Histol Histopath (1992) 7: 23-27

Histology and Histopathology

Effect of ascitic liquid on growth in vitro of embryoid bodies derived from teratocarcinoma

M. Monzó, G. Martínez-Rubio, J.M. de Anta, C. Saltó and D. Ruano-Gil
Department of Morphological Sciences, University of Barcelona, Barcelona, Spain

Summary. Embryoid bodies (EB) derived from teratocarcinoma (TC) OTT6050 were cultured with ascitic liquids (AL) from animals carrying 16-, 22- and 35-day evolved EB. At the same time the presence of fibronectin (FN) in AL were analyzed by immunoblotting. Results indicate the probable existence of growth-stimulatory factors for EB, as well as the presence of FN in the 22-day AL.

Key words: Teratocarcinoma, Embryoid bodies, Fibronectin, Ascitic liquid

Introduction

Teratocarcinomas (TC) are tumors obtained experimentally by the ectopic grafting of young mouse embryos (Stevens, 1970; Damjanov et al., 1987). The tumors thus obtained can be reimplanted intraperitoneally in isogeneic mice, giving rise to the ascitic form of the tumor known as embryoid bodies (EB) (Stevens, 1960). EB are divided morphologically in cystic and simple (Martin, 1978). Cystic EB are formed by an endodermal (END) cell layer that surrounds a cystic cavity with cells of embryonal carcinoma (EC) and are similar to 3.5-day-old mouse embryos. Simple EB form an endodermal layer that surrounds EC cells without cystic cavities and are similar to 5 day mouse embryos (Martin, 1978; Monzó et al., 1985). When EB are injected subcutaneously into isogeneic mice, they develop the original tumor (Pierce and Dixon, 1959; Stevens, 1983). EB are maintained in vivo by intraperitoneal passage in mice of the strain 129/Sv; a volume of ascitic liquid (AL) always appears in relation to the days of evolution of EB in the peritoneal cavity. It is now known that the presence of growth and tumor inhibition factors is detected in the AL in certain experimental tumors (Levine et al., 1984). Little is known, however, about the existence of extracellular matrix (ECM) proteins in AL of experimental TC. The aim of this study is to analyze the influence of AL from TC-carrying animals on the growth of EB in vivo and to investigate the presence of fibronectin (FN) in these AL.

Offprint requests to: Dr. M. Monzó, Departamento de Ciencias Morfológicas, Facultad de Medicina, Avda. Diagonal, 08028 Barcelona, Spain

Materials and methods

EB obtention

EB were derived from the TC 0TT6050 obtained by Stevens in 1970, and were a gift from Prof. F. Jacob of the Pasteur Institute in Paris. EB had been maintained in vivo in our laboratory since 1984 by intraperitoneal passage in 129/Sv isogeneic mice every fifteen days. Animals carrying EB of 12 days of evolution were killed by cervical dislocation and the peritoneal cavity was immediately washed with DMEM (Dulbecco's Modification of Eagle's Medium). EB were spun at 1,000 rpm for 5 min at 4° C and pellets were washed with DMEM.

Ascitic liquid obtention and EB culture

AL was obtained by intraperitoneal aspiration of 129/Sv mice carrying EB of 16, 25 and 35 days of evolution which had previously been injected with 10^6 EB. AL were centrifuged in a microfuge, decomplemented at 56°C for 30 min and filtered through a 0.8 μm membrane (Millipore). EB obtained 12 days postinjection were cultured on Petri Perm dishes (Nunc) and in microdrops of 200 μl, each one of which contained the following culture media:

A) 73% DMEM, 10% FCS, 2% L-Gln, 15% AL (16 days).

B) 73% DMEM, 10% FCS, 2% L-Gln, 15% AL (22 days).

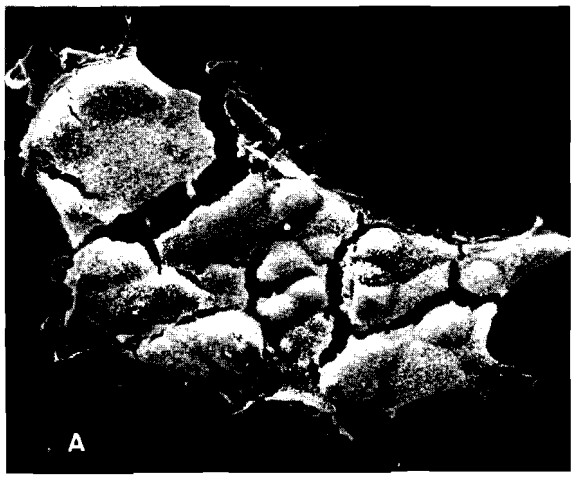

Fig. 1. Aspect of EB cultured with DMEM + FCS for 24 h by scanning electron microscopy (SEM). × 200

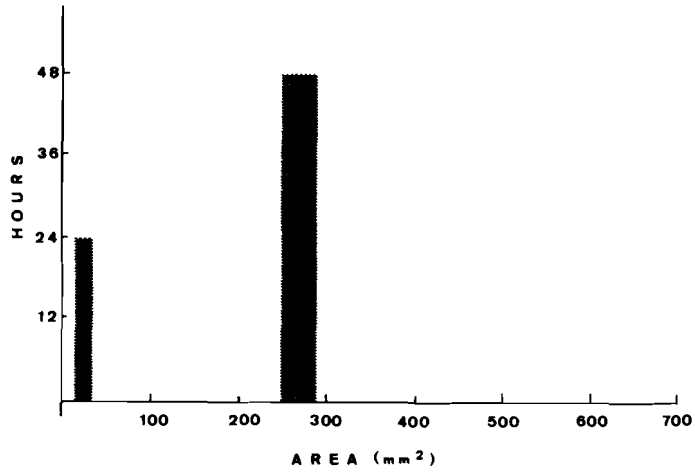

Fig. 2. Growth areas of EB cultured with DMEM + FCS for 24 h and 48 h.

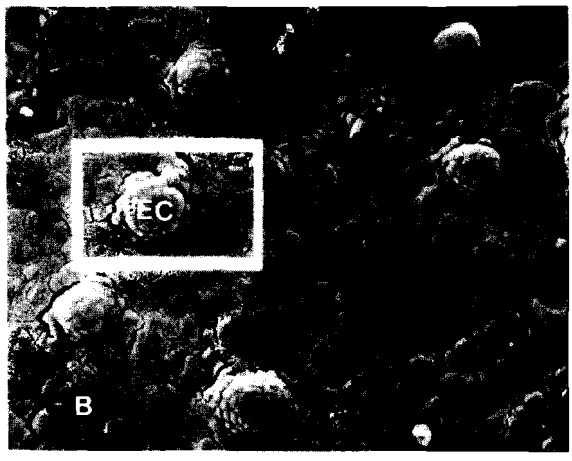

Fig. 1B. Aspect of EB cultured with DMEM + FCS for 48 h by inverted microscope. A great amount of embryonal carcinoma (EC) cell centres are observed.

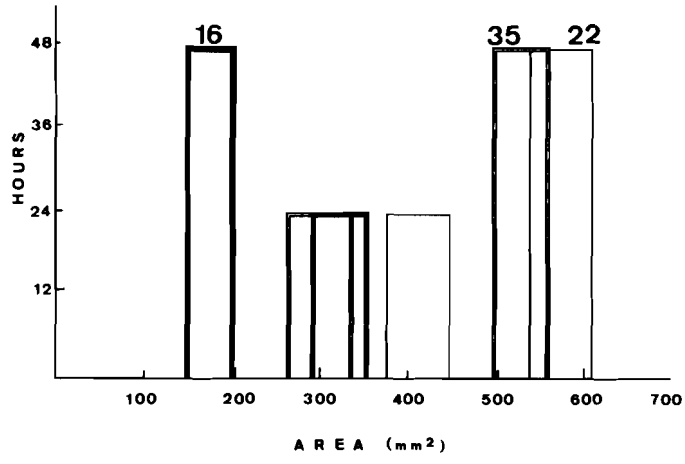

Fig. 3. Growth areas of EB cultured with DMEM + FCS + ascitic liquid (AL) of different days for 24 and 48 h. Thick line, AL of 16 days; double line, AL of 22 days; narrow line, AL of 32 days.

C) 73% DMEM, 10% FCS, 2% L-Gln, 15% AL (35 days).

D) DMEM plus (15% AL [16 days] or 15% AL (22 days) or 15% AL [35 days]).

DMEM plus 10% FCS was used as control medium. Cultures were photographed after 24 and 48 hours and the growth area was calculated by means of a Kontron Mop 20.

After 24 h cultures were processed for scanning electron microscopy (SEM) as follows: they were fixed in 2% glutaraldehyde in cacodylate buffer, postfixed in 1% osmium tetroxide, washed in PBS, dehydrated in acetone series, conserved in isoamyl acetate, and critical-point dried.

Detection of FN in AL

AL of 16, 22 and 35 days of evolution were processed by 7.5% SDS-polyacrylamide gel electrophoresis. Proteins obtained were transferred to nitrocellulose filters by electroblotting at 12 volts for 16h at 4° C. Filters were then blocked overnight in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), incubated with a polyclonal antibody of rabbit anti-FN (Biogenex) diluted 1:200 in 1% BSA in PBS and washed in 1% BSA in PBS. They were then postincubated with peroxidase-conjugated goat anti-rabbit IgG antibodies (Miles) diluted 1:400 in 1% BSA in PBS, washed in PBS and developed with diaminobenzidine (DAB).

Results

AL, at 16 days of evolution, presented a clear, transparent aspect, which contrasted with that of 25 and 35 days which usually appeared more turbid. Some 35-day AL were occasionally haemorrhagic and were thus discarded for culture. Animals carrying AL

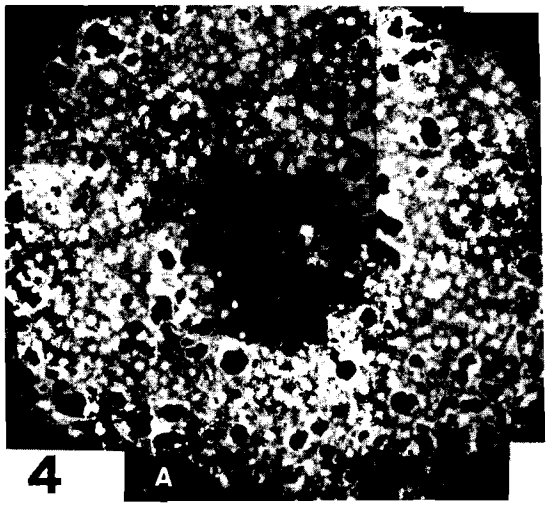

Fig. 4A. General appearance of EB cultured with DMEM + FCS + AL of 22 days for 48 h. × 40

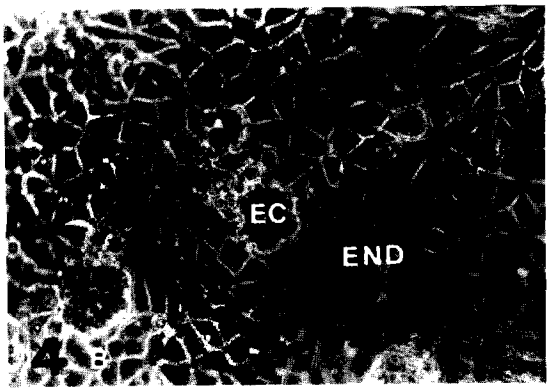

Fig. 4B. Detail of **Fig. 4A.** EC cell centres and endodermic (END) cells are observed. × 400

after day 22 usually presented tumoral implantations in the peritoneal cavity.

Most of the EB cultured with DMEM plus FCS for 24 h had anchored and begun to grow, and END-like cells appeared with microvilli on the surface (Fig. 1A). After 48 hours the culture had increased its growth considerably and nodules of EC cells were observed together with END-like cells (Fig. 1B). Areas of growth corresponding to 24 and 48 h are shown in Fig. 2.

Cultures with DMEM, FCS and AL

EB cultured with 73% DMEM, 10% FCS, 2% L-Gln and 15% AL of 22 and 35 days were completely anchored after 24 h, although the cultures with 16-day AL were still not adhered. The anchored EB presented a similar morphology to that observed in Fig. 1A, as did the areas of growth, as shown in Fig. 3. However, we observed a slight increase in the areas of growth of the cultures with 22-day AL compared to

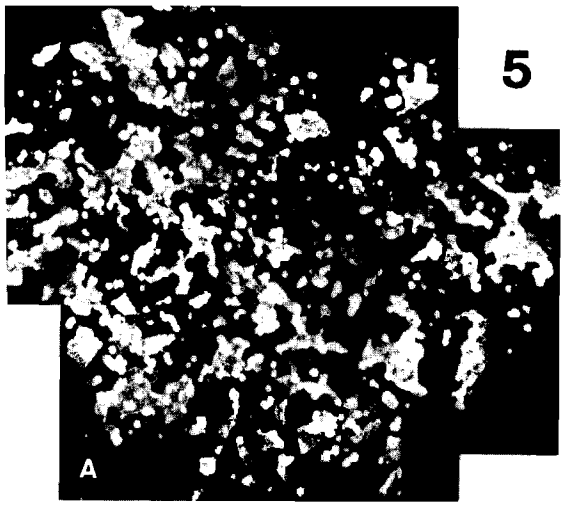

Fig. 5A. General aspect of EB cultured with DMEM + FCS + AL of 16 days for 48 h. × 40

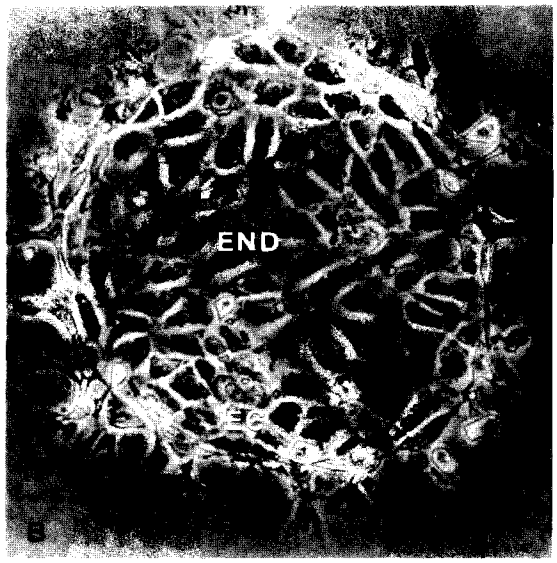

Fig. 5B. Detail of **Fig. 5A.** EC and END cells are observed. × 450

those with 16- and 35-day AL. The same cultures presented important variations in their growth at 48 h (Fig. 3). Maximum area of growth corresponded to cultures with 22-day AL (Figs. 3, 4A, 4B). Cultures with 35-day AL were slightly smaller than those with 22-day AL, (Fig. 3) while those with 16-day AL showed very little growth compared with the others (Figs. 5A, 5B).

Cultures with DMEM plus AL in absence of FCS

EB cultured with 73% DMEM, 2% L-Gln and 15% AL of 16-, 22- and 35-days, but without FCS, showed a higher area of growth (Fig. 6) than controls after 24 h (Fig. 2), but was lower that of cultures with FCS plus AL (Fig. 3). After 48 h we observed that the

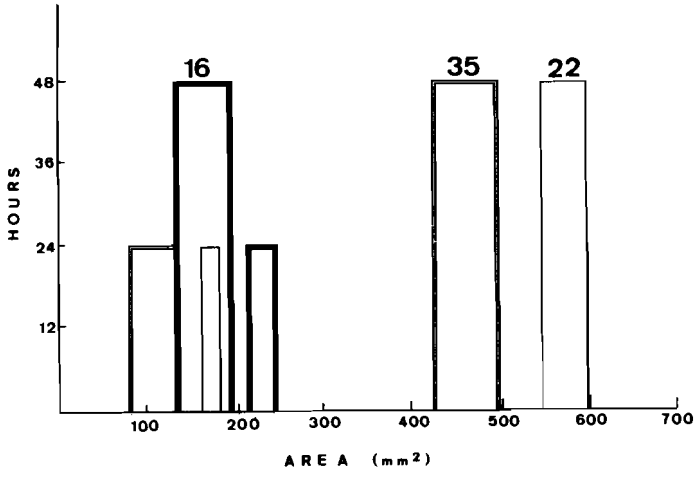

Fig. 6. Growth areas of EB cultured with DMEM + AL of different days and in absence of FCS.

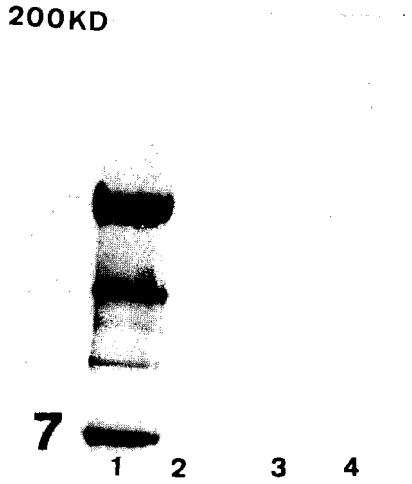

Fig. 7. Immunoblot of AL of 16 days (2), 22 days (3) and 35 days (4) to fibronectin (FN). FN is detected in AL of 22 and 35 days. (1) Standard proteins.

area of growth of cultures with 16-day AL was similar to that of controls and to those of AL plus FCS. Areas of growth of cultures with 22- or 35-day AL were smaller than those obtained with AL plus FCS, and maximum growth area continued to be that associated with 22- day AL (Fig. 6). Immunoblot performed on 16-, 22-and 35-day AL to FN indicated that its presence could be detected from day 22 onwards (Fig. 7).

Discussion

Most cells in culture need a set of exogenous substances to enable them to adhere to and grow on an artificial substrate. Depending on this process of adhesion, cells show varying responses to exogenous stimuli such as growth factors. Thus, cells of the corneal epithelium in vitro respond to the addition of fibroblastic growth factor (FGF) but not to epidermal growth factor (EGF). However, in these same cells are grown in the presence of ECM proteins, they respond to EGF, as is observed in vivo (Gospodarowicz, 1978). In cultures of EB, our results indicate that the maximum adhesion is obtained in cultures of EB with DMEM, FCS plus 22-day AL. The presence of FN is also detected from day 22 onwards. This suggests the possible influence of this protein in facilitating the anchorage of the EB. The function of FN as an anchor protein has already been demonstrated in cultures of pre-implantation embryos (Armant et al., 1986; Tsuiki et al., 1989), in EC cells (Tienari et al., 1989) and observed during EB growth (Monzó et al., 1991). It is also demonstrated in the rat that histiocytoma cells do not adhere to the host animal if they are injected together with an anti-FN antibody (Pande and Khar, 1988). The maximum area of growth is also observed in cultures with DMEM, FCS plus 22-day AL, and it is interesting to note that cultures with 22-day AL but without FCS grow more than control cultures, with only contain FCS. This finding suggests the possible existence of growth factors in AL. In fact Levine et al. (1984) isolated a tumor-stimulatory factor in AL of Novikoff's hepatoma. On the other hand we cannot discard the possibility that FN itself may act as a growth-stimulatory factor as this phenomenon has been demonstrated both in the growth of experimental TC (Wartiovaara et al., 1978; Rizzino and Crowley, 1980; Leivo and Wartiovaara, 1989) and in humans (Maclhinney et al., 1983; Chakrabarty et al., 1988).

Cultures with 35-day AL show less growth than those with 22-day AL. The mechanisms by which this phenomenon may be explained are unknown, although it has been observed that growth-stimulatory and -inhibitory factors can coexist in AL, and that the latter operates by preventing the anchorage of tumor cells, and also that the growth of the tumor depends on the relative concentration of these factors (Levine et al., 1984).

In conclusion, we would argue that the AL of animals carrying TC contains growth-stimulatory factors apart from FN, and that this is thus a suitable model for the study of the relation between tumor cells and anchorage and growth factors.

Acknowledgements. We thank Robin Rycroft for his help in the preparation of the English manuscript, and Ms. Dolors Fuster for her technical assistance. This study was supported by the DGICYT PB87-0163.

References

Armant R.D., Kaplan H.A. and Lennarz W. (1986). Fibronectin and laminin promote in vitro attachment and outgrowth of mouse blastocysts. Dev. Biol. 116, 519-523.

Chakrabarty S., Tobon A., Varani J. and Brattain M.G. (1988). Induction of carcinoembryonic antigen secretion and modulation protein secretion expression and fibronectin/

laminin expression in human colon carcinoma cells by transforming growth factor-beta. Cancer Res. 48, 4059-4064.

Damjanov I., Damjanov A. and Solter D. (1987). Production of teratocarcinomas from embryos transplanted to extra-uterine sites. In: Teratocarcinomas and embryonic stem cells. A practical approach. Robertson E.J. (eds). IRL press. Oxford. pp 1-18.

Gospadorowicz D., Mescher A.L. and Birdwell C.R. (1978). Control of cellular proliferation by the fibroblast and epidermal growth factors. Natl. Cancer. Inst. 48, 109-130.

Levine A.E., Hamilton D.A., Yeoman L.C., Busch H. and Brattain M.G. (1984). Identification of a tumor inhibitory factor in rat ascites fluid. Biochem. Biophys. Res. Commun. 119, 76-82.

Leivo I. and Wartiovaara J. (1989). Basement membrane matrices in mouse embryogenesis, teratocarcinoma differentiation and neuromuscular maturation. Int. J. Dev. Biol. 33, 81-89.

MacIlhinney R. and Patel S. (1983). Characterization of the fibronectin synthesized by human germ cell tumors. Cancer Res. 43, 1282-1288.

Martin G.R. (1978). Advantages and limitations of teratocarcinoma stem cells as models of development. In: Development in mammals. 3. Martin H.L. (ed) Elsevier/ North-Holland Biomedical Press. Amsterdam, New York. pp 225-265.

Monzó M., Serra I. and Ruano D. (1985). Embryological aspects of the study of embryoid bodies. Ital J. Anat. and Embryol. 4, 297-305.

Monzó M., Barnadas A., de Anta JM and Ruano D. (1991). Laminin and fibro nectin expression during in vivo growth of embryoid bodies derived from teratocarcinoma. Virchows Archiv (A) 418, 509-514.

Pande G. and Khar A. (1988). Differential expression of fibronectin in a rat histiocytoma: possible role of fibronectin in tumor cell adhesion. Exp. Cell Res. 174, 41-48.

Pierce G.B. and Dixon F.J. (1959). Testicular teratomas. I. Demonstration of teratogenesis by metamorphosis multipotencial cells. Cancer 12, 573-583.

Rizzino A. and Crowley C. (1980). Growth and differentiation of embryonal carcinoma cell line F9 in defined media. Proc. Natl. Acad. Sci. USA 77, 457-461.

Stevens L.C. (1960). Embryonic potency of embryoid bodies derived from transplantable testicular teratomas of the mouse. Dev. Biol. 2, 285-297.

Stevens L.C. (1970). The development of transplantable teratocarcinomas from intratesticular grafts of pre- and post-implantation mouse embryos. Dev. Biol. 21, 364-382.

Stevens L.C. (1983). The origin and development of testicular, ovarian and embryo-derived teratomas. In: Teratocarcinoma stem cells. Cold Spring Harbor conferences on cell proliferation. Vol. 10. Silver L.M., Martin G.R. and Strickland S. (eds). CSH laboratory press. New York. pp 23-36.

Tienari J., Lehtonen E., Vartio T. and Virtanen I. (1989). Embryonal carcinoma cells adhere preferentially to fibronectin and laminin but their endodermal differentiation leads to a reduced adherence to laminin. Exp. Cell Res. 182, 26-32.

Tsuiki A., Preyer J. and Hung T. (1989). Effects of fibronectin and its peptide fragment of preimplantation mouse embryo. Am. J. Obstet. Gynecol. 160, 724-728.

Wartiovaara J., Leivo I., Virtanen I., Vaheri A. and Graham G.F. (1978). Appearance of fibronectin during differentiation of mouse teratocarcinoma in vitro. Nature 252, 355-356.

Accepted June 1, 1991