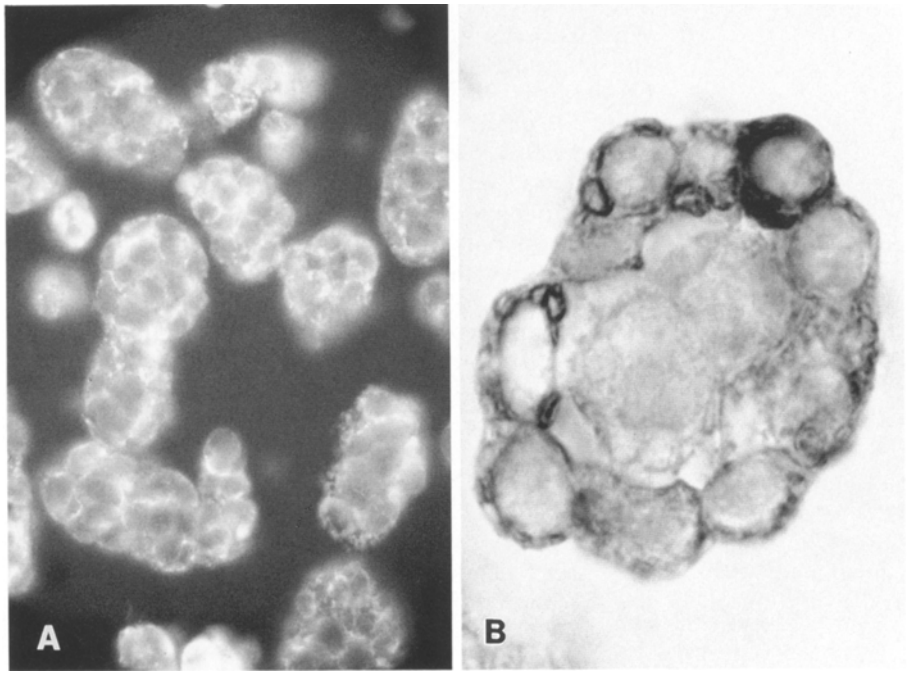

Fig. 5A, B. Immunofluorescence and immunoperoxidase staining for laminin in D7 EB. **A** Paraffin-embedded EB section in which immunofluorescence is seen in both faces of endodermal cells and in some internal EB cells. **B** Immunoperoxidase staining shows laminin distribution in endodermal cells. **A** ×300; **B** ×850

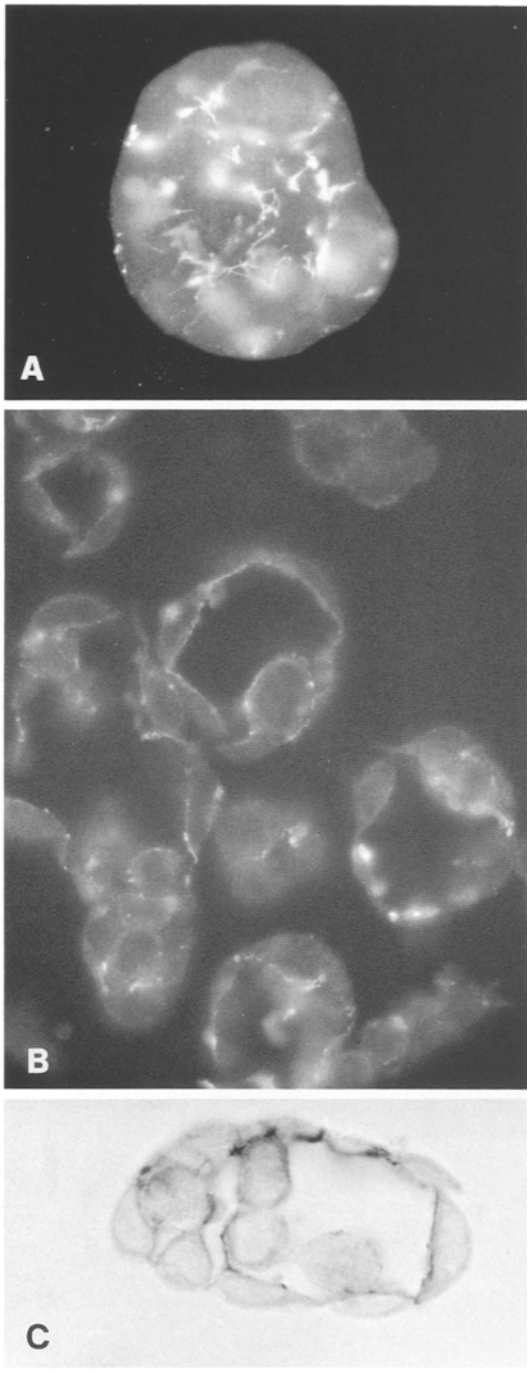

Fig. 6A–C. Immunofluorescence and immunoperoxidase staining for fibronectin in cystic EB. **A** Immunofluorescence in fresh and permeabilized cystic EB. **B** Immunofluorescence sections of cystic EB and **C** stained by immunoperoxidase. **A–C**, ×350

similar to those shown in Fig. 2B. From D6 onwards, the number of EB rose progressively, as seen in Fig. 3.

Careful examination of the whole abdominal cavity did not reveal the macroscopic existence of tumours on D5, although from D7 onwards, small nodules implanted in parietal and visceral peritoneum were observed.

Immunofluorescence in cystic EB incubated with anti-laminin serum presented a uniform distribution of this protein which delimited the whole EB cavity and some internal cells (Fig. 3A). Immunofluorescence and immunoperoxidase in paraffin-embedded EB allowed us to observe that the LAM was not only on the basal face of the endodermal cells which surrounded the EB cavity (Fig. 3C) but also in some internal cells (Fig. 3D).

As stated previously, EB obtained 5 days post-injection had more EC cells and endodermal elongations. With the laminin antisera we observed, in both fresh and permeabilized D5 EB, that this protein delimited the internal EB cavities (Fig. 4A). Moreover, by immunofluorescence and immunoperoxidase techniques we observed LAM in the basal endodermal cell face and also in some internal EB cells (Fig. 4B). We wish to point out that the internal endodermal EB elongations were stained with laminin antisera (Fig. 4C). EB on day 7 had an external endodermal layer which surrounded many EC cells. Immunofluorescence and immunoperoxidase showed LAM to be present in both faces of the endodermal cells and in some internal EB cells (Fig. 5A, B).

Immunofluorescence in cystic EB (Fig. 6A) and D5 EB (Fig. 6B, C) in paraffin-embedded EB showed an FN distribution which was more irregular than in LAM, but some cells which delimited the internal EB cavity presented a slight reaction to FN, as shown in Fig. 6B and C.

In the 7 days following cystic EB injection, immunoperoxidase and immunofluorescence showed FN to be present on the basal face of the endodermal cells which surrounded the EC cells and more slightly in scattered areas inside the EB (Fig. 7A).

Nine days after cystic EB injection the EB obtained were heterogeneous. Most were in the process of dividing and their morphology on immunofluorescence and immunoperoxidase was similar to that of D7 EB. Negative controls are shown in Fig. 7B.

Discussion

When EC cells grow in a suspension system, some generate simple EB which form cavities and become cystic EB (Martin et al. 1977). These EB have been used to study EB growth in vitro. In our study TC OTT6050-derived EB were used and the cystic form was isolated. When cystic EB were injected intraperitoneally, they grew as follows.

Five days post-injection EB had increased in size owing to the growth of EC cells and the endodermal cells presented some elongations within the EB cavity. Cells with a granulocytic appearance occasionally observed next to the endoderm in these EB could be differentiations of the endoderm itself, as it has been shown in vitro that EC cells give rise to endoderm cells and these can differentiate into various types of haemopoietic cells (Cudennec and Nicolas 1977; Cudennec and Johnson 1981).

Seven days post-injection, the EB had increased in size either by endodermal or EC cell division. Nine days post-injection different EB types were obtained, the ma-

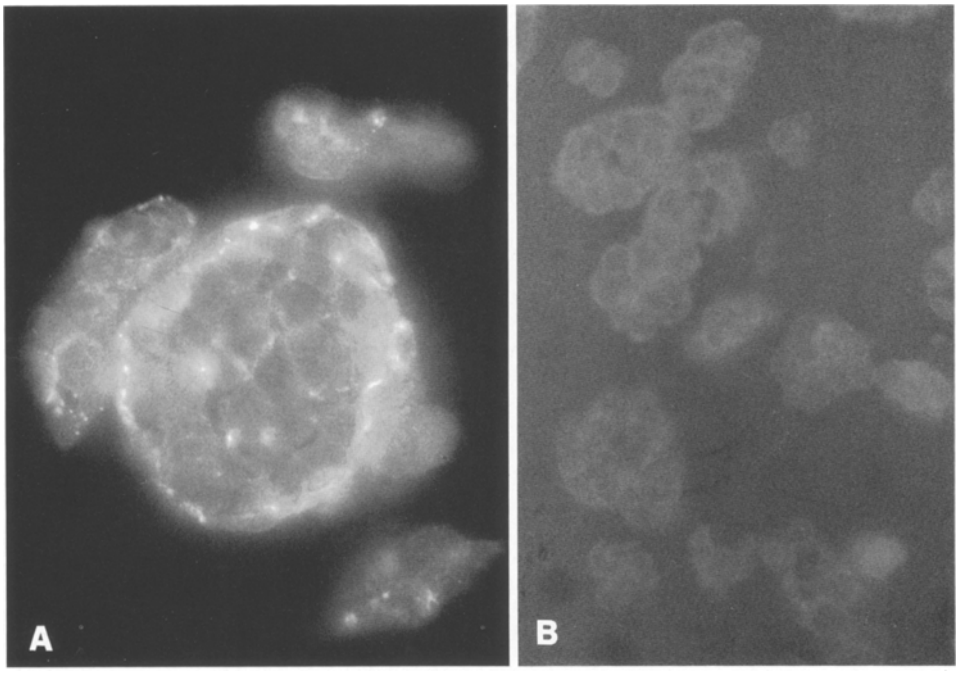

Fig. 7A, B. Immunofluorescence staining for fibronectin in D7 EB and negative control. **A** Immunofluorescence section of D7 EB. Fibronectin is located between endodermal and embryonal carcinoma cells. **B** Negative control in a section of a heterogeneous population of EB. **A**, ×650; **B** ×300

jority in the process of division, which might account for the large number of EB obtained at this stage. Our observations suggest that cystic EB grow in vivo in a synchronous manner for 7 days. At this time, endodermal cells with elongations within the EB cavity are assisting EC cell growth. Finally, when EB have increased their size and cell number, they start to divide, thereby generating different EB types. An alternative pathway for the formation of EB in vivo could be that many EB were simply disrupted by the procedure of intraperitoneal injection and single cells or small clusters thereof restart to differentiate and thus may form this variety of EB stages. We consider this improbable because on examination of the peritoneal cavity a few hours after injection, cystic EB were seen to conserve their morphology intact. However, the fall in the number of EB obtained at D5 suggested that some had attached to the peritoneum, because small nodules were observed from D7 onwards. Moreover, some cystic EB were formed exclusively by endodermic vesicles and these were unable to grow.

LAM is a component of the basal lamina. It is detected in early embryogenesis in 2–4 cell stage embryos (Dziadek and Timpl 1985; Martin and Timpl 1987) in compacted morulas, in extra-embryonic post-implantation membranes (Leivo et al. 1980) and in new-generation basal membranes (Wartiovaara et al. 1979; Wu et al. 1983). The protein has been observed in some cell lines derived from TC; in F9 endodermal differentiated cells induced by retinoic acid or cAMP (Wartiovaara et al. 1978; Hogan et al. 1983; Dziadek and Timpl 1985; Grover and Adamson 1985) and in PYS-2 cells, which resemble embryonal parietal endoderm (Leivo 1983; Hogan et al. 1983). By electron microscopy LAM has been located in the cellular face (lamina rara) of the epidermal cell basal membrane (Osawa 1986).

We observed LAM distributed along the basal face of the endodermal cells which surround the EB cavity in cystic EB. This finding suggests that the basal membrane is organized in this EB type. When EB grew, their endodermal cells presented some elongations within the EB cavity which were heavily positive to LAM. This protein may promote EC cell adhesion in the EB cavity, as has been demonstrated in different systems by several authors (Yamada 1983; Martin and Timpl 1987; Letourneau et al. 1988). Moreover F9 and PC13 cells adhere rapidly in an LAM or FN coated substrate, (Adamson and Grover 1983; Tienari et al. 1989). When EB acquired their simple form, wide LAM distribution was observed. This protein was also found on the external face of endodermal cells, a different pattern from that observed in cystic-EB type. This finding suggests that LAM might be excreted; in fact, EB and the ascitic fluid in which they grow are an important source of LAM (Ozawa et al. 1983), which contributes to basal membrane formation and may permit EB adhesion in host tissues. In vitro, the addition of LAM to F9 cell cultures generates and maintains the basal membrane (Grover and Adamson 1985; Tienari et al. 1989). Moreover, EC cells have LAM receptors all over their cell surface, but normal cells only have these receptors on the basal face in contact with the basal membrane (Liotta 1986). Slight FN expression located in some basal face endodermal cells was observed in cystic EB. However, in simple EB, FN was found in the space between endoderm and internal EB cells, which suggests that FN is expressed when endoderm is well-defined, as observed in early embryonic stages (Wartiovaara et al. 1979) or during endodermal induction of F9 cells in vitro (Wartiovaara et al. 1978; Wolfe et al. 1979; Rizzino 1983). The high expression of LAM in endodermal cell may stimulate the growth of EC cells inside the EB cavity. Indeed, it has been

shown in vitro that the addition of LAM stimulates the growth of EC cells (Adamson and Grover 1983).

In conclusion, it may be assumed that cystic EB in vivo grow and become simple and when they divide form a heterogeneous population. During this process LAM is expressed more intensively and may promote adhesion and EC cell division in EB.

The study of EB derived from TC OTT6050 growth in vivo constitutes a useful model for ascertaining the role of extracellular matrix protein during early embryogenesis and in tumour growth.

Acknowledgments. We would like to thank Christine O'Hara for her kind assistance in preparing the English version of this paper. This study was supported by DGCYT PB87-0163.

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