MULTIPARAMETRIC ANALYSIS OF CELL DIFFERENTIATION IN TERATOCARCINOMA EMBRYOID BODIES

Manuel GARCIA-SANZ, Fernando UNDA, Rafael ATENCIA, Antonia ALVAREZ, Enrique HILARIO, and Juan ARECHAGA
Departamento de Biología Celular y Ciencias Morfológicas, Facultad de Medicina, Universidad del País Vasco
48940 Leioa, Vizcaya, Spain

Murine teratocarcinoma embryoid bodies are thought to mimic certain events linked to early embryonic development, and these multicellular aggregates are considered good models for studying cellular differentiation processes. The ascitic form of CE44 embryonal carcinoma is formed by a heterogeneous population of cells that morphologically resemble morules and early blastocysts (for review see Martin, 1975., Damjanov, I., 1990). One of the most important features of the embryogenes is the presence of fibronectin, a protein that is expressed during differentiation and morphogenic processes and which has been specifically identified as being responsible for parietal endoderm differentiation and growth (Grabel and Watts, 1987).

Flow cytometry is used to recognize characteristics such as size, granularity and the presence of specific markers among cell populations In the present study we have combined flow cytometry and morphological methods to evaluate the differentiation capacity of embryoid bodies multicellular aggregates. Thus we have quantified fibronectin expression in CE44 teratocarcinoma embryoid bodies cultured in the presence and absence of powerful differentiation inductors such as retinoic acid (RA) and dibutyryl cyclic AMP (dbcAMP)

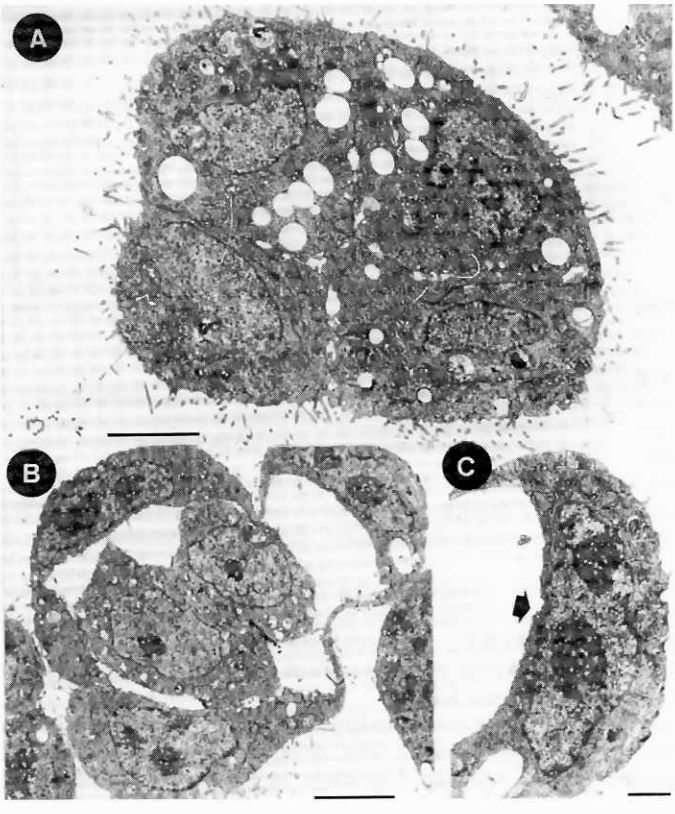

Figure 1. A: simple embryoid body with features of visceral endoderm (Bar 5μM)). B: cystic body of small size showing external flattened cells, resembling parietal endoderm (Bar 5μM). C: magnification of B were flattened cells show an enlarged endoplasmic reticulum (Bar 1μM)

Material & Methods Tumor Cells: CE44 teratocarcinoma embryoid bodies were maintained intraperitoneally by ascitic liquid serial transfers into 129Sv mice. Cell culture and drug treatment: Embryoid bodies were obtained from 129Sv mouse ascitic liquid and cultured in DMEM supplemented with 10% fetal serum. Retinoic acid (RA) and Dibutyryl cAMP (dbcAMP), were added to culture medium for a drug final concentration of 10^{-7}M and $3x10^{-5}$M, respectively. Electron microscopy: CE44 embryoid bodies obtained from 129Sv mouse ascitic liquid were washed with PBS (pH 7.4) and processed as previously described (Hilario et al., 1992). In brief, after centrifugation the tumor-cell were fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate/HCl buffer (pH 7.4) postfixed in 1% osmium tetraoxide in the same buffer, dehydrated and embedded in EPON 812 (Fluka, Switzerland). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips EM 300. Indirect immunofluorescence: E.B were treated for 90 min with antifibronectin antibody developed in rabitt diluted 1:50 in PBS. After three washes the fluorescent secondary antibody, (Goat anti-rabbit IgG FITC conjugated) diluted 1:100 in PBS was added. The samples were observed under fluorescent light microscopy with an Zeiss Axioskop confocal microscope or studied by means of flow cytometry. Flow cytometric analysis was performed by flow cytometry with an EPICS 752 (Coulter, Fl, USA). The indirect immunofluorescence staining with anti-fibronectin antibodies was performed as indicated in the preceding paragraph. The CE44 embryoid bodies were measured simultaneously for three parameters: Forward Angle Light Scatter (FALS), Integrated Side Scatter (ISS) and Logarithm of Integrated Green Fluorescence Light (LIGFL). Usually 10000 embryoid bodies were analyzed per sample using immunoanalysis software from Coulter (Fl, USA). Laser power 250MW, laser wavelength 488, green pmt filter 525BP and flow cell tip exit diameter of 76 μm were used.

Ultrastructural study showed that the simple embryoid bodies were formed by a group of cells resembling embryonal carcinoma cells, which are closely parked and show a few cell junctions, similar to thigh junctions. The cells of the outer surface of the embryoid bodies are flattened and show thin microvilli, profiles of rough endoplasmic reticulum, Golgi complex, refringent vacuoles and phagocytic vacuoles was also observed. These cells resembled primitive or visceral endoderm. These embryoid bodies are called simple embryoid bodies. Some of these were formed by cuboid cells with microvilli and without an outer layer of flattened cells (Fig. 1A).

On the other hand, the cystic embryoid bodies were composed of an outer layer of flattened cells without clear specialized intercellular junctions and scarse microvilli on the cell surface (Fig. 1B). Their most important morphological feature was the distended profiles of endoplasmic reticulum, which frequently contained amorphous material (Fig. 1C). Thus, these cells displayed the basic morphological characteristics of parietal endoderm. Inside the cavity of the cystic bodies, same embryonal carcinoma cells may be seen. The number of these cells decreases when the cystic cavity increase in size.

Cytometric analysis showed that CE44 teratocarcinoma embryoid bodies are formed by two different populations. One of them, corresponding to the simple embryoid bodies, represents 62%-65% of the total embryoid bodies studied; and the other, corresponding to cystic embryoid bodies, represents the 33%-37%. When the embryoid bodies were treated with RA and dbcAMP, we observed a significant increase in the percentage of cystic bodies (46.4% in treated bodies vs 35.5% in controls). Moreover, the average size of treated bodies was larger than in controls (19.6%), and this increase was observed in both simple and cystic bodies (25.5% and 19.4%, respectively).

Table 1. Distribution of C44 Embryoid Bodies with fibronectin expression, cultured alone or in presence of RA and dbcAMP*.

	CONTROL		TREATED	
	Number	%	Number	%
Total E.B. analyzed	30232		30102	
Total fibronectin positive bodies	3929	12.9	12345	41
Simple bodies	19046	63	15353	51
Fibronectin positive bodies	3137	38.3	5046	61.6
Cystic bodies	11189	37	14749	49
Fibronectin positive bodies	791	2.6	7299	59.1

* *Results from three independent experiments.*

Solvent-treated cultured CE44 embryoid bodies showed fibronectin expression in a percentage of 12.9%. However, after treatment with RA and dbcAMP this expression represented the 41%. This rise was basically consequence of an increase in the number of fibronectin-positive cystic bodies, which were increased from 2.6% (in controls) to 59.1% (in treated) of the total number of fibronectin-positive cystic bodies (Table 1).

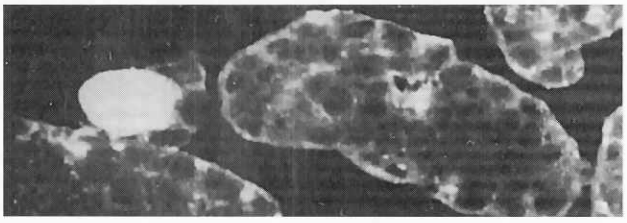

Figure 2 immunofluorescence assays on CE44 E.B. using antifibronectin antibodies

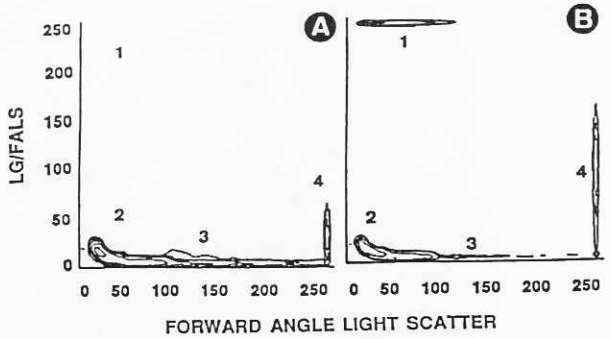

Figure 3 Distribution of embryoid bodies using antifibronectin-FITC conjugate. A: solvent-treated cultured embryoid bodies. B: embryoid bodies cultured in presence of RA and dbcAMP.

Immunohistochemical studies support our cytometric results and reflect the differential distribution pattern of fibronectin. The protein is located, with low-intensity fluorescence, in the intercellular spaces .of. large .simple .bodies, both. before .and after. treatment. However, fibronectin with high-intensity fluorescence is also found inside the cavities of small and medium-sized cystic bodies. (Fig. 2). Relative fluorescence (LG/FALS) and Forward Angle Light Scatter (FALS) showed that RA and dbcAMP induced the appearance of a new cellular population with highly specific fluorescence containing around 20% of the total number of fibronectin-positive embryoid bodies (Figure 3).

Our results are compatible with Dziadek's suggestion (Dziadek and Timpl, 1985) that the differential synthesis of basal membrane molecules provides specific signals for differentiation. In this case, fibronectin synthesis may favor endodermic differentiation and the conformation of cystic cavities in certain embryoid bodies. The absence of fibronectin, even after treatment, in a high number of embryoid bodies, lead us to assume the existence of a population of large, simple embryoid bodies incapable of synthesizing large quantities of this protein and turning to cystic bodies.

The use of multiparametric assays in CE44 teratocarcinoma embryoid bodies makes possible the molecular analysis of the complex events controlling cell differentiation during early mammalian embryogenesis. The isolation of different embryoid bodies subpopulations from heterogeneous samples by cytometric sorting may be important to support the hypothesis suggested above.

Acknowledgements.

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