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Applications of Immortalized Cells in Basic and Clinical Neurology

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Abstract Immortalized cell lines can serve as model systems for studies of neuronal development and restoration of function in models of neurological disease. Cell lines which result from spontaneous or experimentally-induced tumors have been used for these purposes. More recently, the techniques of genetic engineering have resulted in the production of cell lines with specific desired characteristics. This has been accomplished by insertion of a desired gene into a pre-existing immortal cell or by immortalizing primary cells. The production of immortal cell lines using temperature-sensitive immortalizing genes offers an additional method of controlling gene expression, and thereby controlling cell proliferation and differentiation. In the nervous system, these techniques have produced immortal cell lines with neuronal and glial properties.

Key words: immortalization, cell lines, astrocytes, retroviruses, grafts, temperature sensitive

Immortal tumor cells in culture have been widely applied in studies of oncogenesis, development, and differentiation, as well as serving as model systems for studies of the properties they express [1–3]. For example, PC12 cells, originally discovered as a spontaneous pheochromocytoma tumor in rats [4], express many important properties of adrenergic neurons [5,6]. Moreover, the recognition that PC12 cells differentiate in response to nerve growth factor (NGF) [7,8] and basic fibroblast growth factor [9] has provided significant insights into the biochemistry and molecular mechanisms of trophic factors [10]. The retinoic-acid induced differentiation of embryonal carcinoma cells into presumptive neurons has also provided valuable information about the sequence of gene expression in development and the role of differentiating agents in therapy [11,12].

Cell lines have also been used in animal models of neurological disease. Parkinson's disease is a neurodegenerative disorder whose characteristic features are a loss of the dopaminergic neurons in the substantia nigra of the midbrain and a concomitant reduction in voluntary movement. Movement can be restored by the administration of dopaminergic agonists. Several cell lines, known to produce catecholamines or their precursors, have been tested in animal models of Parkinson's disease. PC12 cells express the enzyme tyrosine hydroxylase [1] and produce significant amounts of catecholamines. B16 melanoma cells express the enzyme tyrosinase and produce l-dopa as a byproduct of the production of melanin [13]. We [14] and others [15,16] have implanted PC12 cells into rat brains and have found that, while some cells survive for extended periods, most die off soon after implantation. Other experiments indicate that B16 mouse melanoma cells also do not survive for extended periods when implanted into rats, but form tumors when implanted into mice [17]. Neither of these cell lines was found to ameliorate behavioral consequences of lesions of the dopaminergic neurons.

Genetically-engineered immortalized cell lines offer another line of approach to the problems of neural development and the therapy of neurodegenerative disorders. Two basic strategies have been developed for these studies. The first is to insert desired genes into an already immortal cell line. Genes coding for the tyrosine hydroxylase and nerve growth factor have been inserted

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into immortal fibroblasts such as 3T3 cells [18] (Freed, et al.; Gage, et al., this issue). Skin fibroblasts are not endogenous to the brain and may not contain other critical factors or enzymes necessary for the post-translational activation of transmitter synthetic enzymes. Moreover, they may not be able to integrate well with the normal host brain. Therefore, a second strategy is to immortalize a target neural cell which displays desired characteristics. This is the strategy we and others have adapted and which will be reviewed here.

IMMORTALIZATION STRATEGIES

The unique property of immortalized cells is that they continue to divide for many generations and the progeny are genotypically identical and retain a stable phenotype. Several methods have been used to immortalize brain cells to produce cell lines. The large T antigen of simian virus 40 (Tag), a DNA tumor virus, is frequently used for producing cell lines [19,20]. Insertion of Tag antigen DNA into cells has resulted in the production of transformed and immortalized fibroblasts [21] and the immortalization of neural cells which incorporate and express large T [22,23]. De Vitry and collaborators provided the first evidence that neural cells could be immortalized. By exposing 6-day-old cultures to native SV40 virus, they established a continuous cell line (HT9) of hypothalamic cells [24]. After further passage, several subclones were isolated, some of which were classified as primitive and others as differentiated. One of the latter (C7) expressed neurophysin and vasopressin [22]. Further studies on this clone have revealed that the cells are electrically inexcitable [25]. One primitive clone (F7) could be made to express oligodendrocyte markers by treatment with cholesterol, estradiol, an eye-derived factor and brain extract [26]. These same techniques have produced $GFAP^+$ glial cell lines from primary cultures of cerebellum [27] and mesencephalon [23,28]. Although these methods have succeeded, the success rate is rather low. Transfection is more effective for inserting DNA into cells and has been effective in producing some glial cell lines [29], but the process itself is quite harsh and in our experience fragile cells do not survive following transfection. More recently, several cell lines have been produced by fusing post-mitotic neurons with neuroblastoma cells [30]. Several different cell lines with neuronal characteristics were described. Different subclones express the properties of the parent cells to varying degrees. Whether these fused cells are stable over extended periods in culture is not clear.

Other neural cell lines have been made by incubating primary cells with temperature-sensitive Rous sarcoma virus (RSV), an avian virus. Two clones were isolated from rat cerebellum [31]. At the permissive temperature, the cells were essentially undifferentiated, while at the non-permissive temperature, one clone stopped dividing and was found to demonstrate tetrodotoxin-sensitive veratradine-induced Na^+ uptake (characteristic of a voltage-gated Na^+ channel), while the other continued to divide and expressed GFAP. RSV is much more effective in avian cells and has been used to create a cell line from quail embryonic neural retina which generates action potentials and binds monoclonal antibodies made against chick neural retina [32]. One major problem with the use of these viruses is that infected cells continue to make productive virus which spreads and infects other cells [32].

The third method of inserting oncogenes to create cell lines, which we employ, is the method of retroviral-mediated gene transfer [33,34]. Retroviral vectors have been used by several groups to generate cells which express desired proteins. In these vectors, retroviral RNA is replaced with cDNA coding for a desired protein; the cDNA is integrated into the genome, and viral regulatory elements which normally regulate transcription of viral proteins instead regulate production of the desired protein. Since sequences for viral packaging and replication are missing, these viruses (termed replication-defective) cannot continue to propagate in normal host cells; only those host cells which are originally infected produce the protein. The virus must also be encapsidated by special "packaging" cell lines having the ability to produce the viral envelope protein and package and secrete the retroviral construct [35]. Media from such cells transfected with a given construct therefore contains high titers of replication-defective virus which can be used to infect target cells. We have produced cell lines using retroviruses containing Tag and the neomycin resistance gene, which allows for selecting against cells which did not express Tag. The first described is an astrocyte

line, which we named A7, made with wild-type Tag.

Temperature-sensitive (*ts*) variants of Tag are also available for these purposes. The advantage of *ts* mutations is that Tag is active at a permissive temperature, and conformationally inactive at a non-permissive temperature. One such variant of SV40 large T, *ts*A, is active at 33–34°C and inactive at 39°C [15]. The underlying hypothesis here is that insertion of the oncogene will "freeze" a cell at the stage of differentiation at which the oncogene was inserted (Fig. 1). Thus, immortalization of a more differentiated cell should produce a cell line with differentiated characteristics. For *ts* oncogenes, this model would also predict that differentiation should continue at the non-permissive temperature. McKay and collaborators have produced several cerebellar cell lines using this construct containing the *ts*A mutant [36]. As expected, these lines continue their differentiation program at the non-permissive temperature. A model of the immortalization process with *ts* oncogenes is presented in Figure 1. This model incorporates both the concept that cell lines retain their state of differentiation as well as the hypothesis that cells continue their differentiation program once the oncogene is inactivated.

THE A7 CELL LINE

Our first immortal cell line, called A7, was made by inserting a constitutively expressed Tag into cultured embryonic rat optic nerve cells [37]. The A7 cell line was identified following infection by its resistance to neomycin. After neomycin selection and clonal expansion, several major immunological and functional characteristics the A7 cell line were explored. We have concluded that the A7 cell line displays many antigenic and functional properties of differentiated astrocytes. For example, A7 cells have surface neural cell adhesion molecule (N-CAM), characteristic of astrocytes [37]. They initially showed expression of glial fibrillary acidic protein (GFAP), but this was lost with repeated passage in culture which is very typical for growing astrocyte lines [37]. On the other hand, A7 cells do express the intermediate filament protein vimentin, characteristic of immature nervous system cells and cells in culture [37]. They functionally resemble astrocytes, having the ability to support neuritic outgrowth [37]. Interestingly, certain properties originally observed in A7 cells, such as secretion of platelet-derived growth factor [37] and expression of the *neu* oncogene phosphoprotein, were later confirmed

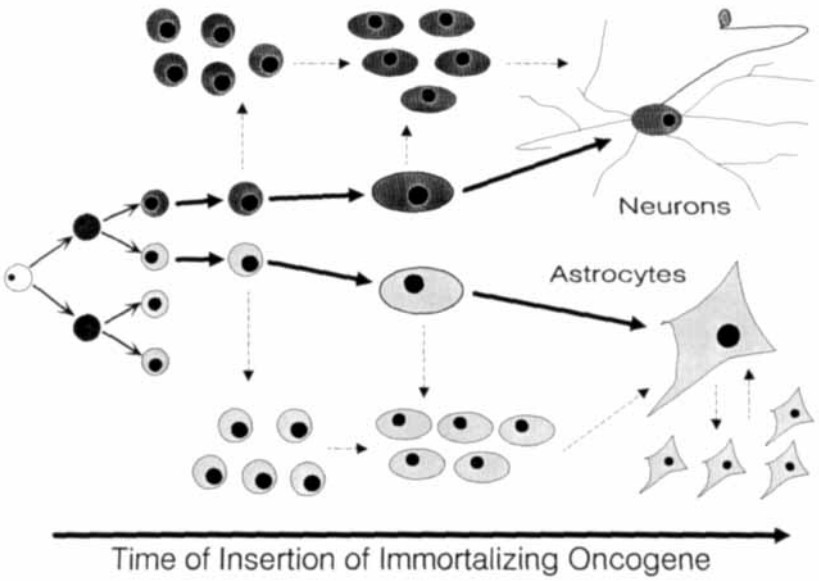

Fig. 1. Schematic diagram of cellular immortalization with oncogenes. Normal cellular differentiation into either neurons or glial is represented in the central part of the figure, as the progressive maturation and acquisition of phenotypic properties. Insertion of an immortalizing gene at a developmental stage causes the production of cell lines with phenotypic properties of the parent cell. With a temperature sensitive immortalizing gene, switching to the restrictive temperature (represented with broken arrows) allows the cell to mature, passing through the same developmental stages as primary cells (represented with solid arrows).

TABLE I. Characterization of A7 Cells as Compared to Protoplasmic Astrocytes

Antigen/enzyme	A7 Cell line	Protoplasmic astrocytes
A2B5	−	−
Galactocerebroside	−	−
Glial fibrillary acidic protein	±	+
Vimentin	+	±
RC-1 antigen	+	±
Fibronectin	+	+
N-CAM	+	+
Thrombospondin	+	+
Laminin	+	+
Tenascin/cytotactin	+	+
Glutamine synthetase	−	+
Monoamine oxidase B	−	+
Platelet-derived growth factor	+	+
Supports neuritic outgrowth	+	+

in primary astrocytes. Table I summarizes the antigenic and functional characteristics of the A7 cells.

FUTURE DIRECTIONS

These experiments provide the seeds for future investigations in this rapidly-moving field. Constitutively immortal cells carry the potential for tumorigenesis, and therefore it would be unlikely that they would ever be therapeutically useful. On the other hand, such cells could be mitotically crippled before implantation by treatment with mitotic inhibitors, such as mitomycin C, or by X-irradiation. Whether such cells would retain the phenotype of the cell line is clearly a matter for further investigation.

The use of temperature-sensitive (*ts*) mutants of immortalizing genes is another area of fertile investigation. We have made our first such cell line using the *ts*A allele of Tag [38]. The interesting aspect of the *ts*A allele is that the non-permissive temperature is 39°C, the normal core temperature of the rodent. Thus, whether these cells retain their phenotype and differentiate following implantation is therefore of considerable interest. Moreover, the evaluation of gene expression at both permissive and non-permissive temperatures should permit a deeper understanding of the process of development.

The original strategy of these investigations was, and still is, to obtain cells which can be used as intracerebral implants for the treatment of neurological diseases. It would appear that the two strategies we have presented at the beginning of this article are still likely to bring success. Because the A7 cell line was produced from a normal glial cell, we expect that it will be able to survive and integrate with the host brain. If so, it may be possible to employ the neurite-promoting activity of the A7 cell line to guide neuronal processed during regeneration [39]. Moreover, the A7 line may also be useful as a suitable recipient for transgenes coding for desired proteins.

Immortalization of a specific neuronal population is the second strategy for somatic cell therapy. The most successful targeted immortalizations are obtained when large purified populations of specific neuronal precursors can be obtained. This has been accomplished in the sympathoadrenal system, where monoclonal antibodies were used to obtain large numbers of precursor cells [40]. No such antibody has been identified thus far for dopaminergic neuronal precursors. The most direct approach would then be to target fetal mesencephalic cells, a significant proportion of which are destined to become dopaminergic and GABA neurons, for these investigations. Given that incorporation and expression of the transgene is a quasi-random event, repeated trials of such immortalization experiments are likely to succeed. Based on evidence from transplants of adrenal cells [41], it appears that reinnervation of the basal ganglia by dopaminergic neurons is not necessary for restoration of function. On the other hand, a line of dopaminergic neurons would be of considerable interest in testing this hypothesis.

Although not strictly deficiency diseases, many other chronic and debilitating neurological and psychiatric diseases are due to alterations in the balance of neurotransmitters. Restoration of function by administering drugs which increase the levels of these transmitters is a mainstay of therapy. The potential to restore neurological function by the administration of cells that produce growth factors is another line of approach [42]. On the other hand, our work has demonstrated that simple exposure of cells to growth factors can produce pleiotropic alterations in cell phenotype, some of which may serve to inhibit recovery of function [43,44]. Clearly, the field of somatic cell immortalization and gene expression is in its infancy, as is our understanding of the basis of most neurological diseases. The role of the immune system in regulating survival of cells implanted into the brain must also be addressed. As our understanding of these

basic processes increases, so will the opportunities for a therapeutic approach using genetically-engineered immortal cells.

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