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Epigenetic modulation of differentiation in CE44 teratocarcinoma

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Summary. Teratocarcinoma is a mixed germ cell tumor histologically composed of embryonal carcinoma cells and embryonic and extraembryonic tissues. In the present work we have used the CE44 teratocarcinoma, which is a tumor cell line derived from the OTT6050 experimental tumor, to appreciate the influence the microenvironment has on the modulation of tumoral differentiation. For this, we have studied the development of CE44 teratocarcinoma in primary tumors (subcutaneous and intrasplenic) and in experimental metastases (hepatic and pulmonary). CE44 teratocarcinoma shows variations in its capacity for differentiation in so far as development is concerned and, in hepatic metastases, we noticed a reparative process of the intratumoral necrotic areas which in the same cases were substituted by loose connective tissue.

Our results clearly suggest that the microenvironment is decisive in the biological behaviour of the teratocarcinoma cells and that epigenetic factors influence the capacity for differentiation of the undifferentiated tumoral cells.

Key words: Epigenetic, differentiation, teratocarcinoma, metastasis

Introduction

Teratomas and teratocarcinomas are tumors that stem from germinal cells with the capacity for generating cells and tissues derived from the three embryonic layers and extraembryonic tissues. They are a type of tumor that spontaneously arise in the gonads and in extragonadal sites of human and animals, and they can be experimentally obtained by implantation of normal early embryos, gonadal ridges or primordial germ cells in extrauterine sites of adult syngeneic recipients (Pierce et al., 1967; Solter et al., 1970; Stevens, 1970; Martin, 1975; Damjanov, 1990).

Teratocarcinoma, a term coined by Friedman and Moore in 1946, is a mixed germ cell tumor that is histologically characterised by a wide variety of tissues in various degrees of maturation. Adult tissues mix with the embryonic tissues of the three embryonic layers and with a population of undifferentiated cells, that are called embryonal carcinoma cells (Mostofi and Price, 1973; González-Crussi, 1982; Rosai, 1996). Embryonal carcinoma cells are the cause of teratocarcinoma malignancy (Kleinsmith and Pierce, 1964) whereas the most differentiated progeny are usually benign (Pierce et al., 1959, 1960; Pierce and Verney, 1961).

Embryonal carcinoma cells are so called because of their close resemblance to the cells of embryonal carcinoma of human testis (Pierce et al., 1959; Stevens and Pierce, 1975). The embryonal carcinoma cells are the multipotential stem cells of the teratocarcinoma (Pierce et al., 1960), and the neoplastic equivalent of the cells of the inner mass of blastocysts (Pierce, 1967; Evans and Kaufmann, 1981). Epigenetic factors play a role in the differentiation and growth of teratocarcinomas (Gaillard, 1974; Damjanov et al., 1983). These factors, and the biological potential of the embryonal carcinoma cells, are demonstrated by the fact that: a) they are competent to originate malignant and destructive tumors concerning the host, and b) they can contribute to the formation of a new living creature, on being placed close to the cells of the inner cell mass of the normal blastocyst (Brinster, 1974; Mintz and Illmensee, 1975; Papaioannou et al., 1975, 1978). Because of the above mentioned characteristics, teratocarcinoma constitutes a good model system to study embryogenesis and neoplasia processes (Pierce, 1961, 1967; Martin, 1975; Damjanov, 1993).

Some solid teratocarcinomas when injected into the peritoneal cavity form solid and cystic aggregates of tumor cells (Pierce et al., 1959; Stevens, 1959), which are called embryoid bodies because they are morphologically similar to morulas and early blastocysts (Pierce et al., 1959, 1960; Stevens, 1959, 1960; Martin, 1975; Martin et al., 1977). The cells located inside the embryoid bodies are the embryonal carcinoma cells, and

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the outer cells express features of endoderm (Pierce et al., 1959, 1967; Lehman et al., 1974; Teresky et al., 1974; Martin, 1975). Thus, embryoid bodies contain the totipotent cellular elements (Stevens, 1960) and they are a highly dynamic structure that makes interesting models for the study of cellular differentiation.

Experimental teratocarcinomas are widely used and several clonal lines have been isolated and some of their culture characteristics investigated (listed in the book edited by Silver et al., 1983). CE44 teratocarcinoma is a tumor cell line (Gaillard, 1976), derived from the OTT6050 experimental tumor, obtained by Dr. Leroy Stevens in 1970 from a graft of a 6-day strain 129 mouse embryo into the testis of strain 129 adult mouse. It is maintained intraperitoneally by serial transfers of ascitic fluid to 129 mouse. It grows in the peritoneal cavity forming simple and cystic embryoid bodies, with the presence of intermediate forms in both types (Parchment et al., 1990; Unda et al., 1994). When the embryoid bodies, or their cells, are grafted in the subcutaneous space, a solid teratocarcinoma results that does not metastatize spontaneously (Gaillard, 1976).

The aim of the present work is to verify the existence of environments capable of infuencing to different degrees the differentiation of the embryonal carcinoma cells. Recently, we have perfected a method with which starting with an in vitro culture of embryoid bodies, we have been able to obtain experimental metastases of CE44 teratocarcinoma (Hilario et al., 1996). Now, we have studied the development of CE44 teratocarcinoma in different micoenvironments: a) as primary tumor in two inoculation sites (subcutaneous and intrasplenic); and b) in two organs that are the common site of metastases (liver and lung).

Materials and methods

Animals

Isogeneic male and female 129/Sv mice (9 to 10 weeks old) from Jackson Laboratory (Maine, USA) were maintained at the Animal facility of the University of the Basque Country (Leioa). Access to food and water was on an ad libitum basis.

Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358. 1, December 12, 1987, and the NIH Guide for the Care and Use of Laboratory Animals, NIH publication 85-23, 1985).

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. The hosts were killed upon the subsequent development of markedly distended bellies and the peritoneal cavity was immediately washed with DMEM (Dulbecco's Modification of Eagle's Medium; Sigma Co, Mo., USA). The peritoneal fluid was aspirated and centrifuged at 200g for 5 min at 4 °C. The resulting precipitate was resuspended in phosphate-buffered saline (PBS) and intraperitoneally reinjected into other intact hosts, in a concentration of 10^6 embryoid bodies/ml.

Cell cultures

Approximately 10^5 embryoid bodies resuspended in 10 ml of DMEN were seeded onto 75 cm^2 tissue culture-treated flasks (Costar, USA), at 37 °C in a humidified 5% CO_2-air atmosphere. All the cultures were maintained in DMEN, supplemented with heat-inactivated 10% fetal calf serum (FCS, Gibco, USA), 200 UI/ml penicillin, 200 mg/ml streptomycin and 2.5 μg/ml amphotericin B (Sigma Co. Mo, USA). The culture medium was replaced every one or two days.

Tumor and experimental metastases production

After 6 days of culture, monolayers were detached with 2 mM trypsin/EDTA. The material obtained was washed with FCS, to inactivate the trypsin, and passed through a 40 μm pore nylon filter (Falcon, Becton Dickinson, USA). Following three consecutive washings and centrifugings (200g), a suspension containing $5x10^6$ viable cells/ml (in PBS) was obtained for inoculation into the animals.

In order to produce solid tumors, mice previously anaesthetized with Nembutal (1.2 mg/mouse, i.p.), were inoculated with 0.1 ml of the suspension containing the tumor cells. Animals were I) subcutaneously inoculated (n=8) to obtain subcutaneous tumor, II) inoculated in the spleen (n=6) to obtain spleen primary tumors and liver metastases, or III) inoculated into a tail lateral vein (n=10) for lung metastases induction, as previously described (Hilario et al., 1996).

Mice were killed by cervical dislocation 21 to 45 days after injection of tumor cells. The subcutaneous tumor and the organs were fixed in formaldehyde buffer for embedding in paraffin for light microscopy. The sections (5 μm thick) were stained with Hematoxylin-eosin or PAS-Alcian-blue (pH 2.5). Some samples were processed for transmission electron microscopy and embedded in Epon (Hilario et al., 1992). Semithin sections (1 μm thick) were stained with Toluidine blue for light microscopy, while adjacent thin sections were examined ultrastructurally.

Results

The study of the primary tumors (subcutaneous and intrasplenic) and of experimental metastases (hepatic and pulmonary) showed that they were made up of undifferentiated cells of embryonal carcinoma and other cells showing different degrees of differentiation. The carcinoma cells were similar to those already described

by Pierce and Beals (1964), with a nucleus of dispersed chromatin with one or more nucleoli and a cytoplasm abundant in ribosomes but lacking in other organelles. It was quite common to find apoptosis images.

Tumor of subcutaneous growth

The tumors of subcutaneous growth (Fig. 1) consisted of cells of embryonal carcinoma and of ectodermic, mesodermic and endodermic derivates, with areas of necrosis.

Ectodermic derivates were represented by neural tissue consisting mainly of neuronal rosettes (Fig. 2A), neural tubes (Fig. 2B) and occasionally, neuronal-like cells (Fig. 2C).

Foci of cartilage of varying degrees of maturation were observed. The presence of cellular differentiation into chondral tissue was observed near necrotic areas and in close contact with small blood vessels (Fig. 3A). Chondral foci showing cells in a degeneration stage were found surrounded by an osteoid cap, similar to those observed in the endochondral ossification (Fig. 3B).

It was relatively common to find tubular structures (Fig. 4) of endodermic differentiation lined by cylindrical or cuboid epithelium. Among the cells lining the tubes, mucous goblet cells (Fig. 4A) showing a positive staining to neutral mucopolisaccharides (PAS-Alcian blue stain) (Fig. 4B) were interposed among the cells linning the tubes. The tubular structures were clearly differentiated, showing a cellular polarity: microvilli at the apical pole, lateral juctions (desmosome and tight junction) and basal lamina (Fig 4D). These structures suggest an endodermic differentiation of the intestinal type.

Papillary structures, with a positive stain for neutral mucopolisaccharides, were also recognised. Some structures resembling the Schiller-Duval bodies were observed, although none of them fulfilled all the morphological criteria for these bodies.

Foci of mesenchymal differentiation were observed among the undifferentiated cells of embryonal carcinoma. One of them resembled muscular tissue (Fig. 5), showing cells with an eosinophil cytoplasm that suggested a rabdoid differentiation, although cytoplasmic striations were not observed. Numerous blood capillaries with prominent endothelium (Fig. 5B) were found in these foci of mesenchymal differentiation.

Tumor of Intrasplenic growth

Intrasplenic tumors were homogeneous with relatively well defined borders (Fig. 6). Scarce, isolated areas of necrosis could be found. The tumors were mainly composed of embryonal carcinoma cells, although small islets of scarcely differentiated nervous tissue and occasional foci of chondral differentiation were recognized.

Pulmonary metastases

Mice innoculated through the lateral vein of the tail showed metastases with a nodular pattern and expansive growth (Fig. 7A). During their growth, the tumoral nodules compressed the cells of the pulmonary parenchyma and the pulmonary blood vessels were incorporated inside. Occasional areas of necrosis were observed.

Metastases consisted of undifferentiated cells of embryonal carcinoma (Fig. 7B), although small foci of chondral differentiation and of immature neural tissue

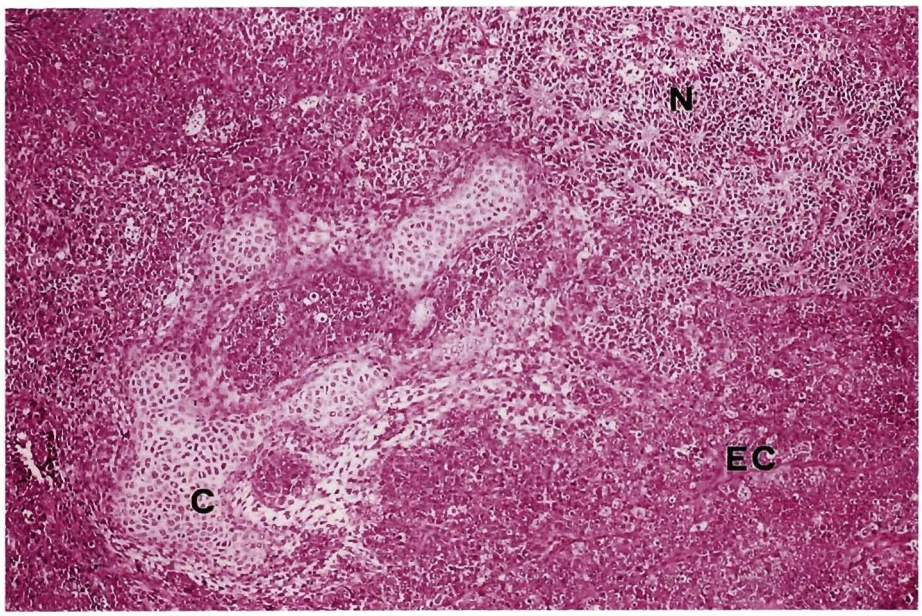

Fig. 1. Subcutaneous CE44 teratocarcinoma. Areas of embryonal carcinoma (EC), chondral differentiation (C) and neural differentiation (N) can be appreciated. Hematoxylin-eosin. x 25

were also observed.

Hepatic metastases

Hepatic metastases presented an expansive growth with lobulated borders marked by the presence of blood vessels (Fig. 8A,B). Tumors consisted almost exclusively of embryonal carcinoma cells. Small areas with tubular and papillary differentiation, both positive to PAS-Alcian blue staining, were frequent. Some areas with immature neural differentiation, similar to those observed in intrasplenic tumor, although in greater

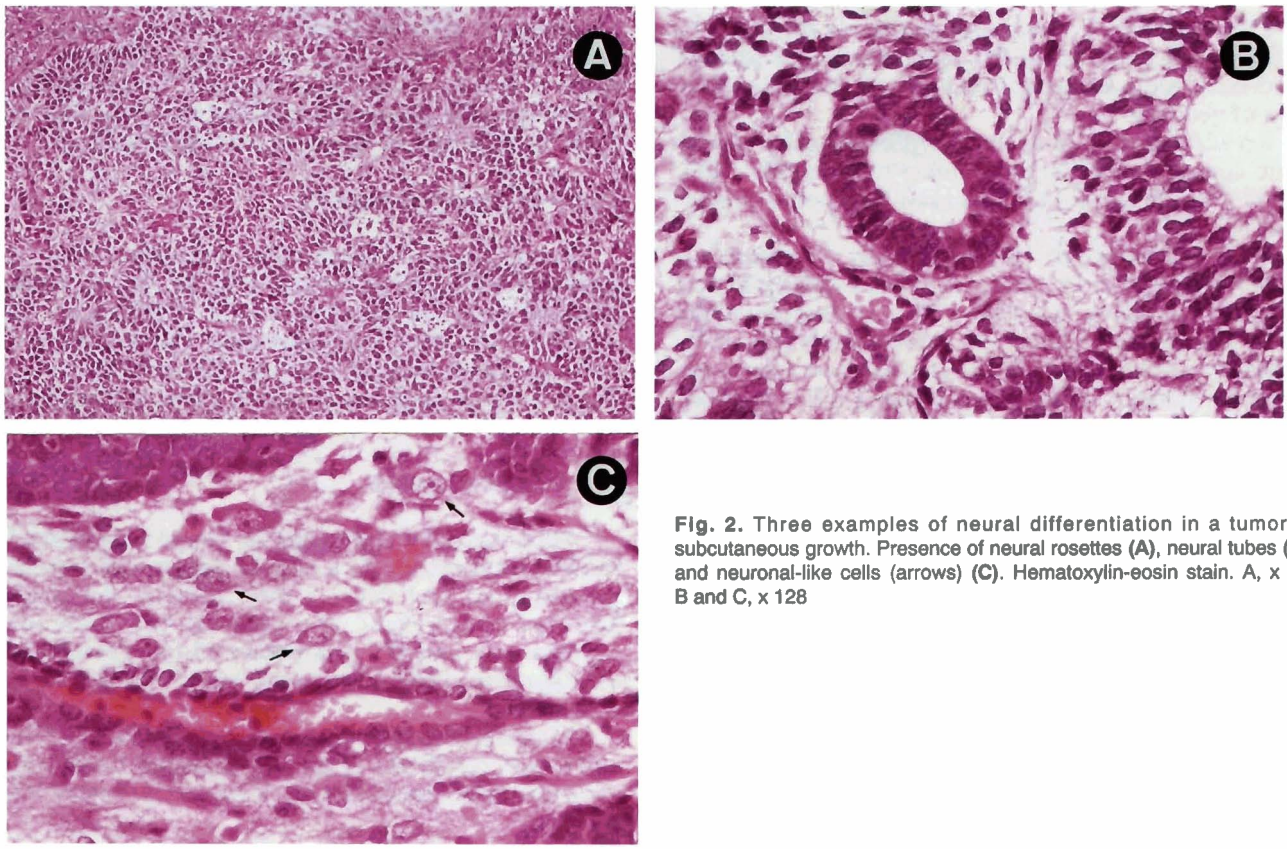

Fig. 2. Three examples of neural differentiation in a tumor of subcutaneous growth. Presence of neural rosettes **(A)**, neural tubes **(B)**, and neuronal-like cells (arrows) **(C)**. Hematoxylin-eosin stain. A, x 50; B and C, x 128

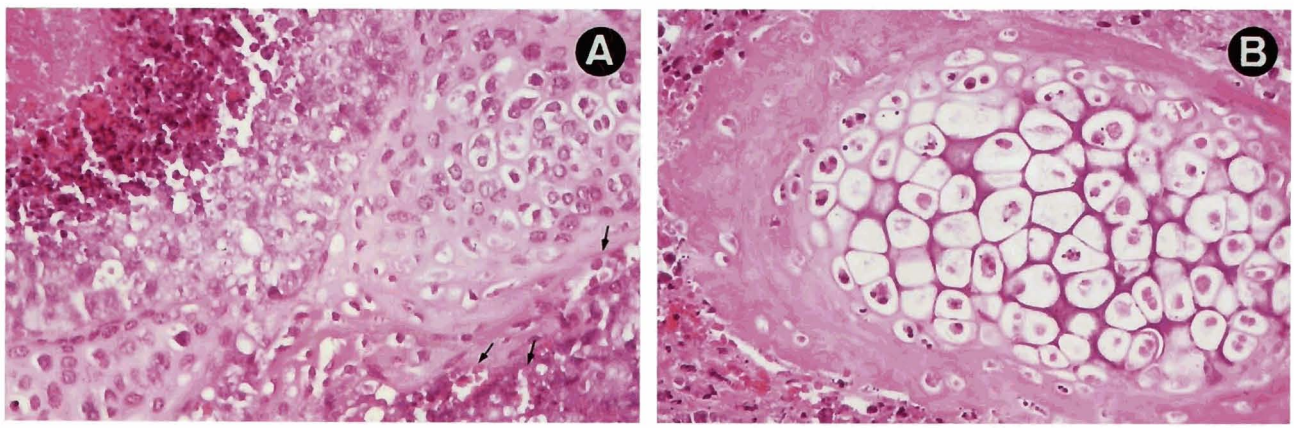

Fig. 3. Foci of cartilagenous differentiation in subcutaneous tumor. In both cases the borders of cartilage are abrupt and surrounded by cells of embryonal carcinoma and blood capillaries. **A.** Observe the presence of capillaries indicated by the arrows and in the upper left angle an area of undifferentiated carcinoma cells with necrosis. **B.** Chondral cells in a degeneration stage are surrounded by an osteoid cap, bringing to mind the process of endochondral ossification. Hematoxylin-eosin stain. x 100

proportion, were recognized.

Frequent areas of intratumoral necrosis were observed (Fig. 8A). Semithin sections enabled us to observe that these areas, located in the parts most distant from the blood vessels (Fig. 8B), were usually surrounded by a crown of seven to ten tumoral cells. In the areas of necrosis, it was common to observe shaded vessels that ran through these areas and went from one viable part of the tumor to the other. In some cases, we appreciated a reparative process in the necrotic areas,

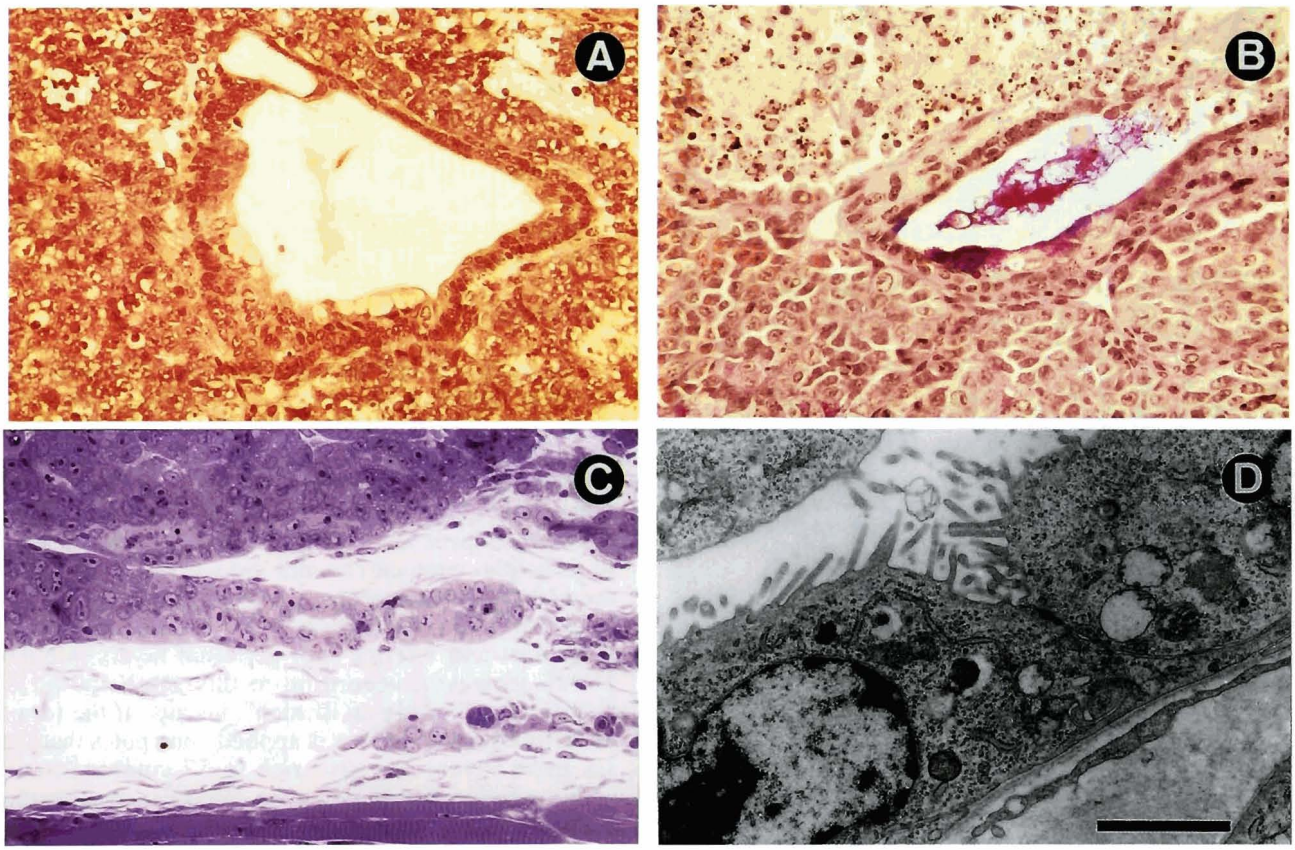

Fig. 4. Subcutaneous tumor forming tubular structures. **A.** Goblet cells are clearly observed in the lining epithelium. **B.** Positive stain for neutral mucopolisaccharides, both in the cells as well as in the inside of the lumen. **C and D.** Cells lining the tubular structures are polarised, showing a basal lamina, tight junctions and microvilli. A, Hematoxylin-eosin stain, x 100; B, PAS-Alcian-Blue stain, x 100; C, Toluidine blue stain, x 100; D, bar: 2 μm.

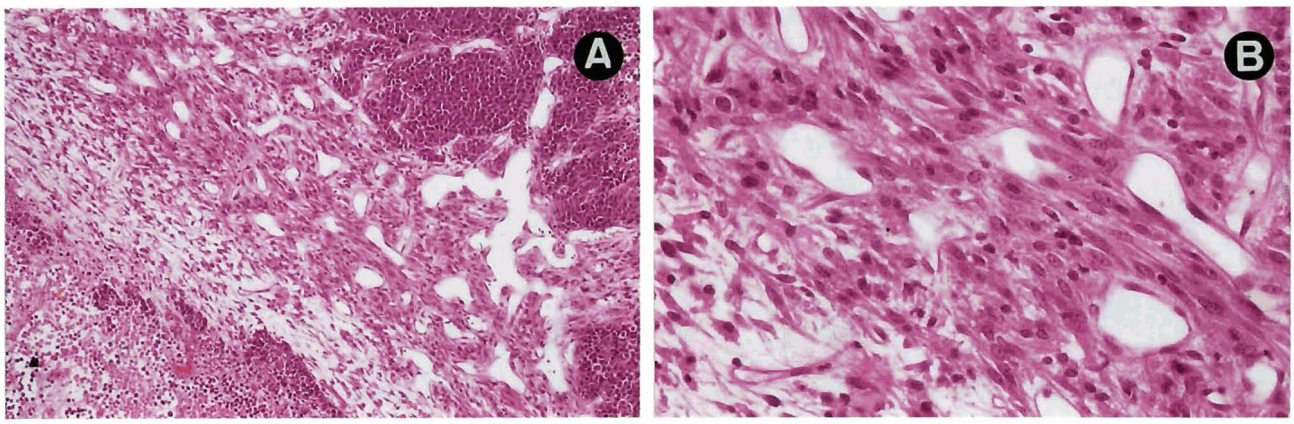

Fig. 5. Subcutaneous tumor. Area of mesenchymal differentiation. **A.** Notice the presence of numerous blood capillaries. **B.** Magnified region showing the mesenchymal aspect of cells and the prominent endothelium of the blood capillaries. Hematoxylin-eosin stain. A, x 32; B, x 100

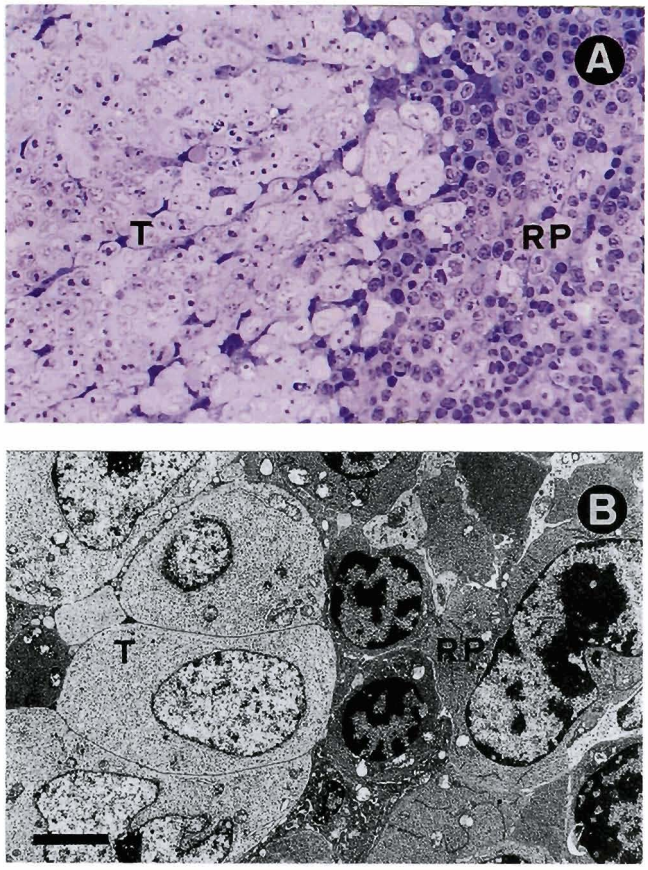

Fig. 6. Intrasplenic tumor of CE44 teratocarcinoma. **A.** Two regions can be observed: a splenic red pulp (RP) and a region showing a homogeneous proliferation of undifferentiated cells of the tumor (T). **B.** At the electron microscopic level, the cells of the teratocarcinoma (T) are clearly identified with respect to erythroblasts and cells of the splenic red pulp (RP). Note the sudden transition between the tumor and the red pulp, an expression of the expansive growth. A, Toluidine blue stain, x 100; B, bar: 2 μm.

which were being substituted by loose connective tissue where nests of undifferentiated cells of embryonal carcinoma persisted inside (Fig. 8C). Some necrotic foci were completely cicatrized (Fig. 8D). There was no sign of an inflammatory response associated with this process. These facts suggest that we were beholding a process of tumoral differentiation which was not haphazard, but was specifically orientated.

In some cases, the animals with experimental hepatic metastases presented pulmonary tumoral nodules consisting of embryonal carcinoma cells. It is interesting to draw attention to the occasional incidence of these pulmonary nodules, in spite of the great size of the hepatic metastases and of the frequent presence of undifferentiated tumoral cells within the intrahepatic blood vessels.

Discussion

In teratomas and teratocarcinomas practically all of the more or less mature tissues, haphazardly intermixed, have been described. By 1952, Dixon and Moore had considered embryonal carcinoma as a tumor of totipotential cells with the capability of giving rise to embryonic derivates (precursors of a teratoma) and to cytotrophoblastic cells (precursors of choriocarcinoma). In principle, teratocarcinomas consist of at least one of the embryonary (ectoblast, mesoblast and endoblast) or extraembryonary (trophoblast, extraembryonary mesoblast and viteline sac) germinal layers (Gaillard, 1974). However, according to this same author, this simplication "is only an illusion", because if the formula of factorial combinations is applied "one notes that these six germ layers can give rise to 57 combinations in addition to the 6 original forms. However, combinations by two's or three's are the most common". The same author points out that "this is in accordance with the order of appearance of these layers during development, as well as with the epigenetic character of this

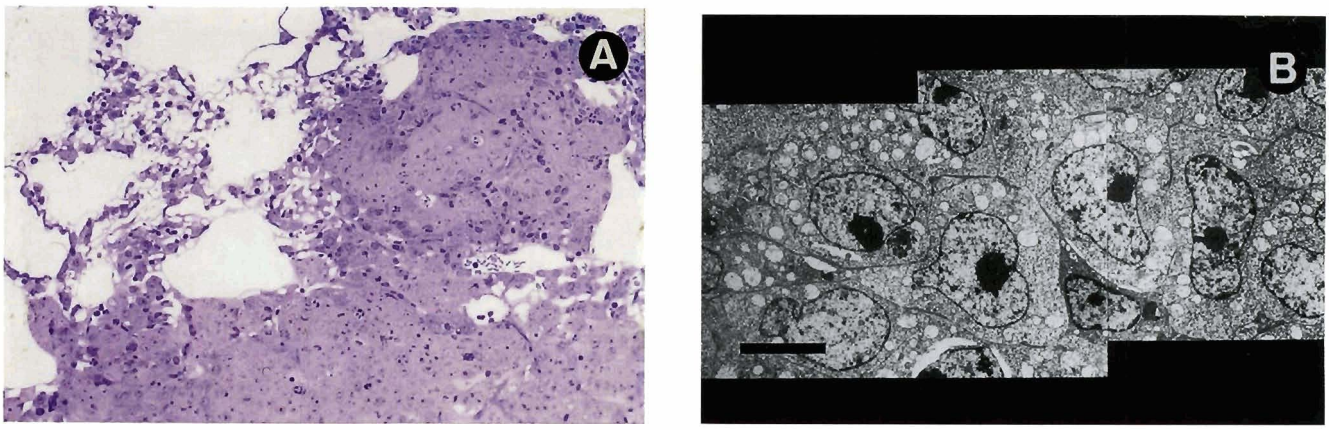

Fig. 7. Experimental pulmonary metastases of CE44 teratocarcinoma. **A.** Nodular growth of the tumoral cells is observed. **B.** Photographic reconstruction shows the cellular homogeneity of the metastatic foci, consisting of undifferentiated cells of embryonal carcinoma. A, Toluidine blue stain, x 50; B, bar: 5 μm.

development".

Pierce and his team were convinced of the epigenetic nature of neoplastic differentiation and, encouraged by the results of Leo Sachs's group (Gootwine et al., 1982), began to study embryonal regulation of other tumors during organogenesis (see Aréchaga, 1993). These studies consider cancer as a problem of cellular differentiation, which is therefore influenced by the microenvironment. In the present work, we have observed that CE44 teratocarcinoma shows variations in its capacity for differentiation in the different organs studied. With respect to the two primary tumors, we observed that while in the subcutaneous tumors there was a differentiation into the three embryonic layers, in the intrasplenic tumors the presence of differentiated tissues was scant; although we can not exclude the fact that in some cases there could be a greater degree of differentiation. Our results also show a greater degree of differentiation in hepatic metastases than in intrasplenic primary tumors. These results support the hypothesis that the microenvironment is decisive in the biological behaviour of totipotent cells of embryonal carcinoma, influencing the expression of certain capacities for differentiation.

In hepatic metastases we have found the presence of areas of tissue differentiation tending to repair areas of intratumoral necrosis, suggesting the existence of a specifically directed process of differentiation. This behaviour of the CE44 teratocarcinoma cells is in accord with the fact that malignancy of teratocarcinoma stem cells can be epigenetically regulated (Damjanov et al., 1983).

When in our laboratory the embryoid bodies of the CE44 teratocarcinoma were exclusively maintained by in vivo serial transfers we observed a loss of their capacity for chondral differentiation in the subcutaneous tumors of the animals injected with these embryoid bodies. On the other hand, when the embryoid bodies were cultivated in vitro and the cells of the monolayers were injected, the subcutaneous tumors recovered their capacity for chondral differentiation. These findings suggest that the loss of differentiation does not necessarily involve a loss of potentiality, but of expression. Moreover, when the tumors were cultured in vitro and later the cells were innoculated subcutaneously, on occasions spontaneous pulmonary metastases

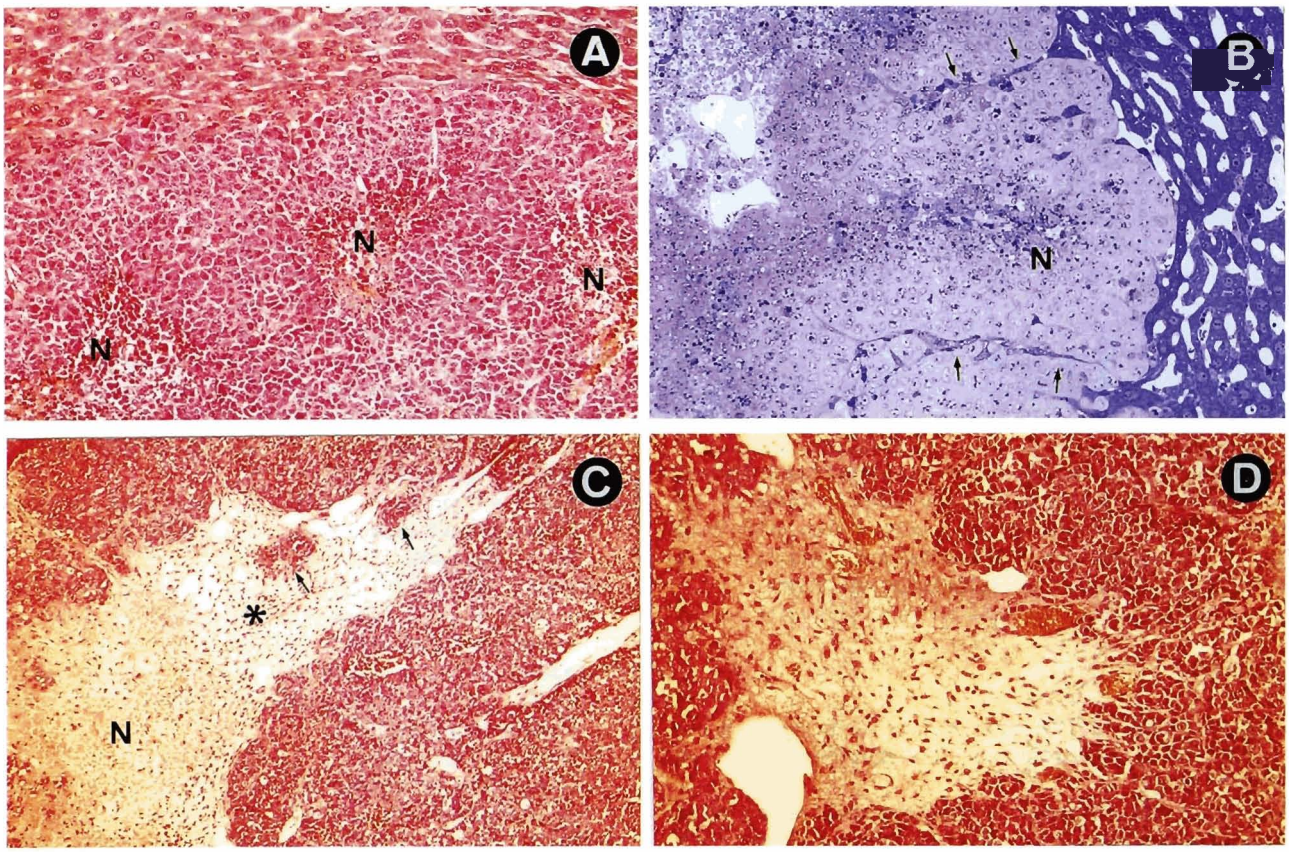

Fig. 8. Experimental hepatic metastases of CE44 teratocarcinoma. **A.** The metastatic foci were mainly composed of undifferentiated cells. Necrotic areas (N) were also observed. **B.** Intratumoral blood capillaries (arrows) and a central necrotic area (N) can be recognized. **C.** Focus of necrosis (N) partially substituted by loose connective tissue (asterisk), where nests of embryonal carcinoma cells persist (arrows). **D.** More avanced (mature) cicatrization of a area of necrosis. A, B and C, Hematoxylin-eosin stain; D, Toluidine blue stain. A and B, x 50; C, x 25; D, x 50

appeared. These facts support the existence of an epigenetic influence on the undifferentiated tumoral cells of teratocarcinoma. The absence of tissues more or less mature or recognizable in teratocarcinoma does not mean that it has lost its identity, since their undifferentiated cells (with a malignant behaviour) have the capacity for differentiating in an appropiate microenvironment (Pierce et al., 1959).

Two cell populations have been described in the embryoid bodies of this teratocarcinoma: one undifferentiated (embryonal carcinoma cells) and the second with endodermic differentiation (Parchment et al., 1990; Unda et al., 1994). The cultures in vitro of the embryoid bodies give rise to monolayers that cytometrically are grouped into four cellular populations (unpublished observations). This could be related to a differential epigenetic selection of certain cell populations or to a differential expression of their potential for differentiation.

Already, in 1967, Pierce postulated that teratomas and teratocarcinomas represented caricatures of normal embryogenesis (Pierce, 1967; Pierce and Speers, 1988; Pierce et al., 1988). Pierce's crucial insight into carcinogenesis was that it was due to a flaw in the normal process of tissue renewal. This idea was developed and published in a series of review articles and lectures (see Aréchaga, 1993). In 1994, Sell and Pierce published a paper in which they reviewed the origins of teratocarcinomas, premalignant epithelial lesions and epithelial cancers. They hypothesized that cancer is the consequence of a maturation arrest of immature "stem" cells and that the phenotype of the cancer is determined by the stage of differentiation at which the majority of the tumor cells are arrested. In this respect, we think that some types of tumors, such as teratocarcinoma, are a result of disturbances in the program of differentiation of their cells and that the malignant character is only an adjective that defines the consequences that its growth has for the host and not an anthropomorphic concept of its behaviour.

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