Correlation Between flow Cytometry and Transmission Electron Microscopy

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Flow cytometry techniques together with morphologic studies were used to perform multiparametric analyses with the aim of studying the dynamic aspects of cell cultures derived from CE44 teratocarcinoma embryoid bodies. The intrinsic cell parameters studied by flow cytometry were size (FALS) and cytoplasmic complexity (ISS). Our results show that CE44 teratocarcinoma yields monolayers whose cells show a marked morphological heterogeneity that can be grouped according to flow cytometric criteria into four populations. These cytometric findings can be correlated with differences in cytoplasmatic traits observed in the electron microscopic study.

Keywords flow cytometry, electron microscopy, integrated studies

1. Introduction

Embryonal carcinoma cell is the distinctive cell type of teratocarcinomas. Moreover, a variety of differentiated cell types representing derivatives of all three embryonic germ layers are usually present. Experimental teratocarcinomas are widely used and several clonal lines with different morphologies and potential have been isolated.

Some teratocarcinomas can grow in ascitic form making up solid (simple) and cystic aggregates of tumor cells which are called embryoid bodies because they are morphologically similar to morulas and early blastocysts. While the outer cells of embryoid bodies express features of endoderm [1-4], the cells located inside embryoid bodies are known as embryonal carcinoma cells because of their close cytologic resemblance to the cells of embryonal carcinoma of human testis [1]. Embryonal carcinoma cells are the multipotent stem cells of the teratocarcinoma, and the neoplastic equivalent of the cells of the inner mass of blastocysts.

Embryoid bodies can be kept *in vitro* by cultivating them in suspension or in monolayers whose cells express a variety of morphologies and differentiation capacities [2, 4, 5]. Consequently, embryoid bodies are a highly dynamic structure that make interesting models for the study of cellular differentiation and epigenetic control of malignancy [6].

Flow cytometry and transmission electron microscopy (TEM) are tools widely used in the study of tumor cells. In the present work, we have taken advantage of the possibilities of multiparametric analysis afforded by flow cytometry in order to study morphological characteristics of the cells such as size (FALS) and cytoplasmic complexity (ISS) and compare them with the observations come from the classic morphological method as TEM.

2. Materials and methods

2.1 Animals

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Isogeneic male and female 129/Sv mice (9 to 10 weeks old) were purchased from Iffa Credo Laboratories (France). Water and food were given ad libitum. The experimental protocols were carried out in accordance with European Union regulations for animal research (EU 86/609).

2.2 Maintenance of Embryoid bodies

In our laboratory CE44 teratocarcinoma has been maintained intraperitoneally by serial transfers of ascitic fluid to 129 Sv mice of either sex, every 21 days (Fig. 1). The hosts were killed upon the subsequent development of markedly distended bellies and the peritoneal cavity immediately washed with DMEM (Dulbecco´s Modification of Eagle's Medium; Sigma Chemical Co., St. Louis, MO). The peritoneal fluid was aspirated and centrifuged at 200 g for 5 min at 4° C. The resulting precipitate was resuspended in phosphate-buffered saline (PBS) and intraperitoneally reinjected in other intact hosts, in a concentration of 10^5 embryoid bodies/100 ml.

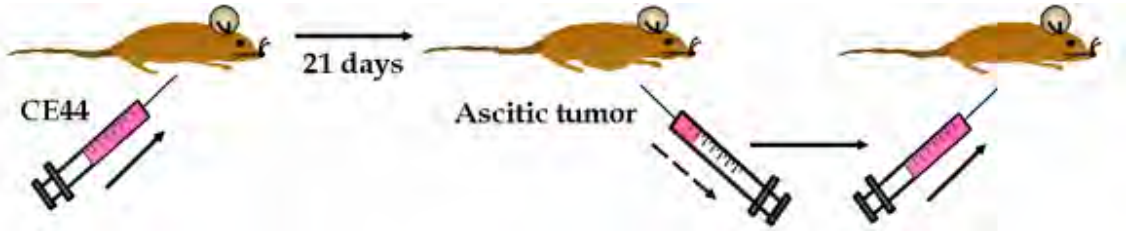

Fig. 1. CE44 teratocarcinoma was maintained intraperitoneally by serial transfers of ascitic fluid to 129 Sv mice of either sex, every 21 days.

2.3 Cell cultures

Mice bearing 21-day-old ascitic tumors were killed and the embryoid bodies obtained as described above. 10^5 embryoid bodies resuspended in 10 ml DMEM were seeded into 75 cm^2 tissue culture-treated flasks (Costar, New York, NY), at 37° C in a humidified 5% CO2-air atmosphere (Fig. 2). All the cultures were maintained in DMEM, heat-inactivated 10% FCS (Gibco, Gran Island, NY), 200 IU/ml penicillin, 200 mg/ml streptomycin and 2.5 µg/ml amphotericin B (Sigma Chemical Co., St. Louis, MO). In general, culture medium was replaced every one or two days. Non-fixed cell cultures were observed over time using an inverted microscope (Labovert FS, Leitz, Germany). After 6 days, monolayers (n=10) were detached with trypsin/EDTA 2 mM. The material obtained was washed with FCS, to inactivate the trypsin, and passed through a 40 µm pore nylon filter (Falcon, Becton Dickinson, San Jose, CA). Following three consecutive washes and centrifugations (200 g) the cells were studied by flow cytometry, light microscopy and transmission electron microscopy.

Fig. 2. Embryonal bodies were obtained from mice bearing 21-day-old ascitic, resuspended in culture medium and seeded into 75 cm2 tissue culture-treated flasks.

2.4 Flow cytometry

For the cytometric study we used an EPICS ELITE Flow Cytometer (Coulter Electronics, Inc., Hialeah, Florida, USA). The readings corresponding to Forward Angle Light Scatter (FALS), Integrated Side Scatter (ISS). Electronic noise and cellular debris were also eliminated from the calculations by ignoring in all measurements the values comprised by the areas of the FALS/ISS biparametric plot delimited by channels between 0 and 5 (resolution of 64 channels). A total of 10,000 cells were recorded for each sample and the data stored in list mode for later analysis. The parameters were represented as biparametric histograms (FALS-ISS).

2.5 Light and transmission electron microscopy

Pellets from monolayers were processed as previously described [7]. Briefly, after a wash in prewarmed PBS, pellets were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate/HCl buffer (pH 7.4) for 2 hours. Next, the pellets were removed from the centrifuge tubes and each one was divided into a number of small pieces. These pieces were then postfixed in 2% osmium tetroxide in the same buffer for 2 hours and subsequently embedded in Epon 812 (Fluka, Switzerland). Semithin sections were stained with Toluidine blue and thin sections stained with lead citrate.

2.6 Statistical analysis

All values have been expressed as the mean ± SE in every experimental group (n = 10). Groups were compared by Student's t-test for unpaired samples, and differences were statistically significant to $p < 0.05$.

3. Results

3.1 Dynamic of monolayers

About 24 hours after being placed in culture, the embryoid bodies attach to the surface of the flask. A nest of cells is produced around the attached embryoid body, becoming easily detectable after some 48 hours. The size of the embryoid body decreases with time, so that after 72 hours have elapsed, it is practically undetectable. By the fourth day of culture, a monolayer has formed, and it is possible to observe clusters of cells that detach like bubbles, forming structures similar to embryoid bodies.

In the nest, cells with either round, epithelioid or fibroblastic morphology are commonly observed. On the periphery of the nest, the cells become more spindle- or star-shaped in appearance, with long cytoplasmic projections with a neuronal-like aspect. Also observable are large round cells. In the center of the colony are small, round refringent cells usually associated with the formation of new embryoid bodies. All these cell types normally remain once confluency is achieved by the cultures.

3.2 Light and electron microscopy

In semithin sections from monolayers (Fig. 3), we observed cells with a loose chromatin nucleus, with one or more nucleoli and clear cytoplasm. The nuclei of other cells had denser chromatin and a cytoplasm of greater density. Numerous intermediate forms were found between the two cell types, and were the most abundant type found. The cells had cytoplasmic vacuoles that were not clearly related to any particular cell type. Figures of mitosis were observed. Over the time of the study, no appreciable differences in monolayers were noted.

Ultrastructurally, the differences between cells from monolayers, in addition to greater or lesser chromatin density, were due fundamentally to variations in their cytoplasmic content. We are

confronting a wide variety of morphology ranging from cells of loose chromatin, evident nucleolus and cytoplasm very scant in organelles, excepting ribosomes, to cells of a more dense chromatin and a great cytoplasmatic content. The existence of many intermediate forms made it not possible, in many cases, to know in which of the cell populations found using flow cytometry a particular cell would be included. In Figure 4 a example that we deem representative of the 4 populations observed in flow cytometry (see below) can be seen. In the cytoplasm of some cells (Fig. 5) there were variable amounts of organelles, residual bodies and, occasionally, vacuoles of different sizes. We were not able to identify morphological details that would enable us to establish a correlation with the cellular forms identified in culture

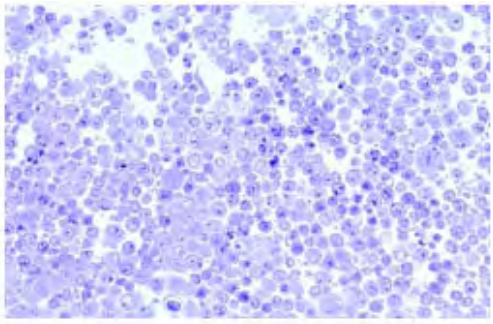

Fig. 3. Semithin section of a 6-day monolayer, in which a great cellular heterogenicity can be observed Stain: Toluidine blue. Original magnification 50x.

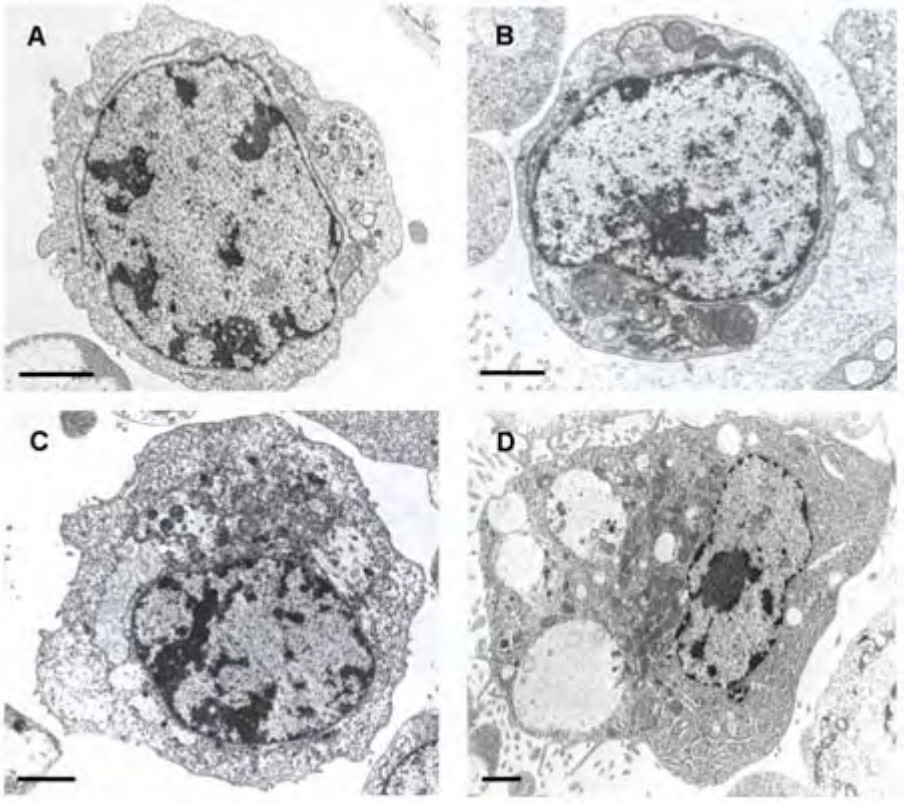

Fig. 4. CE44 teratocarcinoma cells from 6-day monolayers, showing representative microscopic examples of the four populations. A corresponds to population 1, B to population 2, C to population 3, and D to population 4. A shows a cell with loose chromatin, evident nucleolus and cytoplasm consisting preferentially of ribosomes. B shows a cell with more condensed chromatin and a cytoplasm having a large number of structures. B and C show intermediate forms. Bars: A-D: 2 μm.

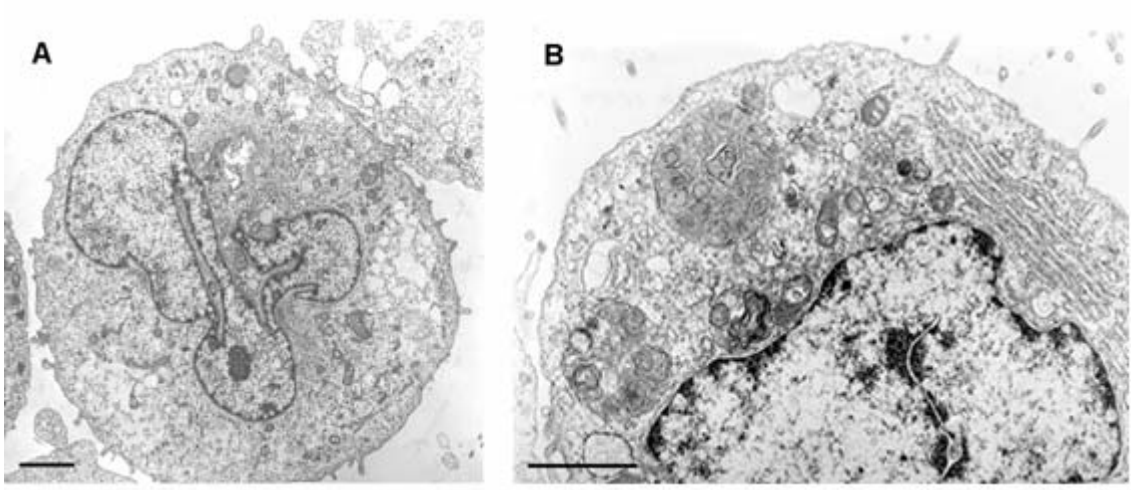

Fig. 5. Ultrastructural aspects of CE44 teratocarcinoma cells from 6-day monolayers. (A) CE44 teratocarcinoma cell with RER and inclusion bodies. In (B) areas of cytoplasmic rarefaction not surrounded by membrane and vacuoles are observed. Bars: A-B:2 µm.

3.3 Flow cytometry

In representing the FALS (Forward Angle Light Scatter) and ISS (Integrated Side Scatter) parameters of cells from monolayers (Fig. 3), we noted that they were distributed in clusters of points occupying 4 distinct regions of the histogram (Fig. 6), and which will be called populations. This distribution in clusters did not show variations in the culture times studied.

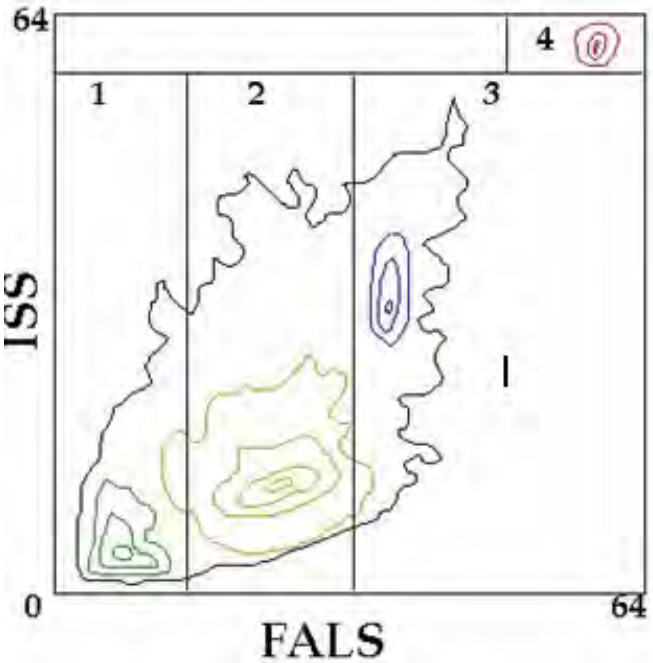

Fig. 6. Flow cytometric analysis of cells from 6-day monolayers derived from CE44 teratocarcinoma. The distribution of frequency of the two parameters of FALS vs ISS is representative of all the monolayers studied. Vertical lines define regions with four predominant populations. N: 10,000 viable cells from a representative analysis in n=10 separate experiments.

Both the size of the monolayer cells (FALS) (Fig. 7A) and their cytoplasmic complexity (ISS) (Fig. 7B) remained stable, without significant variations over time. Population 1 was the smallest and least

complex while population 4 was the largest and most complex. The complexity of population 2 was similar to that of population 1. However, the complexity of population 3 was greater ($p < 0.0001$), while that of population 4 was the most complex of all ($p < 0.0001$). In Figure 8 a schematic correspondence between flow cytometry and TEM studies is showed.

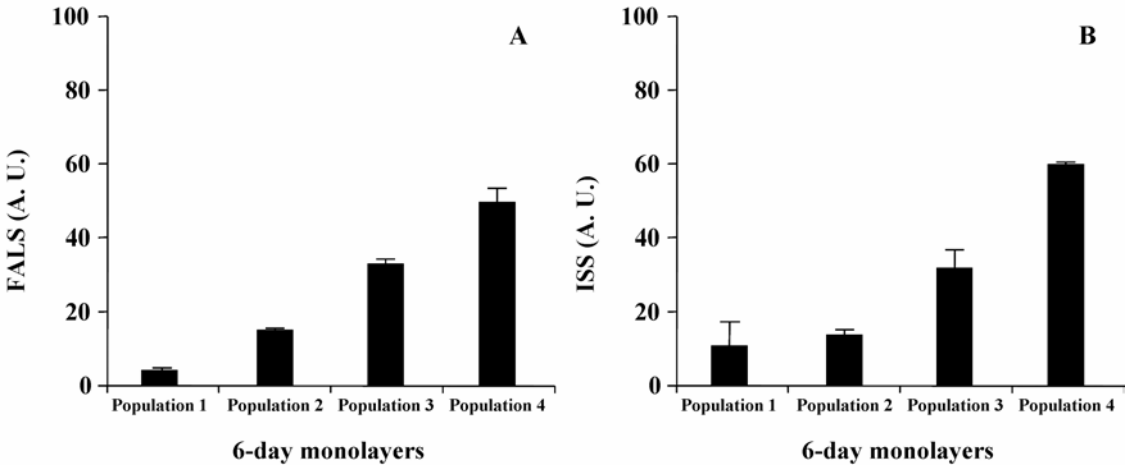

Fig. 7. A)Flow cytometric study of cell size (FALS), expressed in arbitrary units, of the viable cells of CE44 teratocarcinoma 6-day monolayer cultures. On the axis of the abscissas are represented the different cell populations, selected according to the criteria of FALS and ISS. Size is expressed as mean ± SE. B) Flow cytometric study of cytoplasmic complexity (ISS), expressed in arbitrary units, present in the viable cells of CE44 teratocarcinoma 6-day monolayer cultures. On the axis of the abscissas are represented the different cell populations, selected according to the criteria of FALS and ISS. Cytoplasmic complexity is expressed as mean ± SE.

4. Discussion

Our results show that cells from CE44 teratocarcinoma can be grouped, using flow cytometric criteria, into four different cellular populations that can be correlated with differences in the cytoplasmatic traits observed in the transmission electron microscopy study.

The use of flow cytometry techniques together with morphologic studies provide a suitable work tool to study the dynamic aspects of the proliferation process and cellular differentiation [8, 9]. To be able to characterize the cells composing the embryoid bodies using flow cytometry it is necessary to separate them out. The mechanical or chemical dissociation of embryoid bodies is difficult because of the existing tight junctions between the cells [10]. Nevertheless, this drawback is solved by depositing the embryoid bodies in culture flasks, in which spontaneous monolayers begin to form [3, 11, 12].

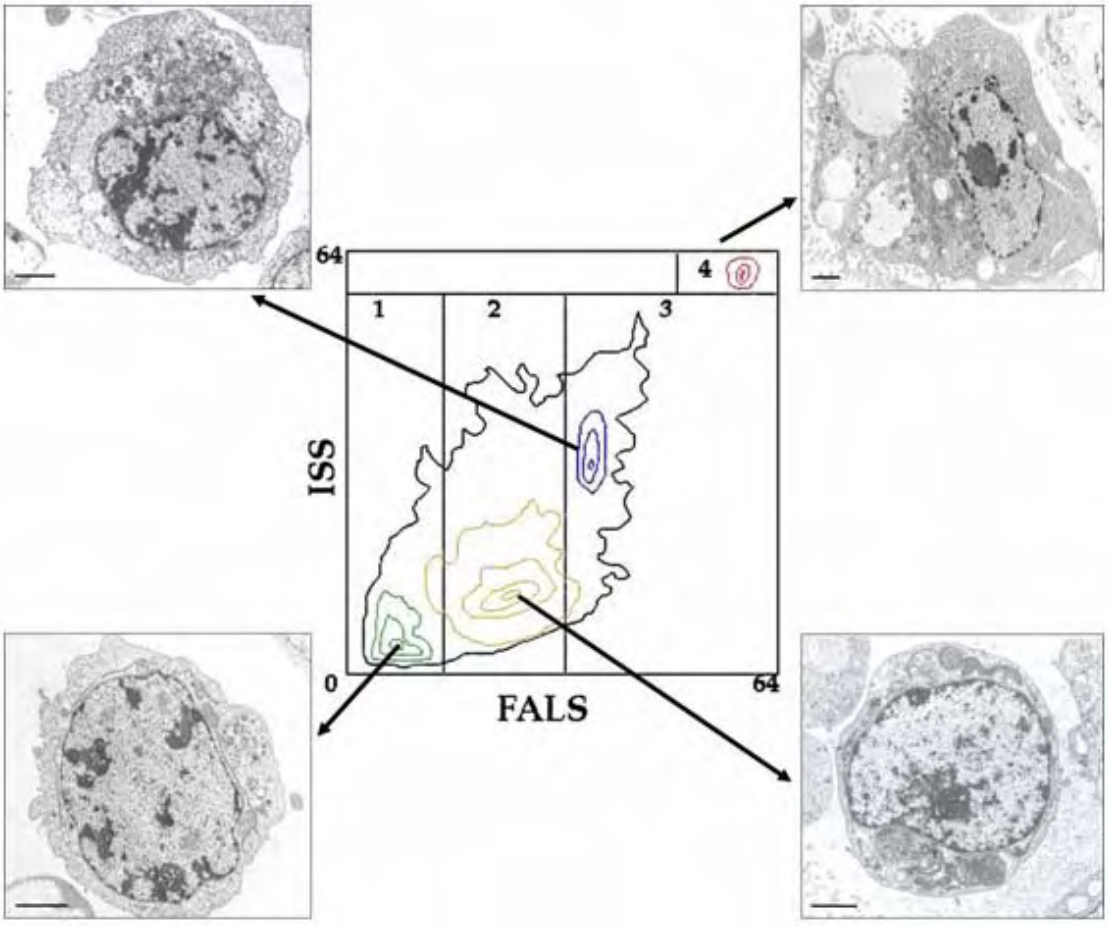

Fig. 8. Schematic representation of the correspondence between flow cytometry and transmission electron microscopic studies.

The morphology of the monolayer cells from CE44 teratocarcinoma is very similar to that described previously for primary cultures derived from other embryoid bodies from OTT6050 tumor [2, 4]. The great cellular heterogeneity observed in the monolayers seems to suggest that they could be composed of cells similar to those located inside the embryoid bodies (the most undifferentiated cells) and cells similar to those lining the outside of the embryoid bodies (showing a greater degree of differentiation) with numerous intermediate forms. However, the utilization of flow cytometry enable us to solve this apparent heterogeneity and to show the existence of four cellular populations. These cell populations are made up of diploid cells with slow proliferation rhythms and with four different duplication times [7]. The presence of several tumor-cell populations could be related to the different potential of embryonal carcinoma cells to differentiate in cells of all three embryonic germ layers [13].

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