Production of Tissue Plasminogen Activator (t-PA) with Differentiated F9-Embryonic Carcinoma Cells Grown as Multicell Spheroids

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INTRODUCTION

Cell-cell and cell-matrix interactions influence the cell differentiation in tissues (2,7), e.g. by mediating changes in the pattern of gene expression during mammalian embryogenesis (3,6). The mouse F9 teratocarcinoma cell line (1) is a nontransfected cell line which cannot only be cultivated as monolayers with few cell-cell and cell-matrix interactions but also as 3-d growing multicell spheroids (4). These so-called embryoid bodies develop tissue-like interactions in a physiological microenvironment which partly simulates the in-vivo situation of embryonic cells. After treatment with retinoic acid and dB-cAMP the F9 cells differentiate to parietal endoderm like cells (8,9,11) and produce substantial amounts of t-PA (5,10), similarly as during embryogenesis when parietal endoderm cells produce t-PA which plays an important role in tissue remodelling and cell migration. In contrast to transfected cell lines, the t-PA gene expression in differentiated F9 cells obeys the physiological gene regulation by responding to the microenvironment around the cells. In our studies we investigated conditions for growing F9 multicell spheroids in spinner flasks and compared the production of t-PA of differentiated F9 cells in monolayer culture with F9 cells cultured as multicell spheroids. Dye injection experiments with differentiated F9 cells growing as monolayers or as multicell spheroids revealed metabolic cell-cell cooperations in form of intercellular communication via gap junctions.

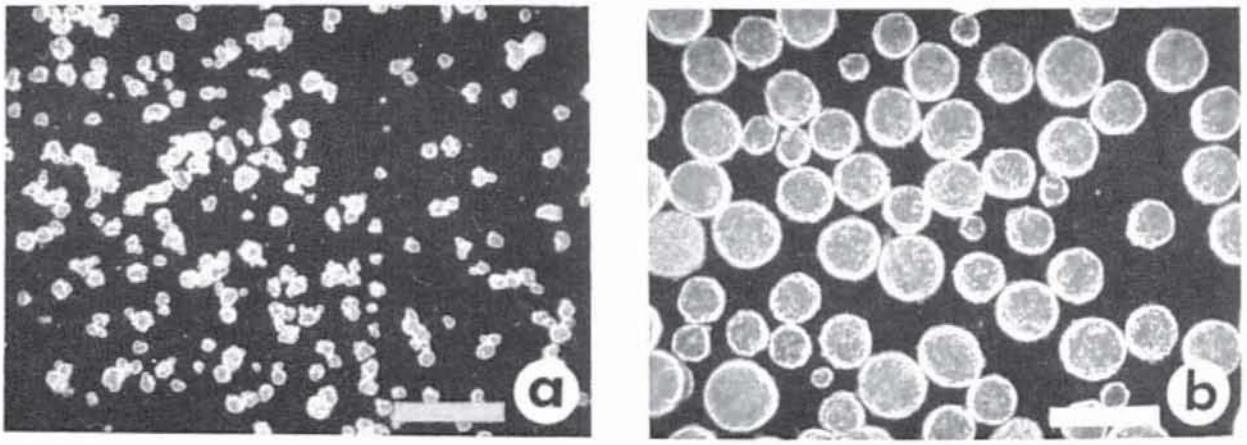

Fig. 1: Formation of multicell spheroids. Small irregularly shaped aggregates form spheroids of uniform size during cultivation a) 24 h, b) 96 h, (bar: 400 μm).

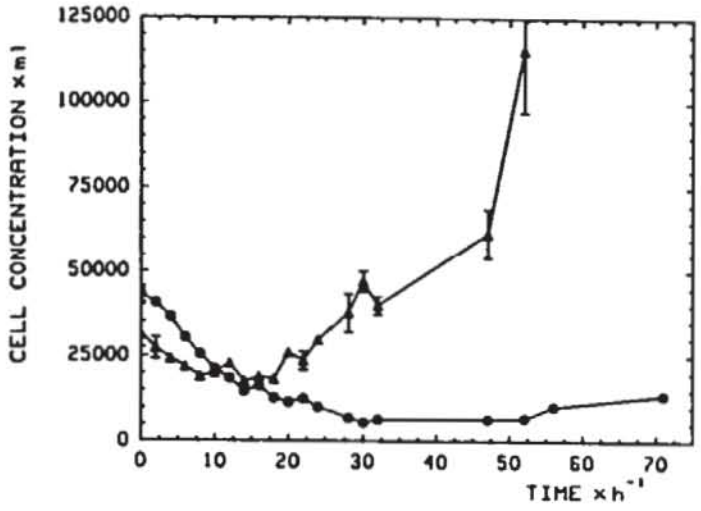

Fig. 2: Aggregation and proliferation of F9 cells in a multicell spheroid culture.
- • number of single cells
- ▲ number of aggregated (proliferative) cells

MATERIAL AND METHODS

Cell Culture

F9 stem cells were cultured as monolayers at 37°C in a CO_2 (8%) incubator in DMEM supplemented with 10% fetal calf serum. Prior to use, the 250 ml tissue culture flasks (75 cm^2, Nr. 658170, Greiner, Nürtingen, FRG) were coated with warm 0.1% gelatine solution and stored at 4°C over night. The cells were passaged three times a week.

For culturing F9 cells as multicell spheroids and their differentiation to parietal endoderm-like cells, we started with a single cell suspension (5-10×10^4 cells/ml) in a 100 ml spinner flask (Bellco Glass, N. J., USA) at 150 rpm. After 3-5 days of aggregation and proliferation the medium was changed and the suspension of multicell spheroids was treated with 10^{-6} M retinoic acid (RA) and 10^{-3} M dB-cAMP. This medium was collected daily and replaced by fresh RA/dB-cAMP-medium. The cell number was determined by centrifuging 5 ml of spheroid suspension, dissociating the spheroids by a trypsin treatment, and counting the isolated cells in a Coulter-Counter (Coulter Electronics, Hialeah, USA).

For culturing F9 cells as monolayers and their differentiation to parietal endoderm-like cells, they were grown in petri dishes (60 mm^2, Nr. 628160, Greiner) or 750 ml tissue culture flask (175 cm^2, Falcon Nr. 3028, Becton Dickinson, Heidelberg, FRG) to almost confluency within 3 days. The cells were passaged after this time in new petri dishes or flasks. The procedure of cell differentiation, medium changes, and cell counting was the same as described for multicell spheroids.

Plasminogen Activator Assay

The collected medium was stored at -20° C and the t-PA activity was determined with a commercially available test (Boehringer Mannheim, Mannheim, FRG). In a first step, the proenzyme plasminogen is activated by t-PA to plasmin. Fibrin fragments serve as stimulator in this reaction. In a second step, the activated enzyme

plasmin converts the substrate chromozym-PL to a chromogenic product which can be detected photometrically at 405 nm (Titertek, Helsinki, Finland). A human t-PA Standard (Boehringer Mannheim) was used for the calibration of t-PA in International Units (I.U.).

Intercellular Communication
With glass microelectrodes the fluorescent dye Lucifer yellow (Sigma, München, FRG) was injected iontophoretically into differentiated F9 cells. Spreading of the dye into surrounding cells was monitored by fluorescence microscopy.

RESULTS

F9 cells can only temporarily be cultivated as single cell suspension. The growth of multicell spheroids from a single cell suspension is shown in Fig. 1 and Fig. 2. Within 24 hours the cells aggregated to multicell spheroids in spinner flasks (Fig. 2). The aggregation was almost finished within 24 h and was independent of the initial cell concentration, but could be influenced by the geometry of the culture flask and the stirring velocity. A significant cell proliferation of the anchorage-dependent F9 cells was only detectable in the aggregated state (Fig. 2). A narrow size distribution of multicell spheroids in a 3 days old culture revealed identical spheroid sizes. Under our conditions, with an inital cell density of 10^5 cells/ml a cell concentration of 6×10^6 cells/ml was reached within 4 days of spheroid culture.
Cells in monolayers and in multicell spheroids were well coupled by gap junctions. Differentiated F9 cells showed a fibroblastoid morphology in contrast to the epitheloid morphology of undifferentiated F9 stem cells. This was not the case for multicell spheroids where the cells were also well coupled (Fig. 3).

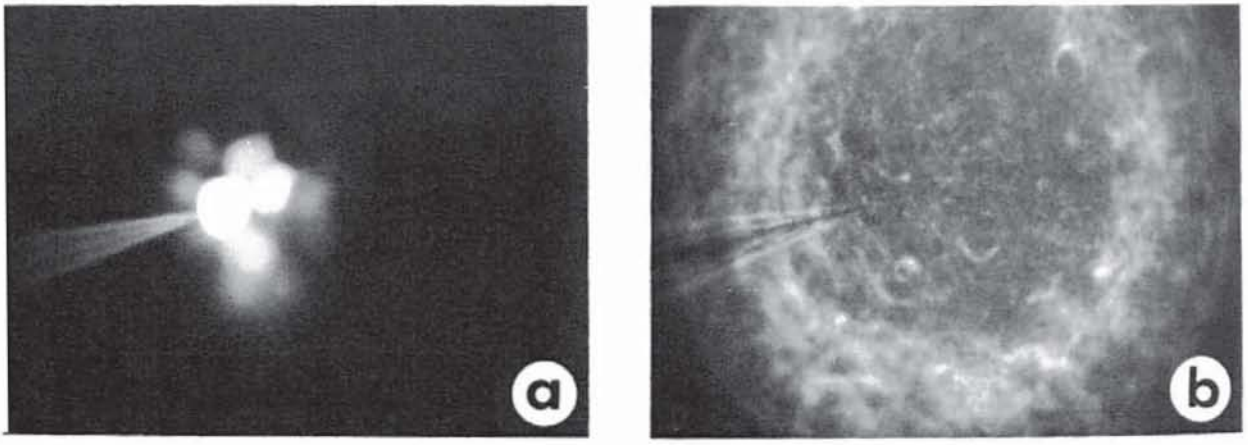

Fig. 3: Intercellular communication between differentiated F9 cells in a multicell spheroid demonstrated with the fluorescent dye Lucifer yellow a) dye spreading into neighbouring cells, b) same cells in phase contrast.

Comparison of the t-PA production of differentiated F9 cells cultured as monolayers and as multicell spheroids revealed a lower t-PA production rate in spheroids than in monolayer cells (Fig. 4). 50 h after cell differentiation with RA and dB-cAMP an

increase of t-PA production was detected in the supernatant of monolayer cells. In spheroid culture, however, an increase was observed only after 100 h.

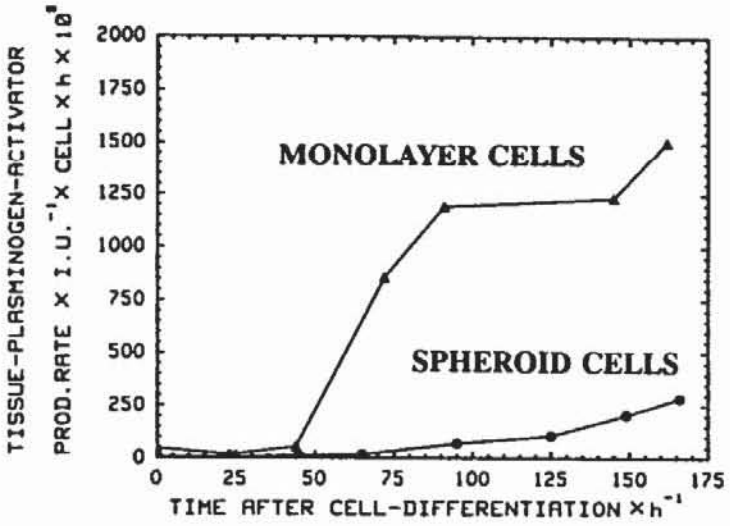

Fig. 4: Production of t-PA in differentiated F9 cells grown as monolayer and as multicell spheroids.

DISCUSSION

This difference in t-PA production may indicate that only the outer cells of the spheroids differentiate to parietal endoderm-like cells and that the core consists of undifferentiated stem cells, which do not express the t-PA gene. This could be due to an effect of gradients of diffusible differentiating agents. We cannot exclude, however, that the undifferentiated cells in the core are not determined by the differentiating agents, but by the environment, e.g. cell-cell or cell-matrix contacts. This specific differentiation of the outer cell layer would correspond to the mouse embryo, where the endoderm cells build only one-dimensional layers within the blastocyst.

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