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Neuronal Properties of Clonal Hybrid Cell Lines Derived from Central Cholinergic Neurons

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Clonal cell lines derived from specific types of central neurons can be used to identify and characterize properties specific to those neurons. With somatic cell fusion techniques, nine clonal hybrid cell lines have been developed from the septal region of the mouse basal forebrain. Two lines express characteristics typical of cholinergic neurons—choline acetyltransferase activity and immunoreactivity, neurite formation with neurofilament protein immunoreactivity, and aggregation in rotation-mediated cell culture. These cell lines may be useful for studying the trophic interactions that support the development and maintenance of central cholinergic connections.

CHOLINERGIC NEURONS OF THE mammalian basal forebrain play an important role in learning and memory and degenerate in Alzheimer's disease (*1*). This degeneration may result from a dysfunction of the trophic interactions that support cholinergic connections (*2*). Recent studies on mediators of central cholinergic trophic processes have focused on the possible roles of nerve growth factor (NGF) and other substances (*3, 4*). Much of this work has used primary cell culture techniques, which should theoretically permit analysis at the cellular and molecular level. However, the use of such techniques to study specific trophic mechanisms is limited because even distinct areas of the mammalian central nervous system contain neurons with various neurotransmitter phenotypes and different synaptic connections. To study cholinergic trophic mechanisms with greater resolution, we developed clonal hybrid cell lines from the mouse septal region. Of nine lines generated, two manifested stable and constitutive properties typical of septal cholinergic neurons. The availability of permanent cell lines such as these could facilitate investigations of cholinergic neuronotrophic processes.

Embryonic murine septal cells were fused with cells of a murine neuroblastoma line, N18TG2, that are deficient in hypoxanthine phosphoribosyltransferase (HPRT; EC 2.4.2.8) (*5*). Neuroblastoma cells are embryologically related to neurons (*5*), and thus are likely to permit the expression of neuron-specific traits (*6*). In addition, the murine origin of both parental cell types minimizes the chromosomal loss that often occurs in xenogeneic fusions (*7*). Finally, this neuroblastoma line is deficient in the specific cholinergic marker choline acetyltransferase (ChAT), the enzyme that catalyzes the synthesis of acetylcholine (*8*).

The septal region was dissected from C57BL/6 mouse embryos at day 14 of embryogenesis (*9*), dissociated, and resuspended in phytohemagglutinin-containing, serum-free, modified Eagle's medium (*10*). The septal cell suspension was then added to a monolayer culture of neuroblastoma cells. Phytohemagglutinin facilitated the adherence of septal cells to the N18TG2 monolayer. After a 15-minute incubation, the medium was aspirated and the cells were fused with a 50% (by volume) polyethylene glycol (PEG 1000, Koch-Light) solution (*11*). The fusion products were plated at 0.05×10^6 cells per 35-mm plate in medium containing 10% (by volume) fetal calf serum, $100 \times 10^{-6}M$ hypoxanthine, $0.4 \times 10^{-6}M$ aminopterin, and $16 \times 10^{-6}M$ thymidine (HAT medium), which selects against HPRT-deficient cells. Individual colonies were isolated with cloning cylinders and expanded in medium without aminopterin. Primary septal cells or N18TG2 cells alone, treated similarly, produced no viable colonies.

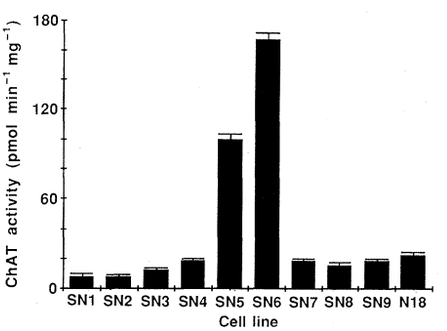

Fig. 1. ChAT activity (picomoles of ACh formed per minute per milligram of protein) of nine cell lines generated by fusion of septal and N18TG2 cells, and of N18TG2 (N18) cells (*26*). The values shown are means ± SEM ($n = 3$).

Nine cell lines from the fusion of septal cells and N18TG2 cells were isolated and screened for ChAT activity with a radiochemical assay (Fig. 1). Most of the cell lines showed ChAT activity similar to that of the N18TG2 parent cell type. Two lines, SN5 and SN6, showed marked ChAT activity and have been further evaluated.

The hybrid nature of the SN5 and SN6 lines is supported by growth in HAT medium and by chromosomal and isoenzyme

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Table 1. Properties of parental cells and cholinergic hybrid cell lines.

Property	Cells			
	CB57BL/6 septal cells	N18TG2	SN5 cell line	SN6 cell line
Chromosome number				
Total	40*	79 ± 2	137 ± 3	162 ± 4
Large metacentrics	0	9†	10†	9†
GPI isoenzymes				
IA (A strain)	−	+	+	+
IAB (hybrid)	−	−	+	+
IB (C57BL/6)	+	−	+	+
Neurite formation	+	−	+	+
ChAT activity	+‡	−	+	+
Immunocytochemistry				
NFP	+	−	+	+
ChAT	+‡	−	+	+
GFAP	+§	−	−	−
Aggregate formation	+	−	+	+

*Established previously. †SEM <1. ‡Septal cholinergic neurons. §Septal cells with astrocytic structure.

analysis. The growth of SN5 and SN6 in HAT medium, in view of the absence of any surviving parent cell colonies on control plates, is consistent with their derivation from septal as well as N18TG2 parent cells. The HPRT reversion frequency for N18TG2 is low (less than 10^{-7}) (*12*); we used only 2.5×10^6 N18TG2 cells in the fusion.

Cells from C57BL/6 mice, which constitute the source of septal parent cells, have 40 chromosomes per cell, all acrocentric or telocentric (*13*). Ten well-spread metaphase cells were examined for each cell line and revealed that N18TG2 cells had 79 ± 2 ($\bar{X}$ ± SEM) chromosomes per cell with an average of nine large metacentric chromosomes. The SN5 cells contained 137 ± 3 chromosomes per cell, with an average of ten large metacentrics. The SN6 cells had 162 ± 4 chromosomes, including nine large metacentric chromosomes.

Isoenzyme analysis provides further evidence that lines SN5 and SN6 represent hybrids between neuroblastoma and septal parent cells. N18TG2 cells, which originated from an A-strain mouse, and brain cells obtained from C57BL/6 mice showed different electrophoretic variants of glucose-phosphate isomerase (GPI; EC 5.3.1.9) (*14*). SN5 and SN6 cells expressed the electrophoretic variants of both parental strains, as well as an intermediate form that occurs in heterozygous strains (*14*). Thus, results of chromosomal and isoenzyme analyses support the hybrid nature of SN5 and SN6 cells.

In addition to expressing significant levels of ChAT activity, SN5 and SN6 cells displayed morphologic characteristics of neurons in monolayer culture. Their somata are round to oval and refractile when viewed by phase-contrast microscopy, and there are prominent neuritic processes (Fig. 2, A and C). In these respects, the structure of these cells resembles that of primary septal neurons grown under our culture conditions that stain immunocytochemically for ChAT. The N18TG2 cells did not show significant process formation in monolayer culture.

Parent and hybrid cells were analyzed immunocytochemically for type-specific antigens, with monoclonal antibodies against neurofilament protein (NFP), ChAT, and glial fibrillary acidic protein (GFAP). Monoclonal antibody $4.3F_9$ recognizes neurofilament subunits NF150 and NF200 (*15*); it reacted immunocytochemically with septal neurons in situ, with murine embryonic septal neurons in primary dissociated culture, and with SN5 and SN6 cells (Fig. 2B), but not with N18TG2 cells. AB8, a monoclonal antibody against ChAT developed in this laboratory (*16*), has been used extensively to immunohistochemically label mammalian central cholinergic neurons, including those of the septal region (*17*). In addition, AB8 reacts with embryonic septal cholinergic neurons in monolayer culture. SN5 and SN6 cells also displayed the AB8 epitope, as shown immunocytochemically (Fig. 2C), whereas N18TG2 cells did not. The hybrid cells did not exhibit the intermediate filament antigen GFAP, expressed by astrocytes. The monoclonal antibody $2.2B_{10.6}$ (*18*), directed against GFAP, labels septal astrocytes in tissue sections and in primary dissociated cultures prepared from embry-

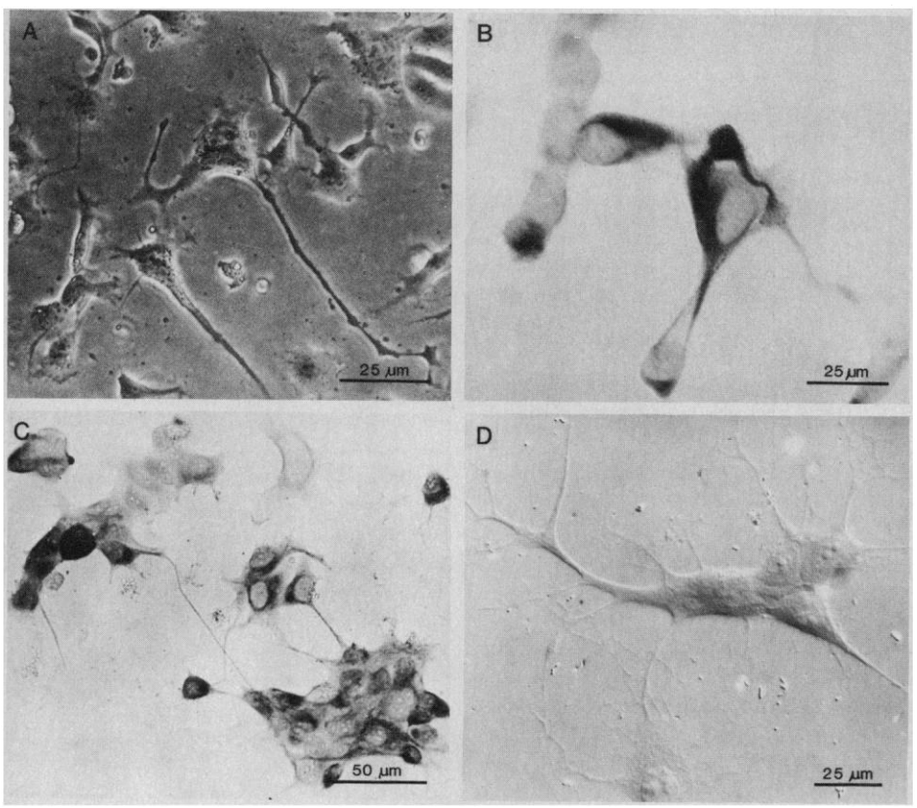

Fig. 2. Photomicrographs of SN6 cells in monolayer culture (*27*). (A) Viewed with phase optics, these hybrid cells exhibit round, refractile somata, and prominent process formation. (B) SN6 cells stained immunocytochemically with monoclonal antibody $4.3F_9$ directed against NFP. NFP-immunoreactivity is seen in perikarya and neuritic processes. (C) SN6 cells visualized immunocytochemically with monoclonal antibody AB8 against ChAT. Perikarya and processes are prominently stained. (D) SN6 cells stained with nonimmune rat immunoglobulin G as a control. Note the absence of significant staining. The use of differential interference contrast optics permits the visualization of the unstained somata and prominent processes that are not well visualized under bright-field optics.

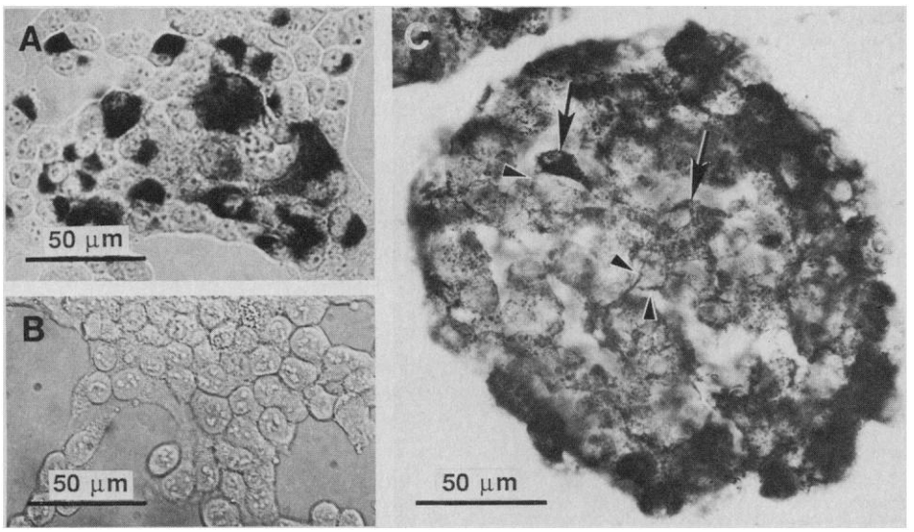

Fig. 3. SN6 cells processed histochemically to show AChE. (A) SN6 cells grown in monolayer, and incubated with iso-OMPA (an inhibitor of nonspecific cholinesterase) (50 μ*M*), demonstrate AChE activity. (B) Staining is completely blocked by the specific inhibitor of AChE, BW284C51 (5 μ*M*). (C) Photomicrograph of a section of an SN6 aggregate grown in rotation-mediated culture (*28*) and processed histochemically for AChE activity (*29*), with iso-OMPA used to inhibit nonspecific cholinesterase activity. Note the positive cell bodies (arrows) and processes (arrowheads).

onic septa, but does not label SN5 or SN6 cells. SN6 cells have also been evaluated histochemically for the presence of specific acetylcholinesterase (AChE); positive staining is observed in the presence of tetraisopropylpyrophosphoramide (iso-OMPA), an inhibitor of nonspecific cholinesterase (Fig. 3A), but is completely blocked by the specific AChE inhibitor, BW284C51 (Fig. 3B).

The cholinergic hybrid cell lines SN5 and SN6 retain an additional trait of normal septal cells, the ability to aggregate in rotation-mediated culture. Under such culture conditions, dissociated primary neural cells from various brain regions are capable of reaggregating and then forming histiotypic patterns similar to those of the regions of origin (*19*). Dissociated primary septal cells can reaggregate and undergo further differentiation when coaggregated with target cells from the hippocampus (*20*). SN5 and SN6 cells also aggregate in rotation-mediated culture, but N18TG2 cells fail to do so. Aggregates formed by the ChAT-positive hybrid cells contain numerous somata and processes that stain intensely for AChE (Fig. 3C).

Thus, we have generated hybrid cell lines from the septal region of the basal forebrain that have properties characteristic of septal cholinergic neurons (Table 1). We have documented the presence of specific neuronal (NFP) and cholinergic (ChAT) proteins in these cells. In addition, the hybrid cells display other neuronal characteristics, including neuritic process formation and aggregation.

It is likely that the neuronal properties of the hybrid cell lines reflect the expression of normal C57BL/6 septal cell chromosomes, since N18TG2 cells do not express any of these characteristics. However, new synthesis of a protein coded by a previously silent gene ("activation") has been noted in somatic cell hybrids (*21*). Therefore, the possibility that the observed properties result from the activation of N18TG2 genes cannot be completely excluded.

Cell lines expressing neuronal characteristics have been generated from fusions of the N18TG2 cells with cells of the sympathetic ganglia (*12*) and with cells of the dorsal root ganglia (*22*). These lines have been useful in evaluating neuron-specific gene products, including neurotransmitter synthetic enzymes. Additionally, some lines expressing ChAT activity have been generated from the fusion of Chinese hamster brain and neuroblastoma cells (*23*) and from other sources (*24*). However, to our knowledge, lines SN5 and SN6 are the first cell lines to be derived from a specific region of the basal forebrain. These hybrid cell lines are especially interesting because they express properties characteristic of a particular neuronal subset of that region. SN5 and SN6 could therefore facilitate the study of the factors influencing septal cholinergic neuron development and selective synapse formation and maintenance. The availability of cell lines derived from septal cholinergic neurons may also provide an in vitro system for evaluating abnormal trophic interactions among these cells, as might occur in Alzheimer's disease (*2, 25*).

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Diminished Response of Werner's Syndrome Fibroblasts to Growth Factors PDGF and FGF

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Patients with Werner's syndrome, an autosomal recessive disorder, undergo an accelerated aging process that leads to premature death. Fibroblasts from such patients typically grow poorly in culture. Here it is shown that fibroblasts from a patient with Werner's syndrome have a markedly attenuated mitogenic response to platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF). In contrast, they have a full mitogenic response to fetal bovine serum. Both PDGF binding and receptor numbers per cell are unaltered. The Werner's syndrome cells express high constitutive levels of collagenase in vitro. Although PDGF enhances collagenase expression through increased levels of hybridizable collagenase messenger RNA in normal skin fibroblasts, no induction of collagenase occurs in the Werner's syndrome fibroblasts. Moreover, the failure to respond to this agonist effect of PDGF is not restored by fetal bovine serum. The data suggest that failure of one or more PDGF-mediated pathways in Werner's syndrome cells may contribute to the phenotypic expression of the disorder.

WERNER'S SYNDROME IS AN AUTOsomal recessive disorder that is generally characterized by an apparent acceleration of many of the processes associated with aging. Some of the principal features, as defined by Thannhauser (*1*), are short stature with thin extremities and stocky trunk, premature graying of hair, premature baldness, patches of stiffened skin (especially in the face and lower extremities), trophic ulcers of the legs, juvenile cataracts, hypogonadism, tendency to diabetes, calcification of the blood vessels, osteoporosis, metastatic calcifications, and a tendency to occur in siblings (*1*). Growth of skin fibroblasts from Werner's syndrome patients in vitro is more difficult than that of normal skin fibroblasts (*1*). Furthermore, Werner's syndrome fibroblasts have a reduced life span in vitro and lengthened mean population doubling time (*2*). These observations, coupled with biochemical evidence for disordered connective tissue metabolism (*3*), suggest that a substantial component of the Werner's syndrome phenotype is manifested in mesenchymal tissues.

The mechanism underlying Werner's syndrome has not been identified, but the apparent premature aging has suggested that abnormal cellular growth and differentiation may be important in the phenotypic expression of Werner's syndrome. A number of peptide growth factors have been identified as being capable of regulating cellular growth and differentiation. The platelet-derived growth factor (PDGF) may be important in these processes, because in vitro it initiates cell proliferation, cell migration, and cellular activation associated with seemingly diverse processes such as inflammation, wound healing, and perhaps athero genesis (*4*), and possibly—by homology to the putative transforming protein of the simian sarcoma virus, $p28^{v\text{-}sis}$—in the molecular events that govern malignant transformation (*5*).

Skin fibroblasts synthesize collagenase as one of their principal gene products (*6*); this enzyme functions at the rate-limiting step in initiating collagen degradation and remodeling in a wide variety of morphogenic events, including wound healing (*7*). Because of the profound effects of PDGF on mesenchymal cells, we postulated that this protein also might influence certain biochemical events in fibroblasts essential to remodeling and wound healing. It was thus of interest to observe that PDGF stimulates the synthesis and secretion of collagenase by normal human skin fibroblasts under conditions in which no apparent increase in DNA synthesis is observed (*8*). Here, we used skin fibroblasts cultured from a patient with Werner's syndrome and from the offspring of a patient with Werner's syndrome (obligate heterozygote) to address several questions: (i) Are two agonist functions of PDGF—mitogenesis and stimulation of collagenase synthesis—altered in Werner's syndrome? (ii) Are the putative alterations specific to PDGF? (iii) What mechanisms are involved?

Initially we examined the replicative capacity in vitro of fibroblasts from age- and sex-matched normal subjects and patients with Werner's syndrome (Human Genetic Mutant Cell Repository, AG780). The mean population doubling times of each were not significantly different when low passage ($\leq$12) cells were cultured in 10% fetal bovine serum (FBS). Further, saturation densities of normal and Werner's syndrome cultures were equal. Confluent, quiescent fibroblasts in culture from normal individuals and from patients with Werner's syndrome were next stimulated with 100 ng/ml of PDGF. The incorporation of [^{3}H]thymidine into DNA of PDGF-stimulated control cells was 480 to 760% of that in nonstimulated cells, whereas [^{3}H] incorporation into Werner's cells stimulated by PDGF was only 108 to 144% of that in unstimulated cells in repeated experiments (Table 1). In cells from a Werner's syndrome heterozygote, PDGF stimulated [^{3}H]thymidine incorporation 153% above control cells (Table 1). In contrast, the Werner's syndrome cells responded normally to the mitogenic stimulus of 20% FBS as did the Werner's syndrome heterozygote cells. Whole human serum (WHS; 20%, pooled samples from adults) was a less effective mitogen than 20% FBS in the cultures of Werner's syndrome cells, perhaps because PDGF is the principal mitogenic protein in human serum for cells of mesenchymal origin. This suggests that the Werner's cells cultured in FBS may be responding to fetal growth-promoting activities that, as yet, are uncharacterized.

The difference in responsiveness of Werner's syndrome fibroblasts and normal control fibroblasts is not due to observable

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