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Original Article

A neural precursor cell line derived from murine teratocarcinoma

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ABSTRACT A cell line NT with phenotypic features of neural precursor cells has been established from an embryo-derived teratocarcinoma in Swiss mouse where, on serial transplantation, the developmental potential becomes restricted to neural pathway. All the cells are positive for nestin (a marker of neuroepithelial stem cells). Many of them are also positive for NFP and/or GFAP. Moreover there is a gradual decrease from 75% to 50% in reactivity for alkaline phosphatase, a marker for EC cells with repeated passages. The bipotential nature, and the probable decline of EC cells suggest that NT is a neural precursor cell line. The cells have doubling time of 12 h with a plating efficiency of 50%. The cells form colonies in soft agar within 7 days and tumorigenicity in syngeneic mice is lost after 70th passage. However, after 70 passages cells do form tumors in nude mice within 5 days and these tumors exhibit better differentiated morphology than the tumors in syngeneic mice. All the other characteristics remain stable. The myc and ras family of oncogenes do not show any alterations in early or late passages. This cell line may therefore be considered as a differentiated cell line derived from teratocarcinoma.

KEY WORDS: *neural precursor cell line, teratocarcinoma*

Introduction

During embryonic development, neuroepithelial cells forming the neural tube generate different cell types which include neurones and glial cells of the central nervous system. The regulation of the process of lineage commitment and differentiation of primitive neuroepithelial cells (neural precursor cells) has evoked keen interest amongst developmental biologists (McKay, 1989; McConnell, 1991). Nestin (neuroepithelial stem cell intermediate filament) serves as a general marker for neural stem cells in rat central nervous system from the developmental stage of newly closed E11 neural tube to the postnatally developing cerebellum (Lendahl *et al.*, 1990). Neural stem cells have the potential to serve as vehicles to carry the appropriate genes for neurotransmitters or growth factors in gene transfer experiments to rescue the affected neurones in neurodegenerative disorders such as Alzheimer's and Parkinson's disease (Cattaneo and McKay, 1991; Geller *et al.*, 1991). Multipotential neural precursor cells can be cloned from developing brain (Kilpatrick and Bartlett, 1993) and they can be immortalized (Cepko, 1989; Lendahl and McKay, 1990). Alternatively, cell lines derived from teratocarcinomas, known as embryonal carcinoma cell (EC) lines, which differentiate along the neural pathway, can provide a continuous source of committed neural stem cells (Andrews, 1984; Lang *et al.*, 1989).

At our institute we had induced transplantable teratocarcinoma in Swiss mouse by grafting embryos under the kidney capsule of syngeneic recipients. The original tumor was composed of various types of tissues originating from all three germinal layers. On serial transplantation, the differentiating potential of this tumor got restricted to neural lineage and the tumors now consisted of mostly medulloepithelial ribbons, neuroblastic and ependymoblastic rosettes. After 90th transplant generation the tumors were so poorly differentiated that no histological architecture was discerned on light microscopy. However, ultrastructurally, the characteristic features of neuroblastoma, e.g. numerous cellular processes rich in microtubules, were seen (Thakare and Lalitha, 1988; Hasgekar and Lalitha, 1991). We report here the establishment and characterization of a cell line from the above mentioned tumor belonging to 130th transplant generation.

Results

The tumor which gave rise to the cell line, when examined by light microscopy, was seen to consist of sheets of poorly differ-

Abbreviations used in this paper: ABC, avidin biotin complex; E, embryonic day; EC, embryonal carcinoma; GFAP, glial fibrillary acidic protein; IF, intermediate filament; IGF-1, insulin-like growth factor; NFP, neurofilament protein; P, passage; RA, retinoic acid; SSEA-1; stage-specific embryonic antigen; TBE; tris buffer EDTA.

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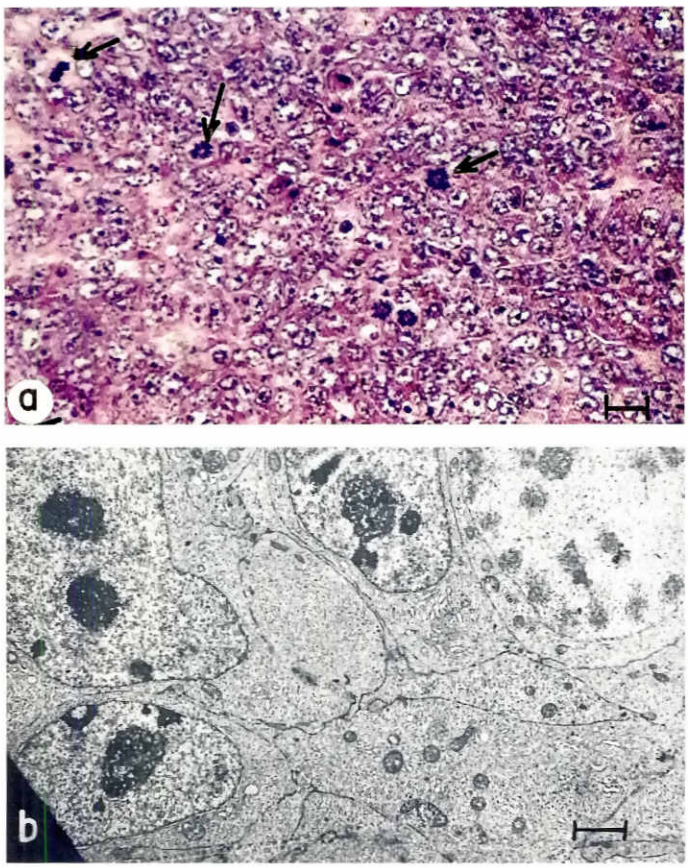

Fig. 1. Tumor belonging to 130th transplant generation. (a) *Shows poorly differentiated cells without any organization with many mitotic figures (arrows). Hematoxylene and eosin. Bar, 20 µm.* **(b)** *Fine structural features with interdigitating cellular processes, probably neuritic. Bar, 200 nm.*

entiated cells without any architecture (Fig. 1a). Electronmicroscopic examination revealed numerous cellular processes (Fig. 1b). The cell line could be maintained in an identical fashion in medium containing either 20% human adult serum (HAS) or 10% fetal calf serum (FCS) and we have used the former as it is more economical. Besides, when the cells were grown in low serum (0.1%) containing medium they could survive for several weeks and put forth long processes (data not shown). In high cell density cultures, the cells appeared polygonal and flat with vesicular nuclei bearing nucleoli (Fig. 2a), while in low density cultures they put forth short processes (Fig. 2b). The striking fine structural features were rough endoplasmic reticulum and mitochondria (Fig. 2c). All cells were positive for nestin by immunofluorescence staining. Nestin was seen in the cytoplasm adjacent to the nucleus and extending into processes (Table 1, Fig. 3a). The cultures were stained by immunoperoxidase method after confirming the specificity of the antisera on paraffin sections of adult rat cerebellum (Sawant *et al.*, 1994). The number of cells positive for NF 200, NF 150, and GFAP (Fig. 3b) were more in early passages while for NF 68 they were more in late passages (Table 1). The intensity of NF 68 was much more than that for NF 150 in late passages and it was the reverse in early passages (Fig. 3c,d,e and

TABLE 1

MEAN NUMBER OF POSITIVE CELLS@PER FIELD

	Nestin	NF-200	NF-150	NF-68	GFAP
Passage 50	10.88±1.00	13.52±0.86	17.68±1.67	10.00±0.98	18.60±0.77
Passage 90	9.20±0.78	7.72±0.80	13.00±0.99	16.72±0.77	13.00±0.96

* P<0.02; ** P<0.01; ***P<0.001. @Mean number of cells±S.E.

f). These findings were consistent with the observations in immunoblots (data not shown). In double labeled preparations, coexpression of nestin and GFAP or NF subunits was observed frequently (Fig. 4a and b). Surprisingly, colocalization of GFAP and NF 150 (Fig. 4c and d) or NF 68 was also noticed occasionally (Fig. 4e and f). In serum deprived cultures, cells expressed nestin and NFP but not GFAP. Alkaline phosphatase was present in 75%, 50% and 5% of cells at P50, P75 and P155, respectively.

The doubling time of cell population was 12 h in the exponential phase (Fig. 5) and the cultures reached confluence at day 3. The plating efficiency was 50%. The modal chromosome number for cells in early passages was 58 and it was 56 in late passage cells. When 50 metaphase plates were scored, it was seen that 32-34% of chromosomes/metaphase were metacentric and the rest were acrocentric. Gaps were observed in 58% of metaphases and deletion of fragments in 84%. Cells of various

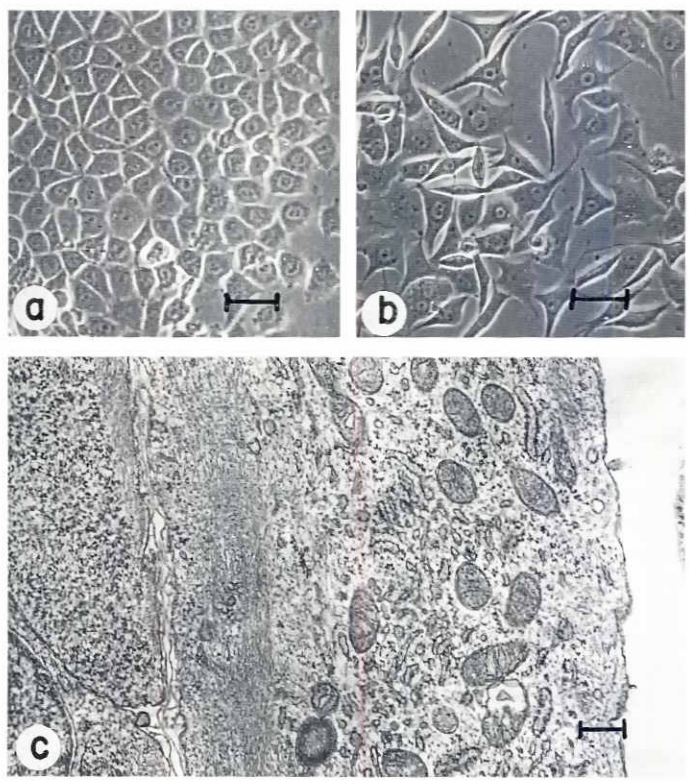

Fig. 2. Morphology of NT cells in culture. (a) *Epithelial cell like morphology in high cell density* **(b)** *process bearing cells in low cell density, Phase. Bar, 50 µm.* **(c)** *Ultrastructural-numerous mitochondria, rough endoplasmic reticulum and filaments in bundle. Bar, 3 nm.*

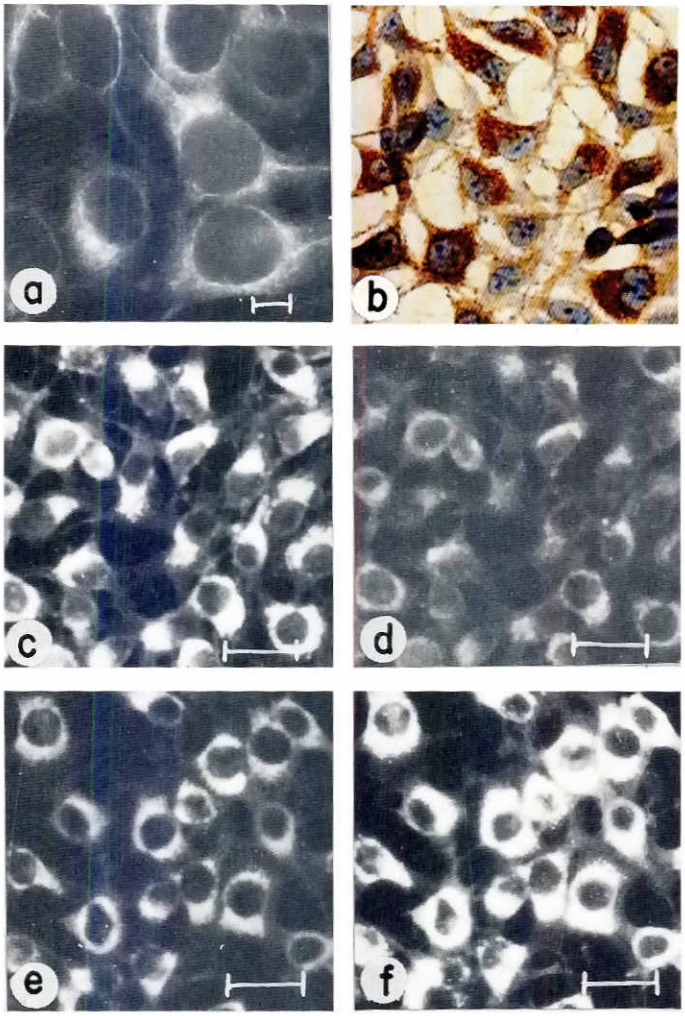

Fig. 3. Immunostaining of NT cells staining. (a) *Early passage cells with nestin positivity in cytoplasm and processes, FITC. Bar, 5 µm.* **(b)** *Early passage cells with GFAP positivity in some cells, ABC. Bar, 20 µm.* **(c and e)** *Early passage cells showing bright staining for NF 150 and weak staining for NF 68 respectively, FITC. Bar, 25 µm.* **(d and f)** *Late passage cells showing weaker label for NF 150 and stronger for NF 68, TRITC. Bar, 25 µm.*

passages could form colonies >0.2 mm in soft agar by 7 days. The colonies were large and necrotic by 21 days. The mean number of colonies per field was 2.5 and 3.05 in early and late passages respectively and the difference was highly significant ($p<0.001$). Up to 70th passage, the cell line was tumorigenic in syngeneic mice forming tumors within 4 weeks. These tumors consisted of poorly differentiated cells with high nuclear cytoplasmic ratio (Fig. 6a). The organelles in the cytoplasm of tumor cells were sparse except for free ribosomes (Fig. 6c). The genomic DNA of cells belonging to 40th and 90th passage, restricted with EcoR1, was checked for alterations such as amplification or rearrangement of N-*myc*, c-*myc*, N-*ras*, H-*ras* and K-*ras* oncogenes. Presence of specific fragments of N-myc with 8.0 kb, c-*myc* with 18.0 kb, N-*ras* with 7.2 kb, H-*ras* with >23.0 Kb and K-*ras* with 2.8 kb sizes (Fig. 7) were observed in both the passages.

Intraperitoneal injection of tumor cells up to P70 resulted in the formation of solid tumors within 6-8 weeks but they could not adapt to grow in suspension as ascitic form. In later passages, the cells failed to form tumors in syngeneic recipients even after irradiation and/or thymectomy on subcutaneous, intratesticular or subrenal inoculations. However, they were tumorigenic in nude mice with a latency of 5 days. These tumors were composed of cells with much smaller nuclei (Fig. 6b) than those seen in tumors in syngeneic mice. The striking fine structural features were rough endoplasmic reticulum and mitochondria (Fig. 6d).

Discussion

Cell lines derived from teratocarcinomas fall in two categories, (a) those containing EC cells expressing markers for the multipotent stem cells of the early embryo and (b) those consisting exclusively of cells committed for differentiation along a single

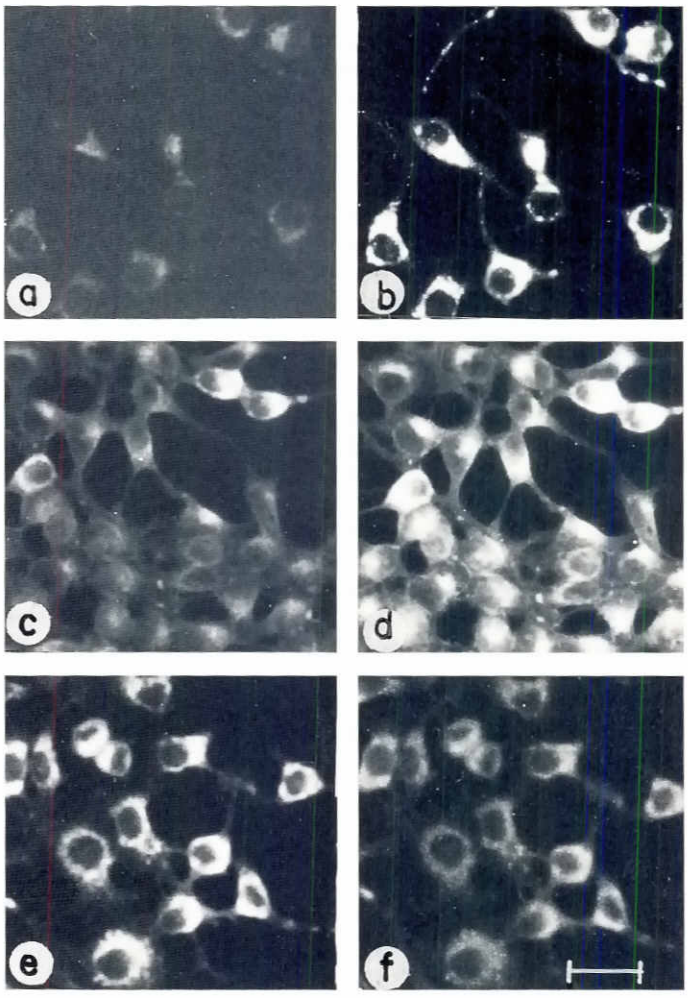

Fig. 4. Coexpression of nestin, GFAP and NFP in NT cells. (a and b) *Nestin and GFAP positive in all cells FITC & TRITC respectively. Bar, 25 µm.* **(c and d)** *GFAP and NF 150 positive cells of varying intensity in same cells, FITC &TRITC respectively. Bar, 25 µm.* **(e and f)** *NF 68 and GFAP positive cells of varying intensity in same cells, FITC & TRITC respectively. Bar, 25 µm.*

pathway (Jacob and Nicolas, 1987). Most of the teratocarcinoma-derived cell lines belong to the first group and contain a few differentiated cells, which in general, stop dividing after an initial period of proliferation. However, about a dozen differentiated cell lines have been established on cloning EC cell lines which are devoid of EC cells and differentiate spontaneously or on exposure to maturation inducers to a single lineage (Jacob and Nicolas, 1987). The immunopositivity for nestin in all the cells in the present study indicates that they are neural stem cells. The cell line therefore can be designated as NT (N for neural precursor and T for teratocarcinoma) rather than EC cell line because EC cells do not express nestin.

The presence of neurofilament and/or glial filament protein in NT cells indicates their ability to differentiate along both neuronal and glial lineages (Table 1). It differs from NT2/D1 (a clone derived from human teratocarcinoma cell line - Tera 2) which expresses only nestin and neurofilament protein but not GFAP and is equivalent to neuroblastoma. NT cell line being bipotential (when grown in the presence of 20% HAS), on the other hand resembles a medulloblastoma. Out of the several (over 50) cell lines derived from murine teratocarcinomas, neural differentiation has been documented spontaneously in two, either on aggregation or on serum deprivation (PCC7-S, clone 1003 derived from C17S1) (Pfeiffer *et al.*, 1981; Darmon *et al.*, 1982) and also on exposure to retinoic acid (P19, PCC7-S-Aza R1) (Jones-Villeneuve *et al.*, 1982; Levin and Flynn, 1986; Paulin *et al.*, 1990). However, the spontaneous bipotential differentiation observed in monolayer cultures of NT cells is comparable to RA induced differentiation of P19 cells in aggregate cultures grown in the presence of serum (McBurney, 1993). The exclusive neuronal differentiation in NT cells (which is a spontaneously differentiated parent cell line) by serum deprivation is very similar to the findings in a clonal cell line 1003, derived from C17S1. The fact that the tumor (teratocarcinoma) belonging to 130th transplant generation displays throughout fine structural features of a primitive neuroectodermal neoplasm explains the neurogenic potential of the NT cell line.

Differentiated cell lines were reported to be aneuploid and incapable of growing in ascitic form in contrast to EC cell lines which are diploid or near diploid and grow as embryoid bodies in the peritoneal cavity (Nicolas *et al.*, 1981). The ploidy status (aneuploidy) and inability of the NT cell line to grow in ascitic form suggest that it belongs to the former category. To rule out

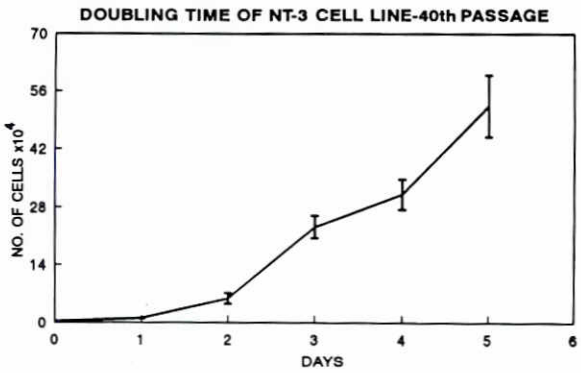

Fig. 5. Growth kinetics of NT cells at P40. *Each point is the mean of three values.*

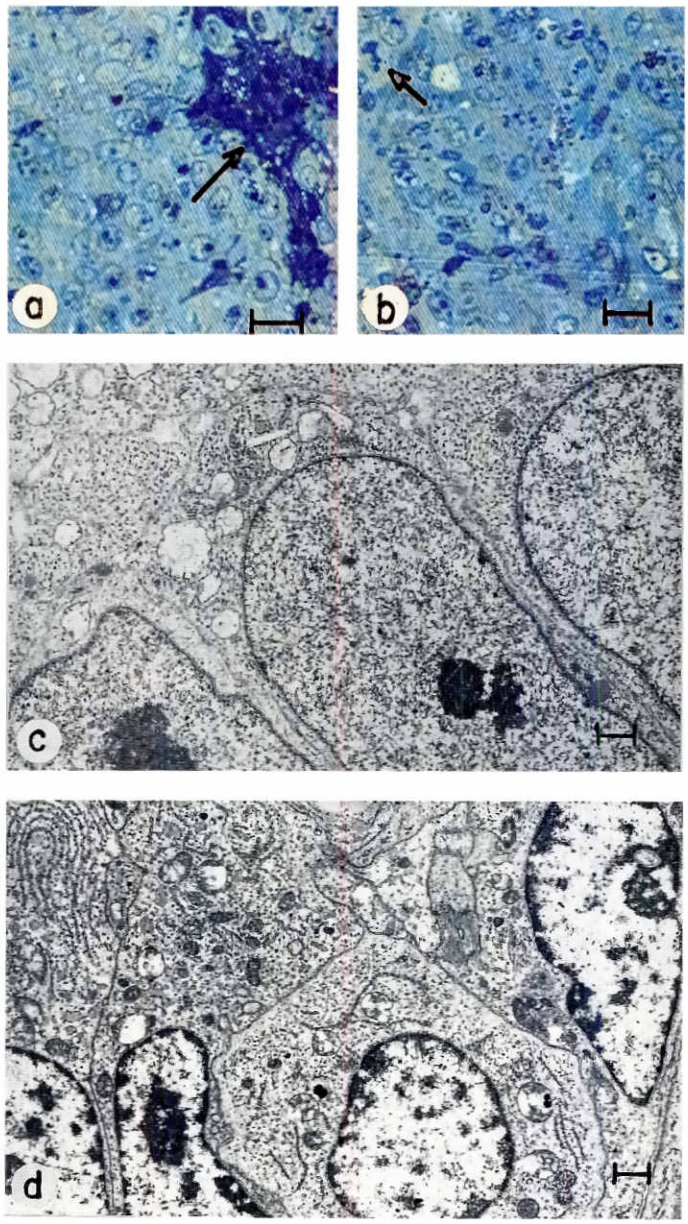

Fig. 6 Ultrastructural features of cell line-derived tumors. *Semithin sections of* **(a)** *syngeneic mouse tumor shows large vesicular nuclei with prominent nucleoli. and a proliferated blood vessel is seen (arrow),* **(b)** *nude mouse tumor shows smaller, darker and pleomorphic nuclei. A mitotic figure is seen (arrow), Toluidine Blue. Bar 20 µm. Ultrathin section of tumor* **(c)** *in syngeneic mouse-shows no heterochromatin, prominent nucleoli, abundant ribosomes in clusters and high nuclear cytoplasmic ratio* **(d)** *in Nude mouse-smaller nuclei with peripheral heterochromatin, well differentiated endoplasmic reticulum and mitochondria in cytoplasm. Bar, 100 nm.*

the presence of EC cells in NT cell line, histochemical localization of alkaline phosphatase was carried out. (Antibodies to SSEA-1, a more preferred specific marker for EC cells were not available to us). Surprisingly, at passage 40 despite nestin immunopositivity in all the cells, alkaline phosphatase reactivity was seen in 75% of cells which further declined on more

passaging to 5% in P155. Possibly there is a positive selection of differentiating cells on repeated passaging. As early as 1976, Nicolas *et al.* documented that 39-75% of cells in EC cell lines showed positive alkaline phosphatase reactivity in contrast to the differentiated cell lines which are generally negative. A single exception was a myoblastic line (PCD2) which exhibited the enzyme reactivity in 60-70% of cells. It is likely that our cell line NT belongs to the same category as PCD2, in being differentiated but expressing alkaline phosphatase activity variably.

The stability of the NT cell line is reflected in the similar pattern of expression of specific intermediate filaments (both neuronal and glial), the generation time (12 h), cloning efficiency (50%), ability to form colonies in soft agar and the presence of myc and ras family of protooncogenes in both early and late passages. The coexpression of NF 68 and NF 150 (Fig. 3c,d,e and f) observed in NT cells is very similar to that in NT2/D1 clone derived from human teratocarcinoma cell line (Pleasure and Lee, 1993). The decline of immunoreactivity for NF 150 with a concomitant increase for NF 68 in late passages indicates that these neuronal cells are more immature than those of the early passages. Similarly, the coexpression of nestin and neurofilament protein in NT cells also resembles the physiological situation. In normal human fetuses, nestin disappears in neuroepithelial cells once they make the commitment towards neuronal differentiation with the onset of NFP expression (Tohyama *et al.*, 1992). On the other hand, in the rat fetal nervous system coexpression of these intermediate filaments has been documented (Friederiksen and McKay, 1992). The fainter staining for GFAP in immunoblots also suggests that astrocytes are less differentiated in late passages. The coexpression of nestin and GFAP observed in NT cells is reminiscent of a situation in developing radial glia in rat (Cameron and Rakic, 1991). The finding of GFAP and NFP in some NT cells is probably because they are neoplastic cells and ectopic expression of specific proteins is not uncommon in tumor cells. It is pertinent at this juncture to recall that NFP expression has been reported in glioma cell lines though the primary glioma tumor showed only GFAP expression (Tohyama *et al.*, 1993).

The involvement of myc and ras family of protooncogenes in embryonic development as well as in some of the tumors is well documented (Gonos and Spandidos, 1993). In the developing nervous system the c-myc gene is associated with cell proliferation. The N-myc gene seems to maintain the committed neural precursor cells in proliferative state and it is shown to be amplified in human neuroblastomas (Brodeur *et al.*, 1984). The role of ras oncogene in neuronal differentiation has been investigated extensively in PC12 cell line exposed to Nerve Growth Factor (NGF). It has been shown to mediate the signal transduction when NGF induces PC12 cells to differentiate into neurones (Wood *et al.*, 1992). Southern blot analysis of NT cells of both early and late passages reveals single copy oncogenes with specific fragments that are not rearranged. This implies that these protooncogenes which are implicated in growth and differentiation of immature neuronal cells remain stable in NT cells even on repeated transfers.

The loss of tumorigenicity in syngeneic mice after 70th passage is exactly opposite of the situation in a cell line obtained from a human malignant ovarian teratoma where the late-passage cells (after P100) were reported to be highly tumorigenic in nude mice (Zauthen *et al.*, 1980). It can be speculated that (1) the tumor cells have undergone differentiation. A correlation between activated N ras and enhanced tumorigenicity has been reported in a human teratocarcinoma cell line (Tainsky *et al.*, 1984). However, both the tumorigenic and non-tumorigenic (in syngeneic mice) passages of the cell line in the present study failed to show any alteration in N ras. The formation of better differentiated tumors in nude mice (Fig. 6a,b,c and d) may be solely due to immunodiffusion state resulting from lack of some cytokines which encourage differentiation. The finding that NT cell line is tumorigenic in nude mice suggests that the loss of tumorigenicity in syngeneic mice is probably related to immunologic mechanisms; (2) the NT cells of late passages express a neoantigen which is recognized by the immune system of the

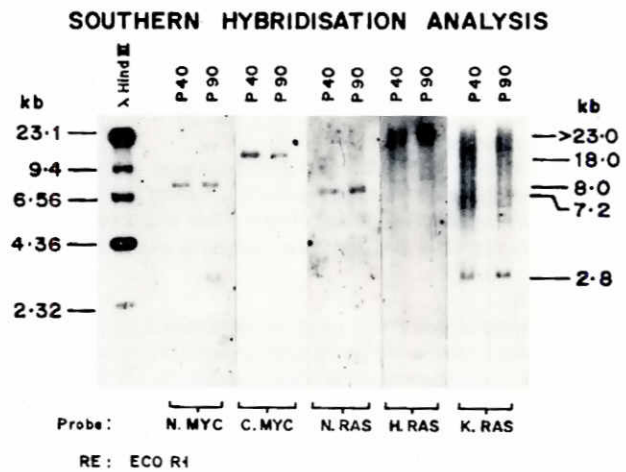

Fig. 7. Myc and Ras family of genes *in early (P40) and late (P90) passages of NT cell line.*

host, or have lost some factor which helps the tumor cells to evade the immunosurveillance as reported in an EC cell line (PCC3) where shutting IGF1 gene off has led to loss of tumorigenicity (Trojan *et al.*, 1994).

In conclusion, the NT cell line which is the neoplastic counterpart of normal neural precursor cells can be used to understand neuronal and glial differentiation, study its response to various maturation inducing chemical agents, growth factors and extracellular matrix, and also investigate whether NT cells transplanted in the brain of syngeneic mice integrate with the neural tissue of the host. NT cells can also be used as a vehicle in experiments on gene delivery.

Materials and Methods

Cell cultures

Explant cultures of the teratocarcinoma belonging to 130th transplant generation were set up on plastic surface in Basal Medium of Eagle (BME) supplemented with 20% human AB serum or 10% fetal calf serum (FCS) and antibiotics (penicillin and streptomycin) (Hasgekar and Lalitha, 1991). The effect of serum starvation (0.1% HAS) was studied. The passages which retained tumorigenicity in syngeneic mice (up to

P70) were referred to as "early passages" and the later ones beyond P70 as "late passages".

Growth kinetics

The cells belonging to 40th, 50th and 90th passages were seeded in 35 mm Nunc plastic Petri plates at a concentration of $3x10^3$ cells/dish and harvested daily up to 5 days. For each time point, cells from 3 Petri plates were removed separately after trypsinization, checked for viability and counted using a Neubar chamber.

Plating efficiency

Nunc Petri plates, 5 each for different cell densities, were seeded with 100, 200, 400, 600, 800 and 1000 cells belonging to 50th passage. On day 10, they were washed with phosphate buffered saline, fixed in prechilled methanol for 45 min and stained with Giemsa stain. The colonies consisting of more than 25 cells were counted using a stereomicroscope (Bausch and Lomb).

Chromosome analysis

Chromosome preparations were made from monolayer cultures at 90% confluence using a standard protocol. Fifty metaphases were counted.

Soft agar colony assay

Cells belonging to 40th, 50th and 90th passages were trypsinized, dispersed into single cells, suspended in 0.3% molten agar at 37°C and overlaid on 0.5% agar in Petri plates. The number of colonies in 60 random fields were scored and subjected to statistical analysis.

Tumorigenicity

Cells belonging to every 10th passage were tested for their ability to form tumors. As a routine, 10^6 cells were injected subcutaneously and intraperitoneally in syngeneic mice. When tumors did not develop, after subcutaneous injections, other procedures such as intratesticular inoculations or implantations under the kidney capsule or in thymectomized syngeneic mice which received 1000 R of gamma radiation in 5 doses or Swiss nude mice (Swiss nu/nu) were used. All tumors were fixed in 10% neutral buffered formalin for light microscopy. Representative samples were taken for ultrastructural studies.

Immunophenotyping

Cells at various passages from P40-P100, grown on coverslips were fixed in prechilled methanol for 5 min at -20°C for immunofluorescence staining (Rudnicki and McBurney, 1987) and in a mixture of methanol and acetic acid (3:1) overnight at 4°C for immunoperoxidase staining (Sternberger *et al.*, 1970). Antibodies used were against (a) nestin (b) glial fibrillary acidic protein (GFAP) and (c) 3 subunits of neurofilament protein (NFP). Antibodies were produced by us in rabbits against human GFAP extracted from spinal cord (Bignami *et al.*, 1980) and NFP extracted from rat brain (Shaw and Weber, 1982). The dilutions used for immunofluorescent staining were 1:300 for nestin, 1:20 for NF-200, 1:10 for NF 50, 1:5 for NF 68 and 1:20 for GFAP. For immunoperoxidase staining by Avidin Biotin Complex method, the dilutions were 1:300 for nestin, 1:250 for NF 200, 1:100 for NF 150, 1:50 for NF 68 and 1:1000 for GFAP. For immunoperoxidase method, positively stained cells were counted in 25 random fields and subjected to statistical analysis. For immunofluorescence staining FITC-labeled and TRITC-labeled antirabbit antibodies were used and the cultures were double-labeled in various combinations of the above mentioned antisera.

Immunoblotting

100 (g of cytoskeletal protein, extracted from both early and late passage cells (Chiu *et al.*, 1981), was separated by 7.5% polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membrane (Towbin *et al.*, 1979) and the blots were stained with antibodies against NF200 (1:250), NF 150 (1:100), NF68 (1:50) and GFAP (1:250) by immunoperoxidase method.

Alkaline phosphatase activity

For alkaline phosphatase, the cells were fixed in absolute methanol and neutral formalin (9:1) for 30 sec, washed in running tap water and allowed to dry. The slides were incubated with 0.3 mg/ml naphthol-As-phosphate in 0.3 M Tris HCl, buffer, pH 9 containing 1 mg/ml fast blue BB (diazolium salt) for 15 min, rinsed in 3-4 changes of tap water and subsequently counterstained with neutral red for 5 min (Kaplow, 1963). After drying, the cells were scanned under oil immersion lens and the percentage of positive cells was computed.

Electron microscopy

Representative samples from (a) original teratocarcinoma (b) the cell line at various passages and (c) the cell line derived tumors in syngeneic and nude mice were fixed in 3.25% glutaraldehyde, post fixed with osmium tetroxide (OsO_4), dehydrated and embedded in araldite. Semithin survey sections and thin sections were cut with Ultratome V (LKB) and viewed with Zeiss 109 electronmicroscope at 80 KV.

Genomic analysis

$2x10^8$ cells from 40th and 90th passages were frozen at -70°C. DNA isolated from these cells by the standard protocol (Sambrook *et al.*, 1989) was digested with ECOR1 restriction enzyme under conditions recommended by the supplier (Amersham, UK). The digested DNA was fractionated by electrophoresis through a 0.7% agarose gel in TBE (0.089 M Tris, 0.089 M Boric acid, 0.002 M EDTA) buffer; denatured, neutralized and transferred to nylon membrane by the method of Southern (1975). Prehybridization, hybridization and washing using N-myc, c-myc, N-ras, K-ras and H-ras as probes were carried out (Saranath *et al.*, 1989). The washed filters were exposed to X-ray films for autoradiography at -70°C using intensifying screens for a period of 1-4 days.

Statistical analysis

Student's *t* test was employed for all statistical analysis.

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