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Short Contribution

Co-expression of laminin and a 67 kDa laminin-binding protein in teratocarcinoma embryoid bodies

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ABSTRACT The synthesis of laminin chains is usually correlated to specific functions of laminin during embryo development. In this study we show that the CE44 teratocarcinoma embryoid bodies synthesize B1 and B2 chains of laminin as well as a 67 kDa laminin-binding protein while simultaneously differentiating into parietal endoderm. The intracystic presence of laminin and the 67 kDa cell surface laminin-receptor in teratocarcinoma differentiated cells suggest that the B chains of laminin play an important role in induction and/or mediation of cell differentiation and confirm the importance of laminin A chain in cell polarization and the supramolecular rearrangement of definitive basement membrane.

KEY WORDS: *teratocarcinoma, embryoid bodies, laminin, 67 kDa laminin-binding protein*

The extracellular matrix is generally considered to be one of the factors involved in the control and in the maintenance of the differentiated phenotype. Laminin is a basement membrane glycoprotein involved in a numerous biological functions affecting different cell types during embryo development, mainly in cell differentiation processes, cell migration (Dziadek and Timpl, 1985; Grabel and Watts, 1987) and morphogenesis (Ekblom *et al.*, 1990; Klein *et al.*, 1990; El Ouali *et al.*, 1991). The laminin chains (A, B1 and B2) are transcribed from independent genes during early stages of embryo development in mammals (Cooper *et al.*, 1983; Martin and Timpl, 1987; Glukhova *et al.*, 1993) and the whole molecule is synthesized at the morula compaction stage (Hogan *et al.*, 1987). During organogenesis, the whole structure of this molecule depends on both the type of organ and its stage of development (Laurie *et al.*, 1989; Kücherer-Ehret *et al.*, 1990; Simo *et al.*, 1991).

Different laminin cell receptors, related or not to the family of integrins have been shown to mediate the effects of laminin (reviewed by Mecham, 1991; Engel, 1992; Mercurio and Shaw, 1992). Laminin and the 67 kDa laminin-binding protein, a non-integrin membrane protein, are expressed in tumor cells and play an important role in tumor cell adhesion and lung metastasis (Malinoff and Wicha, 1983; Terranova *et al.*, 1983; Wever *et al.*, 1987; Aliño *et al.*, 1989, 1990; Shi *et al.*, 1993). This 67 kDa cell-surface laminin-receptor has also been observed in normal cells with a low degree of differentiation (Lesot *et al.*, 1983) as well as during epithelial-mesenchymal interactions, mainly in cell adhesion and polarization phenomenon (Laurie *et al.*, 1989; Clement *et al.*, 1990). Recent work has demonstrated the existence of a variety of laminin molecule isoforms, which in part explains the diverse functions of basement membranes in different tissues (Tryggvason, 1993).

One of the major questions concerning laminin and its laminin-binding proteins is how these interactions mediate functions in different types of cells, from the earliest stages of development. Teratocarcinoma stem cells prove to be an excellent model system for studying the histogenesis of these tumors, the nature of their undifferentiated stem cells (recently reviewed by Damjanov, 1993) and certain events of early mouse embryogenesis (Martin, 1978; Hogan *et al.*, 1983; Grabel *et al.*, 1987). In this work, we use the CE44 teratocarcinoma embryoid bodies as an experimental model of embryo development, and characterize the cell structures of this tumor in relation to cell differentiation using electron microscopy. Moreover, we analyze the expression of laminin and 67 kDa laminin-binding protein in CE44 embryoid bodies by immunofluorescence and immunoblotting. Finally, the relation between the presence of these molecules and the degree of cell differentiation in the embryoid bodies containing endodermal differentiated and non-differentiated embryonal carcinoma cells is discussed.

The microscopic study of CE44 teratocarcinoma showed that the CE44 embryoid bodies from peritoneal cavity of 129 Sv mouse are heterogeneous with cell populations of different size, morphology and cell differentiation stage (Fig. 1A). At the ultrastructural level, the embryoid bodies are shaped by an external layer of

Abbreviations used in this paper: PBS, phosphate buffered saline solution; BSA, bovine serum albumin; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate; SDS, sodium dodecyl sulfate (lauryl sulfate); SSEA-1, stage specific embryonic antigen.

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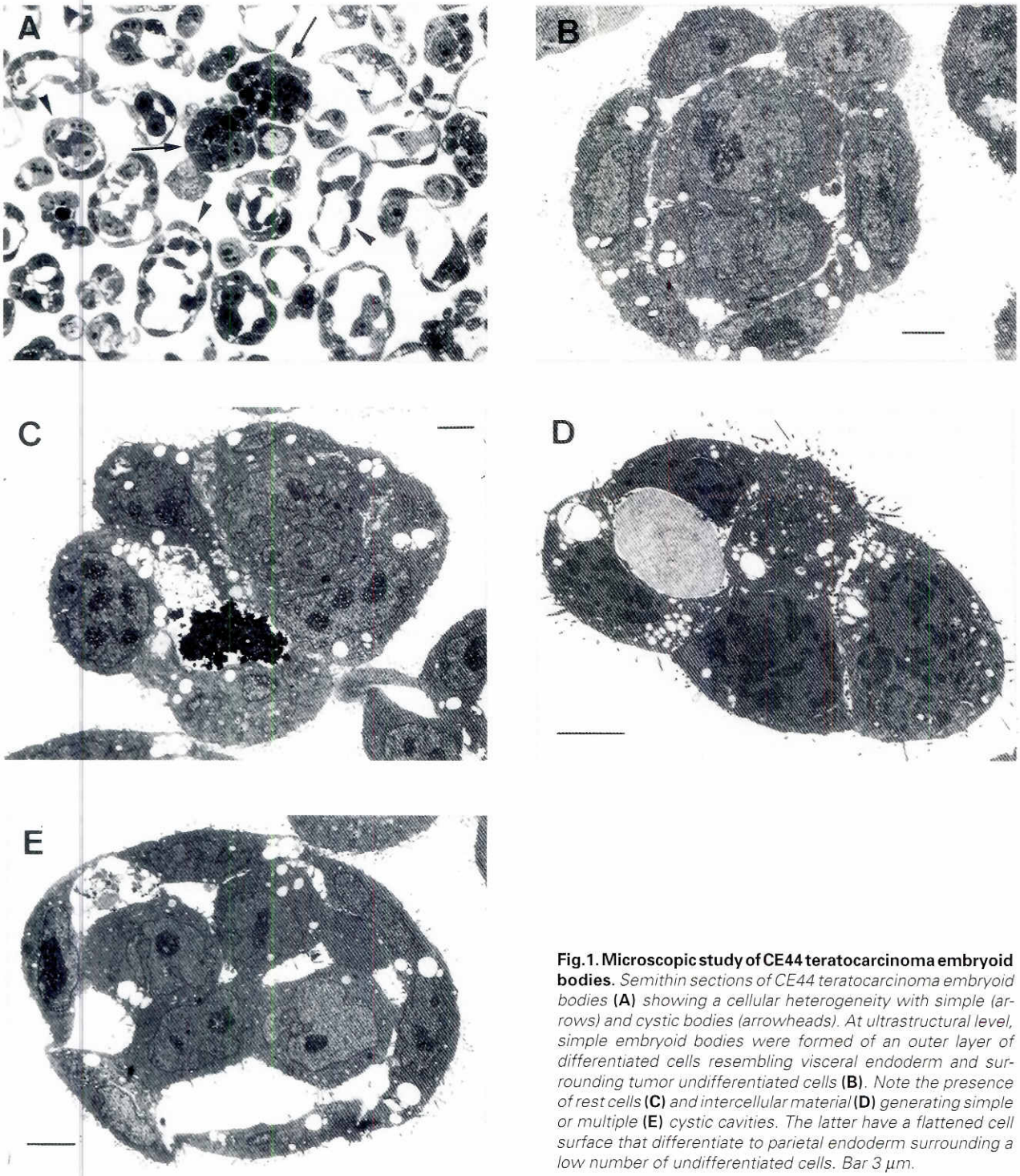

Fig. 1. Microscopic study of CE44 teratocarcinoma embryoid bodies. *Semithin sections of CE44 teratocarcinoma embryoid bodies* **(A)** *showing a cellular heterogeneity with simple (arrows) and cystic bodies (arrowheads). At ultrastructural level, simple embryoid bodies were formed of an outer layer of differentiated cells resembling visceral endoderm and surrounding tumor undifferentiated cells* **(B)**. *Note the presence of rest cells* **(C)** *and intercellular material* **(D)** *generating simple or multiple* **(E)** *cystic cavities. The latter have a flattened cell surface that differentiate to parietal endoderm surrounding a low number of undifferentiated cells. Bar 3 μm.*

endodermic cells, similar to primitive or visceral endoderm, surrounding a variable number of undifferentiated embryonal carcinoma cells. These structures are usually named simple bodies (Fig. 1B). Also cell rearrangement, cell death (Fig. 1C) and extracellular fluid (Fig. 1D) are frequently observed in numerous cellular structures and induce the formation of embryoid bodies having one (Fig. 1C-D) or several (Fig. 1E) cystic cavities. The latter consist of an outer cellular layer of flattened cells resembling differentiated parietal endoderm.

The immunofluorescence analysis for laminin protein showed that cystic cavities were often full of laminin, both in CE44 embryoid bodies containing a low cell number (Fig. 2A) and large embryoid

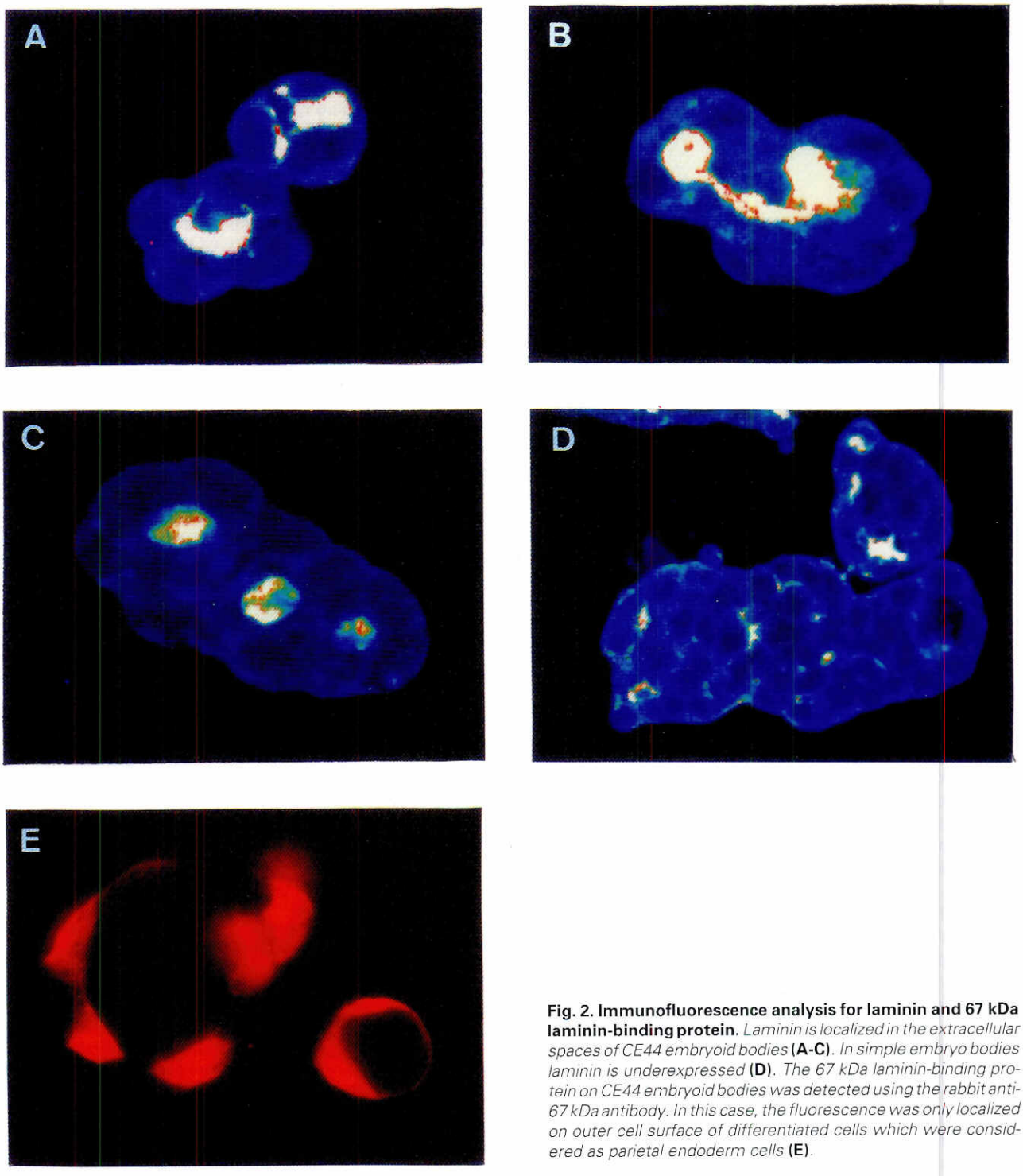

Fig. 2. Immunofluorescence analysis for laminin and 67 kDa laminin-binding protein. *Laminin is localized in the extracellular spaces of CE44 embryoid bodies* **(A-C)**. *In simple embryo bodies laminin is underexpressed* **(D)**. *The 67 kDa laminin-binding protein on CE44 embryoid bodies was detected using the rabbit anti-67 kDa antibody. In this case, the fluorescence was only localized on outer cell surface of differentiated cells which were considered as parietal endoderm cells* **(E)**.

bodies (Fig. 2B-C). The presence of laminin in simple bodies was scarce and only observed in intercellular spaces (Fig. 2D). Immunofluorescence for the 67 kDa laminin-binding protein showed that this cell-membrane protein only appeared on the surface of outer flattened cells of embryoid bodies (Fig. 2E) which were considered as parietal endoderm differentiated cells. Moreover, we observed the absence of fluorescence in simple embryoid bodies composed of undifferentiated or visceral endoderm differentiated cells (data not shown).

The results obtained with CE44 teratocarcinoma proteins by Western-blotting (Fig. 3) showed a 200 kDa band corresponding to B1 and B2 glycosilic chains of laminin and a light 400 kDa band

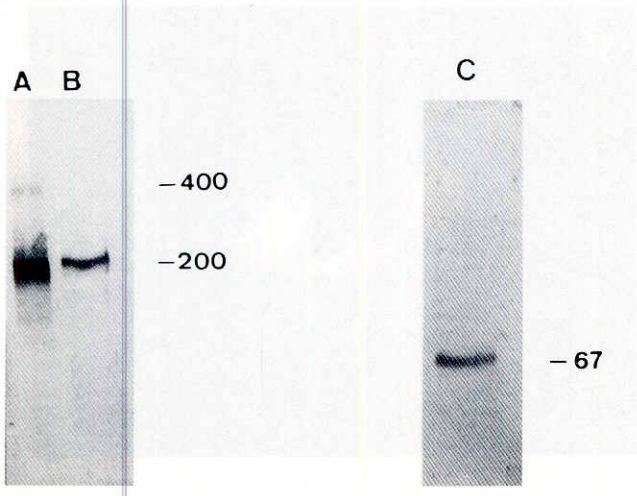

Fig. 3. Immunoblotting of proteins from CE44 teratocarcinoma embryoid bodies. Lane A *represents the CE44 proteins labeled for laminin chain detection.* **Lane B** *shows the protein sample previously reduced with mercaptoethanol.* **Lane C** *represents the proteins labeled for 67 kDa laminin-binding protein.*

which may be considered as B chain dimers (lane A) since the 400 kDa band disappeared when protein samples were treated with a reducing agent such as mercaptoethanol (lane B). The 67 kDa laminin-binding protein was clearly detected in proteins from CE44 teratocarcinoma (lane C).

The heterogeneity in morphology and evolution of CE44 teratocarcinoma embryoid bodies growing in 129 Sv mouse ascitic liquid (Parchment *et al.*, 1990) has important advantages as an experimental model to study the expression of different cell markers in relation to cell differentiation. In previous studies the identification of the cell type in embryoid bodies from teratocarcinoma stem cells has been based upon the morphology of the cells as well as their staining pattern by indirect immunofluorescence for a number of markers. For instance, SSEA-1 (stage specific embryonic antigen) is present on stem cells (Solter and Knowles, 1978), laminin is synthesized by parietal endoderm (Howe and Solter, 1980; Leivo *et al.*, 1980; Cooper *et al.*, 1983) but does not synthesize fibronectin (Wolfe *et al.*,1979), and alpha-fetoprotein is specific for visceral endoderm cells (Adamson *et al.*, 1977).

Previous work has shown the presence of extracellular matrix components in CE44 teratocarcinoma (Monzo *et al.*, 1991). Our immunofluorescence analysis results on CE44 embryoid bodies showing laminin deposits are consistent with the identification of the outer cells of cystic bodies such as parietal endoderm. It has been suggested that cell interactions with matrix molecules as a substrate may play a role in directing the differentiation to parietal endoderm from primitive endoderm (Gardner, 1983). More recent data have showed that these interactions are mediated by matrix protein receptors such as 140 kDa fibronectin receptor (Grabel and Watts,1987). Our finding of 67 kDa laminin-binding protein on cells resembling parietal endoderm suggests that both matrix extracellular molecules and their receptors may be involved in cell migration and differentiation during early embryogenesis. However, further experiments may be performed in order to confirm this possibility.

The absence of laminin A chain synthesis has been observed in several cell types associated with the inability to form the basement membrane. This absence or its presence in late stages during embryo development, suggests that laminin A chain plays an important role in the incorporation of laminin B chains into the basement membrane (Kücherer-Ehret *et al.*, 1990; Simo *et al.*, 1991). It may also be very important during embryo development, mainly in the formation of specialized extracellular microenvironments (Ekblom *et al.*, 1990; Klein *et al.*, 1990; Simo *et al.*, 1991). Since it has been suggested that laminin A chain may play an important role in cell polarization (Ecay and Valentich, 1992), we hypothesize the absence of polarization in cells surrounding cystic cavities from CE44 teratocarcinoma is due to the absence of laminin A chain synthesis. The synthesis of B1 and B2 chains in embryo development (Cooper and MacQueen, 1983) may be related to cell activation and differentiation (Dziadek *et al.*, 1985). Moreover, B1 chain and its related molecules are important in joint and exocytosis of laminin (Chung, 1993).

The 67 kDa laminin-binding protein was detected only in cells forming the outer side of cystic bodies. The morphological characteristics define these cells as parietal endoderm cells. This laminin receptor has the ability to bind domain III of laminin B1 chain (Beck *et al.*, 1990) and we show that this chain is expressed in CE44 embryoid bodies. The 67 kDa laminin-receptor was also described in several teratocarcinomas that evolve to pluridifferentiated cell aggregates (Wu *et al.*, 1991). The absence of significant quantities of laminin in simple embryoid bodies and the presence of the 67 kDa laminin-receptor only in outer cells (parietal endoderm cells) suggest that the expression of these proteins is a time-process dependent on the differentiation of embryoid bodies.

In summary, our results suggest that the 67 kDa laminin-binding protein may be involved in endodermal differentiation. The presence of soluble laminin in cystic cavities of embryoid bodies may be due to the inability of laminin to form basement membrane because of the absence of either laminin molecule A chain or the interaction between laminin and different basement membrane components. It may play a critical role in the rearrangement and association of basement membrane molecules.

Experimental Procedures

Tumor cells

CE44 is a teratocarcinoma tumor line derived from OTT60/50 experimental carcinoma of Stevens (1970). These tumor cells have been maintained intraperitoneally by serial transfers of ascitic fluid in 129 Sv mouse.

Light and electron microscopy.

CE44 cells obtained from 129 Sv mouse ascitic fluid were processed as previously described (Hilario *et al.*, 1991) for electron microscopy. Briefly, after centrifugation (206xg for 5 min) the supernatant was removed and the tumor-cell pellets were washed in pre-warmed PBS (phosphate buffered saline, pH 7.4) and fixed with 2.5% glutaraldehyde at 4°C for 6 h. The fixative was then removed and replaced with 0.1 M sodium cacodylate/HCl buffer (pH 7.4) containing 5% sucrose and the pellets were washed overnight. The pellets were then postfixed with 1% osmium tetraoxide, dehydrated in ethanol and embedded in EPON 812 (Fluka, Switzerland). Semithin sections were stained with toluidine-blue and observed by means of a visible light microscope, and ultrathin sections were treated with uranyl acetate and lead citrate and then examined in a Philips EM 30 electron microscope.

Indirect immunofluorescence

CE44 embryoid bodies were extended on slides and fixed with cold methanol for 20 min. Then, the slides were washed with PBS and incubated with 1% bovine serum albumin (BSA, Sigma Chem. Co., USA) for 1 h in

order to block non-specific antibody binding. Next, cells were incubated for 90 min with rabbit anti-laminin antibody (diluted 1:100 in PBS), from Chemicon, or rabbit anti-67 kDa laminin-binding protein antibody, kindly supplied by Dr. G. Martin and Dr. Y. Yamada, (diluted 1:50 in PBS). After washings with 0.1% Tween-PBS, we incubated the samples with a goat anti-rabbit IgG-FITC/TRITC conjugate antibody, purchased from Sigma Chem. Co. (diluted 1:100 in PBS) for 90 min. The samples were washed with 0.1% Tween-PBS and mounted using Fluoromount-G (Southern Biothechnology Associates Inc., USA). The samples were observed under flourescent light in a Zeiss Axioskop confocal microscope.

Electrophoresis and immuno-blotting

The samples of CE44 embryoid body proteins were prepared according to the procedures described by Kawata *et al.* (1991). Proteins of embryoid bodies migrated on a 5% or 7.5% SDS-polyacrilamide slab gel to detect laminin or the 67 kDa laminin-binding protein, respectively. Gels were transferred onto nitrocellulose membranes which were incubated with a rabbit anti-laminin antibody (dilution 1:100) from Sigma Chem. Co. or a rabbit anti-67 kDa laminin receptor antibody (dilution 1:100). We used a goat anti-rabbit peroxidase conjugate antibody (dilution 1:1000) as a secondary antibody. The membranes were developed with diaminobenzidine and H_2O_2.

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