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Cells from the inner mass of blastocyst as a source of neural derivates for differentiation studies

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Summary. Our results show that cells derived from the inner cell mass (ICM) show a clear tendency to differentiate into the neural lineage, showing both cells and structures in different degrees of differentiation. Among the experimental paradigms used to learn about neural differentiation, there have been several lines of investigation on stem cells, including embryonic stem (ES) cells isolated from the inner cell mass of embryo and also stem cells derived from embryonic carcinoma (EC). In this work, we have used a cellular line obtained from the inner cell mass of a blastocyst. The cells were cultured and after inoculated subcutaneously in syngenic mice. The neural differentiation was predominant, and could be observed both by morphological and immunohistochemical methods. It was represented by neural-tubes, neurons and glial cells, as expressed by the presence of Microtubule-associated protein-2 (MAP-2) and glial fibrilary acidic protein. Moreover, tyrosine hydroxilase positive labelling was found in neuron-like cells, which suggest the chatecolaminergic differentiation. These results show that isolation of cells from the inner mass of blastocyst represents an easy, reproducible and cheap source of neural derivates suitable for both in vivo and in vitro differentiation studies.

Key words: Stem cells, Differentiation, Neurogenesis, Embryonal carcinoma

Introduction

Among the experimental paradigms used to learn about neural differentiation, there have been several lines of investigation on stem cells. The possibility of transplantation of these stem cells into an adult or developed nervous system to develop, integrate and rebuild destroyed nervous pathways has encouraged the study of the mechanisms of neural differentiation.

These processes leading to neuronal differentiation are now of ever increasing interest because of the recent development of research into neural stem cells (NSC). These cells are nowadays being called a panacea in every neurological disease and, although their functional role in the living human brain is still largely unknown, they have been found present in several areas of the adult human brain (Cameron and McKay, 1998; Eriksson et al., 1998; Marmur et al., 1998; Gould et al., 1999; Roy et al., 2000; Arsenijevic et al., 2001; Manev et al., 2001; Nunes et al., 2003). In experimental animals they have been demonstrated to divide and, also, to integrate in normal or lesioned tissue and to mature into fully developed neurones in rodents (Snyder et al., 1997), and even in the primate brain (Gould et al., 1999). Morover, other cells besides NSC, are being used in studies on the development of the nervous system, i.e. embryonic stem (ES) cells isolated from the inner cell mass (ICM) of embryo and also stem cells derived from embryonic carcinoma (EC). ES cells isolated from the ICM of mouse embryos can be cultured in vitro. When reintroduced into an early embryo they can give rise to all cell types in the recipient. However, transplantation of undifferentiated ES cells into animals results in the formation of teratocarcinomas. The study of neural differentiation of EC cells has been performed mainly along predetermined lines which can be induced to undergo neural differentiation. One of the pioneers was the P19 embryonic carcinoma cell line (McBurney and Rogers, 1982), which differentiates easily into neural tumor and muscular pathways in vitro (Jones-Villeneuve et al., 1982; McBurney et al., 1982). However, in most of the experimental designs, the neural differentiation is the result of the treatment of cell lines growing in vitro; retinoic acid being one of the most popular agents promoting differentiation (Edwards and McBurney, 1983; Jones-Villeneuve et al., 1983).

The studies on neural differentiation performed with the P19 EC, have given a number of results on the chronological landmarks of the processes leading to the neuronal development. There have been enlightening studies on microtubule building, and appearance of

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microtubule associated proteins (MAP) (Tanaka et al., 1992), neurite growth and branching (Berger et al., 1997), etc. Moreover, most characteristics of the mature neurons have been demonstrated in cells from EC (Alvarez et al., 1999), and, when fully developed, these cells contain neurotransmitter producing enzymes like choline acetyltransferase (Parnas and Linial, 1997a) and nitric oxide synthase (Gath et al., 1999) and they also show neurotransmitter receptors (Reynolds et al., 1994; Chen et al., 1999). Most interesting, ultrastructural demonstration of normal synapses (Staines et al., 1994) as well as eletrophysiological maturation has been described (Reynolds et al., 1994, 1996).

The EC P19 has also been shown to differentiate into the glial strain, on the same inductor as the neuronal strain. Glial fibrillary acidic protein (GFAP) has been demonstrated in P19 cultures in astrocyte like cells (Gath et al., 1999) but can be absent from the progeny of other EC lines (Wartiovaara et al., 1984).

However, one of the drawbacks of the P19 EC is that it only develops in vitro, and, although it has been later transplanted and found to survive in the mature CNS, previous in vitro development and differentiation under exogenous induction is required. In the present study, we have studied an embryonal cell line, developed from blastocyst internal mass as described by McMahon and Bradley (1990) and later transplanted into mice. The so formed tumor is characterized by in vivo development of areas of neuronal-like differentiation inside a solid tumoral mass, even in the absence of any exogenous inductor.

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 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).

Obtaining of cell line

We have used a cellular line obtained from the inner cell mass (ICM) of a blastocist. The best procedure to obtain blastocists is from pregnant females (normally, to increase the number of embryos, the artificial hormone-stimulation is used). Adult female mice (129/Sv) are paired with males and checked daily for vaginal plugs. 3.5 days after coitum (vaginal plug). Pregnant females were killed by cervical dislocation and uterus dissected.

The blastocists are extracted by perfusion of the uterus and maintained in M2 medium (Sigma Co. Mo, USA) for three days, until the zona pellucida break and the blastocists rise out from them.

Culture medium (MSC-1) was made with Dulbecco's modified Eagle´s medium (Sigma Co. MO, USA) containing 10% foetal bovine serum with 6 mM glutamine (Sigma Co. Mo, USA), 1% sodium piruvate (Sigma Co. MO, USA), 0.1% non essential amino acids (Sigma Co. MO, USA) and 1% nucleosides (Sigma Co. MO, USA) and 0.5 % penicillin-streptomycin (Sigma Co. MO, USA) added. Culture conditions were 37 ºC in an atmosphere of 5% carbon dioxide in air. After 12 hours in culture, trophoectoderm cells appeared attached to the surface of the flask, while inner cell mass remained as a clump.

To remove inner cell mass, the best way is to add 1 mL (every 5 blastocysts) of trypsin-EDTA (Gibco, USA) for 3 minutes and hold it with a glass pippet. Then, add trypsin (2 mL) and maintain it at 37 ºC for five minutes. Centrifuge the cells at 390 g for 5 minutes, discard supernatant and place them in a flask, previously treated with gelatine 0.1% (Sigma Co. MO, USA).

The maintenance of the cell line consist of the addition of fresh medium (MSC-1), with 20 ng/mL of leukemia inhibitory factor (LIF) (for maintenance of the undifferentiated stage) every two days and transfer of the subculture to another flask every four days.

Tumor 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) were obtained for inoculation into the animals.

Mice (n=5), previously anaesthetized with Nembutal (1.2 mg/muose, i.p.), were subcutaneously inoculated with 0.1 ml of the suspension containing the cells, as previously described (Alvarez et al., 1999).

Mice were killed by cervical dislocation from 21 to 45 days after injection of tumor cells. The subcutaneous tumors were fixed in phosphate buffered formaldehyde, embedded in paraffin and processed for light microscopy. Histological sections (5 μm thick) were obtained and distributed in 6 series for immunohistochemistry and routine histological staining with hematoxylin-eosin or with cresyl-violet.

Immunohistochemical procedures

Paraffin embedded sections were cleared of paraffin in two steps in xilene (10 minutes each), re-hydrated in descending series of ethanol-water (2 minutes each step) and washed in two steps in distilled water (5 minutes each).

Sections were rinsed for 10 minutes in phosphate buffered saline (PBS) and pre-incubated for 1 hour in blocking solution consisting of 10% normal horse serum (NHS) and 0,6% Triton-X in PBS. Immunohistochemical incubation was performed as follows: one section out of 5 was incubated with antibodies against Microtubule-associated protein-2 (MAP-2) (Sigma Co. MO, USA) diluted 1:250, as a marker for neuronal differentiation, another section was tested with antibodies against glial fibrilary acidic protein (GFAP) (Intergene, NY, USA), as a marker for glial differentiation, diluted 1:500 and a third section was incubated with antibodies against tyrosine hydroxilase (TH), an enzyme present, although not exclusively, in neurones using chatecholamines as neurotransmitters, (Diasorin), diluted 1:1000. All antibodies were diluted in 10% NHS and 0,6% triton-X in PBS.

Tissue bound antibodies were detected using a commercial kit to enhance immunohistochemistry on mouse tissue (MOM, Vector), with biotinilated secondary antibodies and developed using commercial ABC complex and DAB kit (Vector). After development of the immunoreaction, sections were photographed, counterstained with cresyl violet, dehydrated and coverslipped.

In every series, one was kept as a negative control and processed together with the rest of the immunotested sections through all the steps, except for the addition of the primary antibody, which was omitted and substituted for the buffer.

Results

Morphological study

The study of the tumors showed that they were made up of undifferentiated cells of embryonal carcinoma and other cells showing different degrees of differentiation towards the three embryonal layers (Fig. 1). The carcinoma cells were similar to those already described initially by Pierce and Beals (1964), having a nucleus of dispersed chromatin with one or more nucleoli and a cytoplasm abundant in ribosomes but lacking in other organelles. Some areas of necrosis and apoptotic images were present.

The neural derivates were the most common representing about the 70% of the tumor surface. They consisted mainly of neural tubes and neuronal-like cells (Fig. 2). Sometimes, pigmentary epithelium, resembling the pigmentary layer of the retin, was present. Wide areas of neural derivates, without the presence of other germ layer derivates, were observed in many cases. Between undifferentiated and more differentiated neural elements a soft gradient was usually present, although a clear cut border could also be observed. Other derivatives from the ectoderm were represented by small foci of stratified squamous keratinizing epithelium, with presence of a granular cell layer.

Foci of cartilage were occasional and no endochondral ossification were associated. Foci of mesenchymal differentiation resembling muscular tissue was present showing cells with an eosinophil cytoplasm although cytoplasmic striations were, in any case, observed. Blood capillaries with prominent endothelium were found in these foci of mesenchymal differentiation, although they were also present in the neural areas.

The endodermic differentiation was represented by tubular structures lined by cylindric or cubic cilliated epithelium. Mucous goblet cells were interposed among the cells lining the tubes.

Neural and glial differentiation

Immunoreactive tissue was found for the three tested

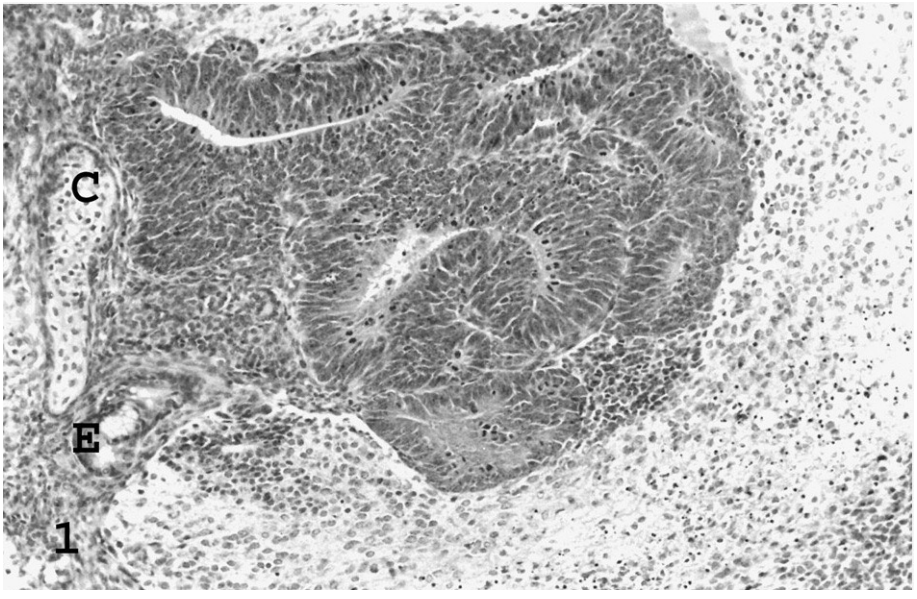


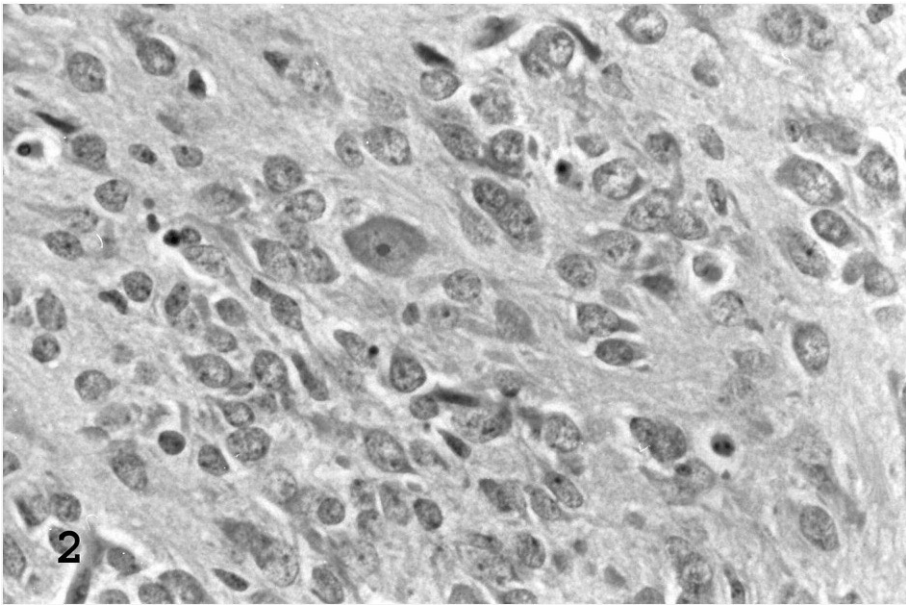
Fig. 1. Subcutaneous tumor obtained from the inner cell mass of a blastocyst. Wide areas of neural differentiation, represented mainly by neural tubes and isolated cells, can be observed. Condral (C) and endodermic differentiation (E) are also appreciated. Hematoxylin-eosin. x 200

antigens, as distinct DAB precipitate inside areas of neural like differentiation. This precipitate was differently arranged for the different antibodies. Also, no specific labelling was found in the negative controls processed without the primary antibody.

Even at low magnification, wide areas of MAP-2 positive labelling could be seen, intermingled with immunonegative fields, but always a clear-cut boundary was present (Fig. 3). The reaction was thus mostly found as wide labelling, encompassing huge groups of cells. Immunoreactive tissue was coincident with areas where neuron-like cells were located.

When observed in detail, MAP-2 positive labelling was strong enough to clearly label the cytoplasm of individual cells inside the labelled patches and neuron looking cells were demonstrated (Fig. 4). Unlabelled nuclei could be seen, round and wide inside the labelled cytoplasm. However, on occasions, cells were found with features different from the classic central neuron type (e.g. dots of condensed cromatin adjacent to the nuclear membrane, or clear cytoplasm) and degree of staining was not uniform for all the cells inside the labelled patch.

In strongly labelled cells, the MAP-2 immunoreactivity could be seen in the perikarya and even in the main dendrites, when sectioned in the correct



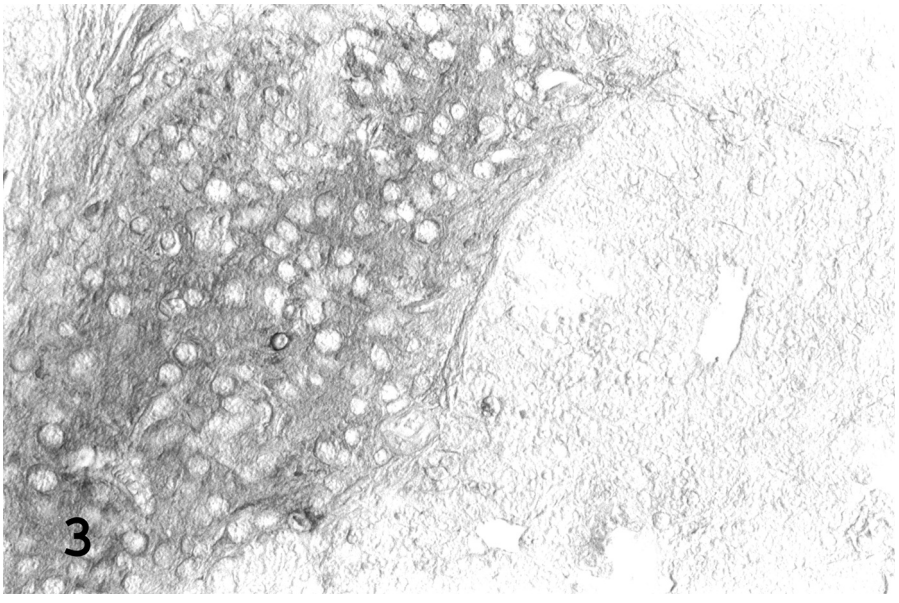


Fig. 2. Isolated cells showing a neuronal-like phenotype. Hematoxylin-eosin. x 400

Fig. 3. MAP-2 like immunoreactivity inside the solid tumor mass, labelled with DAB labelled without cresyl violet counterstaining. The area of neural-like differentiation is immunoreactive for MAP-2 showing a sharp contrast against the immunonegative areas. The homogeneous MAP-2 labelling is cytoplasmatic and the big round clear nuclei of neuron-like cells can be seen unlabelled. x 400

direction. Surrounding the neurons and filling the immunopositive patch, fainter labelling appeared in small threads, suggesting labelling of neuropilar structures, mainly thinner dendrites. In a section specially fortunate, a bundle of roughly parallel dendrites is seen (Fig. 4).

Tissue processed to demonstrate GFAP-like immunoreactivity showed areas of distinct positive reaction. The labelling, clearly reminiscent of the normal features of central glia, was distributed in thick, poorly ramified, sinuous processes and small polygonal perikaria in a negative field (Fig. 5), but no diffuse or fainter labelling was found among cells. The labelling was in this respect dramatically different from the diffuse and almost uniform staining obtained with MAP-2.

Tissue reacted to TH-like immunoreactivity showed intense labelling in wide areas of tissue. At the cellular level, intense TH positive labelling was found in the cytoplasm of neuron like cells, including processes, similar to dendrites (Fig. 6). A fainter TH positive signal was found in epitelial looking groups of cells, characterised by a smaller nuclei with condensed cromatin.

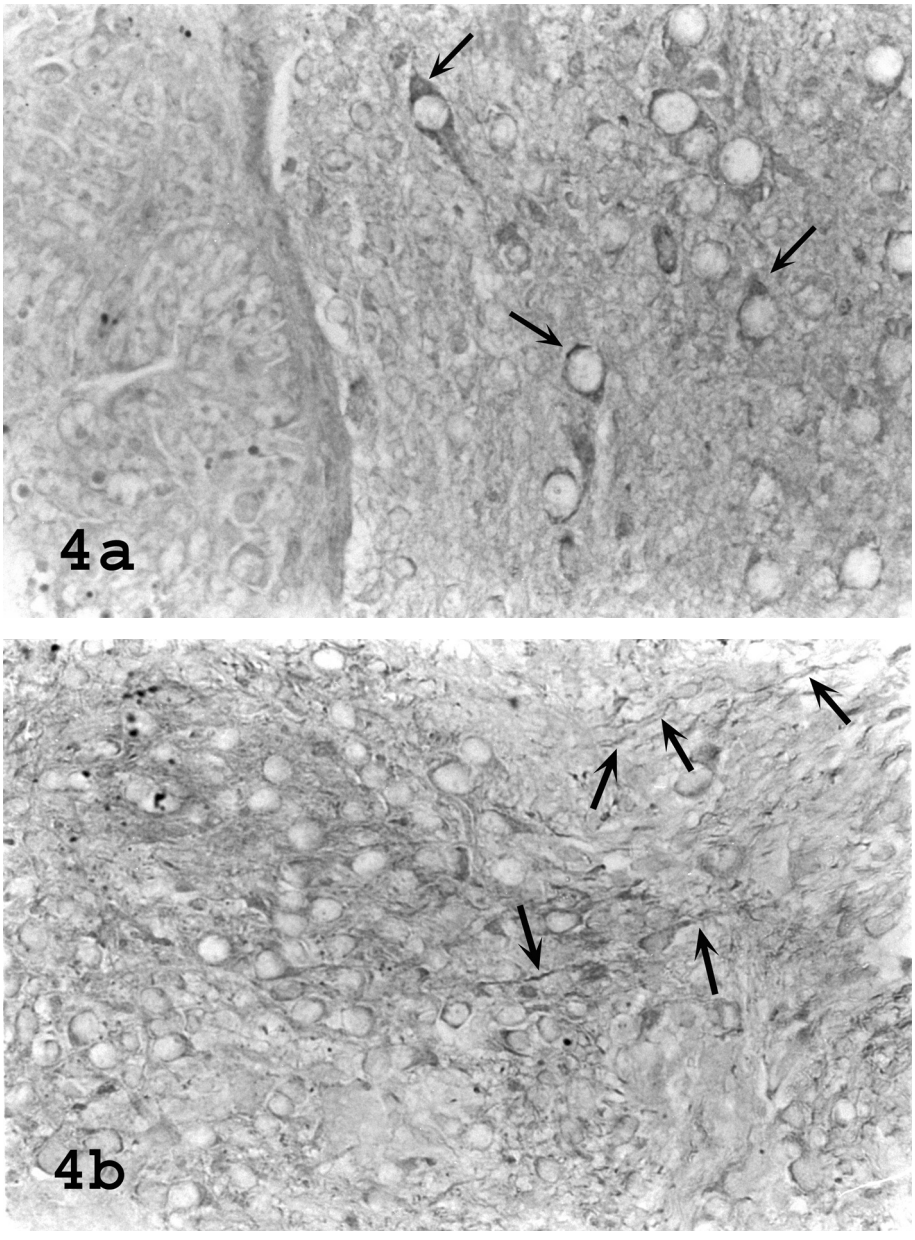


Fig. 4. Demonstration of MAP-2 immunoreactivity. **a.** Area of MAP-2 positive tissue close to an immunonegative area. The cytoplasmic labelling is strong enough around the round immunonegative nuclei to show the cell body and main dendrites of some neurons. The surrounding tissue, presents a fainter labeling and is probably made up of thinner processes. **b.** Another area of MAP-2 labelling where many neuron looking immunopositive cells crowd. Together small bundles of thin processes, presumibly dendrites can be seen (arrows) x 400

Discussion

The results show that cells derived from the ICM are able to differentiate, after their culture and transplantation into mice, into several cell lines. There is a clear tendency for these cell lines to differentiate into the neural line, with preference to other germ layer derivatives. The neural differentiation can be observed both by morphological and immunohistochemical means, for neuron looking cells can be identified inside the solid tumor mass and current markers for neural tissue can be demonstrated, both for neurons (MAP-2) and glial cells (GFAP). These markers are considered to be exclusive of the afore mentioned cell types and, although anomalous protein expressions can be found in tumoral cell lines, the labelled cells display the morphological features expected for each labelling. Besides, TH positive labelling, which is a functional maturation marker for chatecholaminergic neurons were found in neuron looking cells. However, TH positive labelling is not as specific as the GFAP and MAP-2 and can be found in cells different from the neurons, (i.e. melanocytes). Also present in our study were small epithelioid cells outside the areas of neural

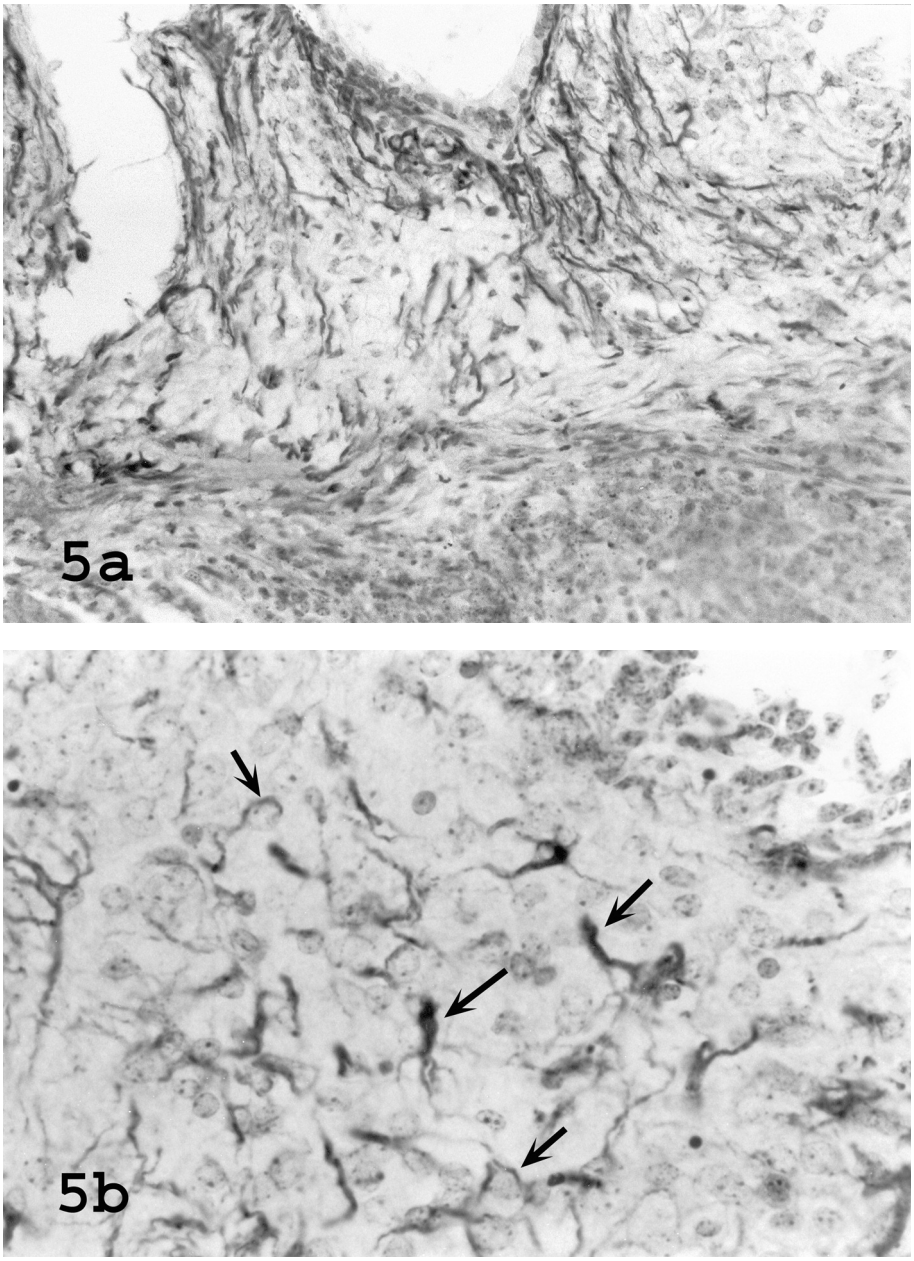


Fig. 5. Picture of the tumor processed to demonstrate immunoreactivity for GFAP and counterstained with cresyl violet. **a.** Picture of a patch of neural differentiation inside the tumoral mass. GFAP immunopositive labelling can be identified in sinuous processes. x 200. **b.** Higher magnification to demonstrate glial morphological landmarks of the GFAP positive labelling. Small poligonal bodies are seen (arrows) and sinuous processes surround neuron looking immunonegative cells. x 400

differentiation. However, inside the neural patches the degree of labelling was strong enough to demonstrate neuronal morphologies for the labelled cells.

The immunohistochemical reaction product was seen to clearly label the immunopositive structures and for GFAP and TH it was an all-or nothing reaction that gave no reign to false positives. It could be argued that the fact that it was carried out on mouse tissue with antibodies produced in the mouse can elicit cross reactions of the labelled secondary antibodies on the tissue, but the use of a neutralization solution gives a clear picture that is by no means unspecific. This was tested on the negative controls that were processed for the secondary antibody with no incubation of primary antibody, and subsequently gave no positive reaction. The difference between the three labellings tested can also be observed in this study, specially by the striking difference between GFAP and the other two labellings.

The differentiation of the cells derived from the ICM into neural tissue, as observed in this study, is spontaneous and not dependent on any pharmacological inductor. This is different from cell lines obtained from embryonal carcinoma and tested for neural development that need the addition of an inductor like retinoic acid (Jones-Villeneuve et al., 1982, 1983), dibutyryl cyclic AMP (Liesi et al., 1983), or NGF (Sharma et al., 1990).

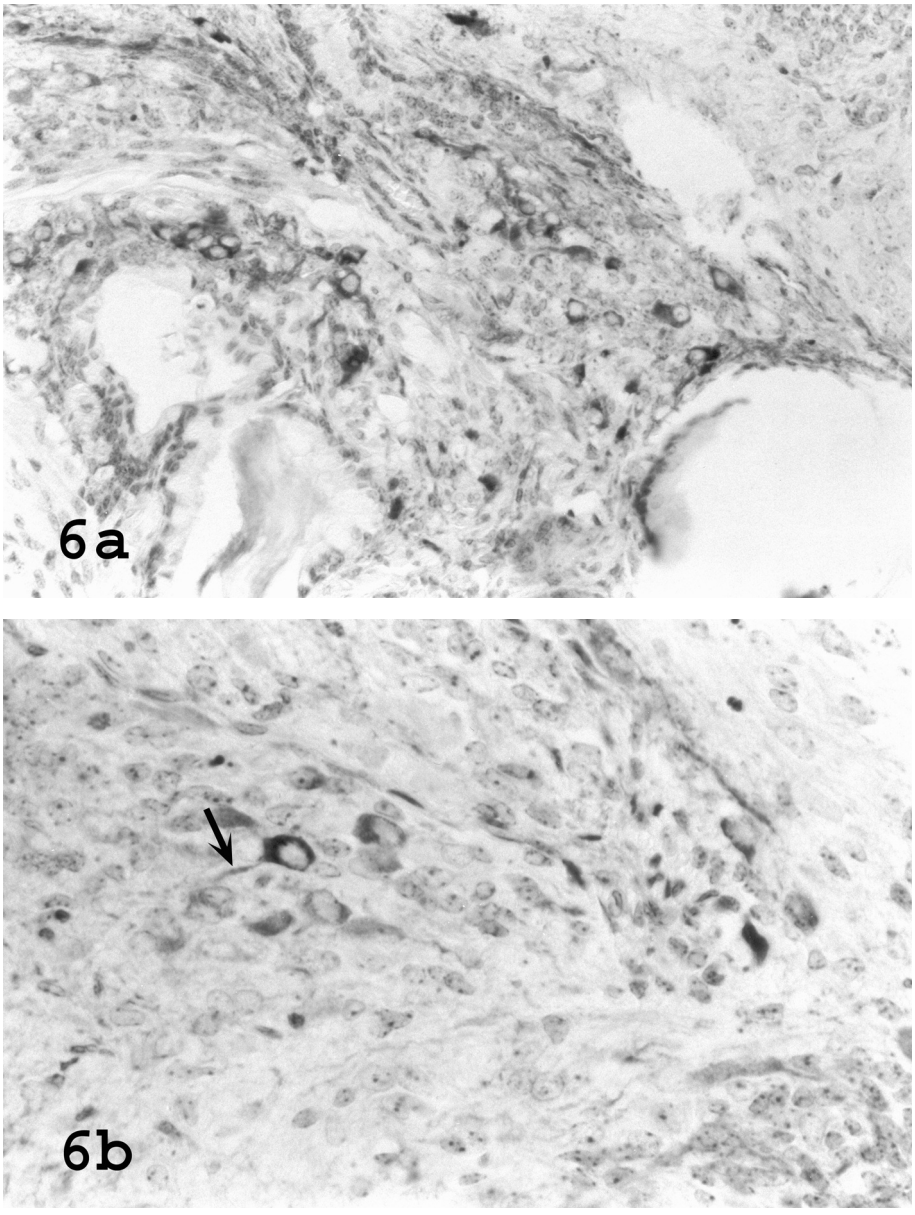


Fig. 6. Demonstration of tyrosine hydroxilase (TH)–like immunoreactivity inside tumoral mass. **a.** Low magnification picture of a particular area of a developed tumor where several cells are positive for TH immunoreactivity. x 200. **b.** Higher magnification of a different area where some other TH positive cells can be seen. The labelling is enough to demonstrate the neuronal features of the labelled cells and even a dendrite can be seen (arrow). x 400

Inside the neural-like tissue, varied types of neural structures can be found from presumed neural tubes to fully developed neuron and glia looking cells. Development of both cell types takes place in the same patches of the tumor mass.

MAP-2 positive cells have not only the markers but also the morphological features and cytoarchitecture of neurons. They appear in patches and are not mixed with any other kind of tumor derived cell except for glial looking ones which are evident when labelled for GFAP. This is similar to the organization found normally in developed tissue of the central nervous system.

For their part, GFAP labelled presumed glial cells do not cluster but intermingle with neurons and their processes seem to surround neural cell bodies. This picture suggests that their appearance is gradual and simultaneous with that of the neurons and that this enables them to achieve a normal location among the neurons. It is likely that both kinds of cells are generated from the very neural tubes detected on the outskirts of the MAP-2 and GFAP positive areas. These characteristics are different from the results obtained with NSC, that are able, when exposed to mitogens in vitro, to give rise to neurons and glia in a successive and clonal fashion. The coincidence of regular neuronal and glial morphologies in the most differentiated areas of the tumor, is an indication that the maturation of the neural tissue from the embryonic carcinoma can be harmonic. The neuronal versus glial differentiation has been proved to depend on a gene Wnt (Tang et al., 2002), and inside the embryonic carcinoma different cells activate or inactivate the pathway to glia or neurons. The implied factors are unknown, but the fact that it happens on a living host makes possible a diffusible factor produced in vivo. On the other hand, cell density improves and accelerates neuronal differentiation in culture (Parnas and Linial, 1997b; Kitani et al., 1997; Alvarez et al., 2001).

Besides the morphological features of mature neurons, the enzyme tyrosine hydroxylase, which is the first step of the chatecholamine synthesis, can be found in neuron looking cells. The presence of this enzyme, strongly suggests the ability to synthesize catecholamines and use them as neurotransmitters and consequently a particular neurotransmitter phenotype for some of the generated neurons. This is coincident with other reports in the literature where cell lines obtained from embryonal carcinoma, have been demonstrated to show functional maturation by detection of neurotransmitter related enzymes, for cell line P19 (Parnas and Linial, 1997a; Staines et al., 1994; Sharma and Notter, 1988) for cell line F9 (Wartiovaara et al., 1984) and for PCC7-Mz1 (Gath et al., 1999). Other kinds of demonstration include detection of neuropeptides (Wartiovaara et al., 1984; Staines et al., 1994) or receptors (Chen et al., 1999; Reynolds et al., 1994, 1996). Morphological features of synapses have also been demonstrated at the electron microscopy level (Staines et al., 1994) and even electrophysiological recordings (Reynolds et al., 1994, 1996).

Besides, the fact that not all the neurons in the neural tissue patch develop the same transmitter phenotype implies diverse differentiation patterns among the neurons derived from the transplanted ICM cells. In the same manner as neural differentiation itself, in the present study, catecholaminergic differentiation is spontaneous, while in tumors like F9 is induced by NGF (Liesi et al., 1983). Once again it remains to be resolved if this differentiation depends on the commitment of the cells or on some external influence. The appearance of transmitter phenotype following the differentiation of cells for neurons and not for glia, makes it possible for generated glia to be responsible for this modulation, either by segregating diffusible factors or by close cell-to-cell interactions. In fact, the cell to cell interactions are susceptible to the control of transmitter phenotype in neurons developed in vitro, where culture density enhances GABAergic over cholinergic differentiation in P19 line (Parnas and Linial, 1997a). In fact, astrocytes have been found to modulate the differentiation of neural stem cell towards neurons, this capability being related to the place of origin of the astrocytes rather than to the origin of the neural precursors (Svendsen, 2002; Song et al., 2002).

These results strongly support the idea that important neuronal characteristics are epigenetically regulated during development. These and other factors (Alvarez et al., 1999; Hilario et al., 2001) determining the specialization of undetermined cells for each specific type of neuron can enable us to unveil the factors involved in cell fate specification. This gives rise to the possibility of modulating pharmacologically the cells from the inner mass of the balstocyst in order to enhance the diferentiation in a particular cell line and to apply them for differentiation studies or therapy purposes.

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