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Unique dopaminergic modulatory polypeptide factors have been obtained form the lysate of an immortalized monoclonal cell derived for the mouse striatum (X61). Partially purified preparations of the lysate are capable of increasing the dopamine content of hybrid monoclonal dopaminergic cells of mesencephalic origin and preventing the loss of primary dopaminergic neurons in the absence of target cells. The monoclonal derived dopaminergic stimulatory factor has undergone a 50,000-fold purification sufficient to permit mass spectrographic and amino acid sequence analysis of the purfied fractions. Based on this data, polypeptide fractions of known amino acid composition have been synthesized and are being examined for stimulatory activity in monoclonal dopaminergic cells and for the ability to prevent loss of primary dopaminergic neurons in the absence of striatal target cells. The availability of polypeptides of know molecular structure will also permit assessment of whether such agents are capable of preventing of reversing the motor deficits seen in experimental models involving loss of the dopaminergic neurons of the nigrostriatal projection. Polypeptide factors of low molecular weight which modulate neuronal monoaminergic function are of considerable interest in terms of their therapeutic potential in a variety of neurologic disorders including Parkinson's disease.

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INTRODUCTION

Parkinson's disease remains a major world-wide health problem particularly in the aging population. In North America alone, over one million individuals suffer from the disease experiencing severe motor dysfunction with muscle rigidity, bradykinesia and tremor. The fact is that the anatomic and neurochemical basis of the disease has been known for over four decades. An array of reasonably successful treatment modalities is available ranging from L-DOPA administration to surgical lesions and stimulation. Nevertheless, the need for new approaches to treatment of the disease remains a significant focus of neuroscience research. Given the fact that at least a major component of the symptomatology of the disease is secondary to an extensive loss of a dopaminergic projection to the corpus striatum arising in the substantia nigra of the mesencephalon, there has been intense interest in the examination of striatal "trophic factors" which may prevent, retard or even reverse some of the consequences of the degenerative process.

As an approach to the identification of unique striatal factors, we undertook a number of years ago to clone the dopaminergic neurons of the mesencephalon and their striatal targets by somatic cell hybridization (Choi et al., 1991; Wainwright et al., 1995). This work resulted in the production of monoclonal cells derived from the striatum which we demonstrated produced "factors" capable of increasing the dopamine content of monoclonal cells derived from the mesencephalon expressing a dopaminergic phenotype (MN9D cells). With the support of Grant #DAMD 17-01-1-0819, it was possible to demonstrate that cell lysate from one of the monoclonal striatal lines (X61) was capable of markedly increasing the dopamine content of the dopaminergic MN9D mesencephalic derived line (Heller et. al., 2000a). It is clear that the monoclonal derived dopaminergic stimulatory activity is polypeptide in nature and Grant #DAMD 17-01-1-0819 permitted an extensive effort at purification and identification of the relevant polypeptide sequences involved. Incubation of the X61 cell lysate under appropriate conditions results in an increased yield and a shift of the stimulatory activity from high to much lower molecular weight polypeptide fractions (see annual progress report of October 2002 for details). These polypeptides have now been purified some 50,000 fold, and the work has proceeded to the actual synthesis of putative polypeptide sequences (see below). In addition, mechanistic studies on the effect of the "stimulatory" polypeptides suggest that the effects on the MN9D cells is secondary to a unique mechanism, namely an increase in cellular dopamine storage. In addition, the factor(s) is capable of preventing the loss of primary dopaminergic neurons in the absence of target cells. Loss of dopaminergic neurons is, of course, the primary anatomic and neurochemical basis of Parkinson's disease (for details see below). Current studies are focused primarily on the further purification and chemical identification of both the stimulatory and an inhibitory factor present in the X61 cell lysate.

Enhanced Survival of Primary Murine Dopaminergic Neurons Induced by a Partially Purified Cell Lysate Fraction From Mouse-Derived Striatal Hybrid Monoclonal Cells (Neuroscience Letters, in press)

The hybrid monoclonal MN9D cell derived from a fusion of mesencephalic dopaminergic neurons with the N18TG2 neuroblastoma has proven to be an essential test object for the identification and purification of striatal trophic factors produced by the monoclonal striatal X61

cell line. However, the critical question with respect to the biological significance and utility of such factors is whether they produce effects on primary dopaminergic neurons comprising the nigrostriatal projection. One of the most effective and flexible methods for studies on primary neurons is three-dimensional reaggregate culture, a system in which dopaminergic, serotonergic and cholinergic neurons can be cultured both in the presence and absence of appropriate target cells (Heller et al., 1992, 1993; Hsiang et al., 1987, 1988). Such preparations allow for the monitoring of the neurochemical status, survival, and differentiation of transmitter phenotypes over developmental time (Heller et al., 1992, 1993). In addition, they provide a means for experimental manipulations which are not practical in the intact animal such as the removal of target cells without confounding lesion techniques. In addition, it is possible to coculture primary cells obtained from different genetic strains (Won et al., 1997). One of the major advantages of the system is that the findings obtained have been demonstrated in a number of instances to be predictive of effects on these neurons in the intact animal (Heller et al., 2000b).

For the purposes of examining the effects of the purified X61 lysate derived dopaminergic stimulatory factor(s) on primary dopaminergic neurons, we took advantage of the fact that it is possible in the aggregate cultures to coculture dopaminergic neurons in the presence or absence of appropriate striatal target cells. In the presence of striatal targets, the dopaminergic neurons selectively make connections with appropriate target cells and demonstrate quantitative survival (Heller et al., 1997; Won et al., 1989). However, in the absence of such targets (fetal mesencephalon co-cultured with optic tectum, an area of brain which does not receive a dopaminergic innervation), fewer dopaminergic neurons are observed, probably secondary to cell death, (Heller et al., 1997). This model culture system is not unlike the dopaminergic neuronal degeneration seen in Parkinson's disease.

Reaggregate cultures were prepared from embryonic day 14 C57BL/6 mouse brains by dissecting the mesencephalic tegmentum (containing developing dopaminergic neurons) and the tectum (a region to which dopaminergic neurons do not project). After 24 hours of culture, the reaggregates were treated with X61 cell lysate (12.5 μl/ml; n = 5 flasks), UF4 (20 μl/ml; n = 6 flasks, a partially purified fraction of the X61 lysate described in the previous progress report of 10/10/02) or with appropriate volumes of phosphate buffered saline (PBS) vehicle (12.5 μl/ml for X61 cell lysate (n = 5) or 20 μl/ml for UF4 (n = 6) treatment). The culture media were changed every 2 days and fresh amounts of X61 cell lysate, UF4 or PBS were added. After 8 days in culture, reaggregates from the flasks for a given treatment group (X61, UF4 or PBS) were combined and redistributed to 5 or 6 experimental flasks. Samples of culture media and reaggregates were collected at 15 days from each flask for HPLC analysis of dopamine (DA) and homovanillic acid (HVA) levels. Protein content of the reaggregates was determined spectrophotometrically (Smith et al., 1985).

The density of dopaminergic cells in the reaggregates, was determined using immunocytochemical methods for tyrosine hydroxylase and estimated based on the procedure previously described by Heller et al. (1993) and validated by computer simulation methods (Heller et al., 2001).

In the case of reaggregates treated with X61 cell lysate or the UF4 ultrafiltrate, it was apparent from gross inspection that many of the reaggregate sections contained considerably more TH-

positive neurons than the corresponding PBS controls (shown for UF4 treatment in Fig. 1). The results of the quantitative cell counting and neurochemical analysis of reaggregate tissue and media are presented in Table 1.

Table 1

Effect of X61-derived dopaminergic stimulatory factor(s) on survival of dopaminergic neurons

	TH Cell Density	Reaggregate DA	Media HVA
Ultrafiltrate Treatment			
UF4	563±131/mm^3*	10.4±0.02***	92.9±1.0***
PBS	196±48/mm^3	7.9±0.22	53.1±0.1
Cell Lysate Treatment			
X61 Cell Lysate	839±85/mm^3**	8.5±0.1***	185.0±20.3***
PBS	415±99/mm^3	6.4±0.3	47.5±1.7

Values given are the mean ± SEM of n = 5 for the UF4/PBS and n = 6 for X61 cell lysate/PBS treatment. Reaggregate dopamine (DA) is expressed as ng/mg protein. Media homovanillic acid (HVA) is expressed as ng/ml of media/mg reaggregate protein. *Significantly different than PBS control p<0.025. **Significantly different than PBS control p<0.01. *** Significantly different than PBS control p<0.001.

It is clear from this study that the X61 cell lysate as well as the UF4 ultrafiltrate are capable of preventing a loss of dopaminergic neurons in the absence of dopaminergic target cells in reaggregate culture. The neurochemical changes observed most probably are a result of the increased density of neurons expressing a dopaminergic phenotype in the treated reaggregate cultures. While dopamine is only increased by 25 to 30%, this may be a reflection of the immaturity of the neuronal population of the reaggregates. In addition, since no target cells were present in mesencephalic-tectal reaggregates of the current study, axonal process formation is, at best minimal, limiting dopamine storage (Kotake et al., 1982). The dopaminergic neurons within the mesencephalic-tectal reaggregates are, however, clearly neurochemically active and release dopamine as evidenced by the 1.8- (treatment with UF4) to 3.9-fold (treatment with X61 cell lysate) increase in media content of the dopamine metabolite, HVA, which provides an estimate of released transmitter (Table 1).

It is a reasonable assumption that in the absence of striatal target cells (mesencephalic-tectal reaggregates), there is an actual loss of the dopaminergic neurons as occurs *in vivo* following

various insults. However, the possibility that the cells are present, but they no longer express a dopaminergic phenotype cannot be excluded. In either case, there are clearly fewer neurons expressing a dopaminergic phenotype in mesencephalic-tectal as compared to mesencephalic-striatal cultures.

X61 cell lysate or partially purified fractions from this material (i.e., UF4 ultrafiltrate) therefore contain an activity capable of increasing the dopamine content of dopaminergic MN9D monoclonal hybrid cells or of three-dimensional reaggregates containing primary dopaminergic neurons in the absence of targets (Heller et al., 2000a). As demonstrated here, it would appear that this latter effect represents an actual increase (2.0 to 2.9-fold) in the density of dopaminergic neurons surviving in the cultures. The ability of the lysate to increase dopamine levels and prevent dopaminergic cell loss has obvious utility in the investigation and treatment of Parkinson's disease in terms of elevation of transmitter level in surviving cells, prevention of progressive cell loss and as an adjunct to dopaminergic cell survival in fetal transplantation.

Purification of Dopaminergic Factors from X61 cell lysate

Cell lysates of X61 immortalized clonal hybrid cells derived from embryonic murine corpus striatum contain factors which have stimulatory and inhibitory effects on both immortalized dopaminergic hybrid cells derived from mesencephalon as well as on primary dopaminergic neurons (Heller et al., 2000a, and see annual progress report of October 2002). As part of the process of purification of these factors, large (preparative) amounts of X61 cell lysate were allowed to "autodigest" at room temperature over two days. This results in the conversion of the stimulatory factor(s) from high molecular weight precursor(s), that are associated with the particulate fraction and can not be readily fractionated, to small-sized polypeptides. The low molecular weight polypeptide fraction can be separated from the crude cell lysate material by pressure-filtration through a 5 kDa (5×10^3 molecular weight) cut-off membrane, and the product obtained has a stimulatory activity (yield) equal to, or greater than, that of the initial crude lysate.

Most of this stimulatory activity from the low molecular weight fraction can be concentrated by ultrafiltration using a 0.5 kDa (5×10^2 molecular weight) cut-off, indicating that its molecular size is approximately between 5×10^2 and 5×10^3 Daltons. This concentrated activity has been further purified by chromatography on a Superdex-peptide, gel-filtration column, resulting in the elution of most of the stimulatory activity in a single peak (approximate mol. wt. 0.4 – 0.8 kDa). In addition, an inhibitory factor was separated (approximate mol. wt. 0.8 – 1.0 kDa) and it decreases dopamine content in MN9D cells. (Additional progress on the purification of the inhibitory factor is described below). The overall purification of the stimulatory factor at this step is on the order of 500-fold, when compared to the crude lysate, and its yield is close to 100%.

Following absorption to washed, activated charcoal, the stimulatory activity, which fails to bind, is purified approximately 50,000-fold, compared to the crude X61 lysate, as judged by the absorbance at 280 nm. In addition, digestion of the stimulatory activity with pronase results in the removal of about two-thirds of the activity, strongly suggesting that this activity resides in

one or more, small polypeptide fragments. However, attempts to purify this activity to homogeneity by reverse phase chromatography under a variety of conditions on C18, C8, C1, and cyano-propyl, HPLC columns and on a C18 column, that is N-capped with polar material to enhance binding of polar compounds, have all failed to bind the stimulatory activity. Nevertheless, the tail-end fraction from one of these chromatographies, when analyzed by mass spectroscopy, gave a result that suggested a possible biochemical composition for the stimulatory activity. We have tested this hypothesized structure by attempting to bind the stimulatory factor to an appropriate affinity ligand that should bind the postulated polypeptide(s). Pilot affinity chromatography resulted in binding of over half of the stimulatory activity. Elution of the bound activity was possible with an appropriate biochemical eluting reagent. This result and mass spectroscopic analysis of the affinity-purified activity strongly suggest that our hypothesis as to the biochemical composition/structure of the stimulatory polypeptide(s) is correct. Although the amount of polypeptide material is small, amino acid sequencing of the affinity-purified activity is at least suggestive of the hypothesized structure. To test our postulated structure(s) more rigorously, these polypeptides are currently being synthesized at The University of Chicago amino acid sequencing core lab facility. We will shortly test the synthesized polypeptides for dose-dependent stimulation of dopamine content in MN9D cells.

As indicated above, as well as in the annual progress report of October 2002, gel-filtration of "autodigested" X61 cell lysate, following ultrafiltration through a 5 kDa cut-off membrane and concentration with a 0.5 kDa membrane, results in the appearance and separation of an inhibitory activity. There was concern expressed by a reviewer of our previous progress report (October, 2002), that the data demonstrating this activity showed only a single data point with only about a 15% reduction in dopamine content. However, when this inhibitory fraction is tested at multiple concentrations (see Table 2), it gives a reproducible, dose-dependent reduction in MN9D cell dopamine content that exceeds 60%.

Table 2

Addition	**Dopamine (ng/mg protein)**
none	**42.8**
3 ul inhibitor	**35.9**
6 ul inhibitor	**25.6**
10 ul inhibitor	**15.1**

Our efforts to purify the inhibitor have shown that it, unlike the stimulatory activity, binds to charcoal. However, elution of bound inhibitory activity has not yet proven feasible. Unlike the stimulatory activity, the inhibitor binds transiently to a C18, reverse phase column, and, consequently, can be considerably purified from most other polypeptides with this chromatographic step. Amino acid sequencing of this step has demonstrated a partial possible sequence, but further purification is required to determine the precise, complete sequence. We believe this will be achieved by further reverse phase chromatography with the other HPLC columns mentioned above. Once a more certain sequence is obtained, it will be synthesized and tested to determine whether or not it is the correct structure for the inhibitor.

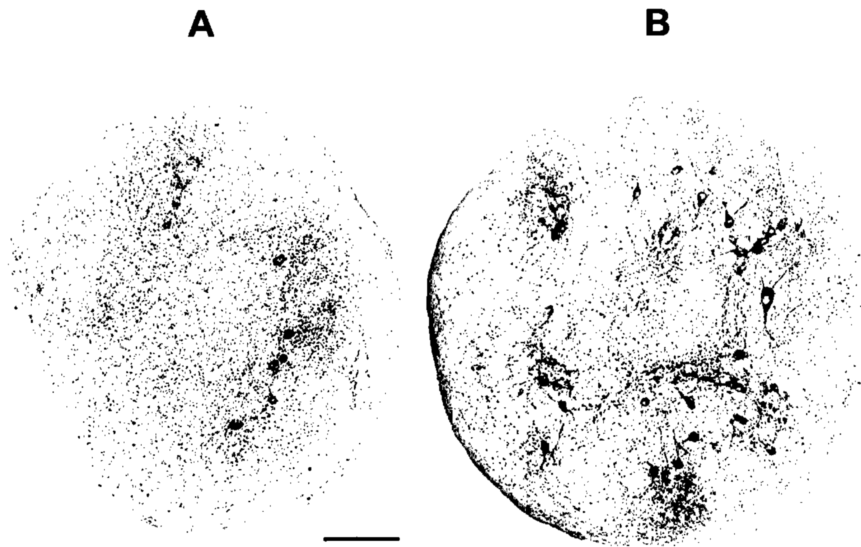

Figure 1: Sections of mesencephalic-tectal reaggregates with dopaminergic neurons visualized by tyrosine hydroxylase (TH) immunocytochemistry. Microscopic examination of section A (PBS-treated reaggregate) showed that it contained 11 TH labeled cells while section B (UF4-treated reaggregate) contained 35 such cells. Scale bar: 100 μm.

KEY RESEARCH ACCOMPLISHMENTS

- The demonstration that a partially purified preparation of the dopaminergic stimulatory activity obtained from the lysate of monoclonal hybrid cells derived from the mouse striatum is capable of preventing the loss of primary dopaminergic neurons grown in aggregate cultures lacking striatal target cells.

- Mass spectrographic and amino acid sequence analysis of a 50,000-fold purification of the monoclonal derived dopaminergic stimulatory factor has been accomplished. This permits the synthesis of putative polypeptide sequences, which may be responsible for the stimulatory activity seen in monoclonal dopaminergic cells and the ability of the partially purified preparation to prevent loss of primary dopaminergic neurons in the absence of striatal target cells.

REPORTABLE OUTCOMES

1. Published manuscript from the New York Academy of Sciences meeting on "Parkinson's Disease: The Life Cycle of the Dopamine Neuron" held on September 18-20, 2002, in Princeton, New Jersey (see Appendix for Heller et al., Dopaminergic stimulatory peptides from immortalized striatal cells, Ann. N.Y. Acad. Sci. 991: 339-341, 2003).

2. Manuscript accepted for publication in Neuroscience Letters (see Appendix for Won et al., Enhanced survival of primary murine dopaminergic neurons induced by a partially purified cell lysate fraction from mouse-derived striatal hybrid monoclonal cells, Neuroscience Letters, in press).

3. Abstract submitted for the Society for Neuroscience Meeting held November 8-12, 2003, in New Orleans (see Appendix for Won et al., Enhanced survival of dopaminergic neurons induced by a partially purified cell lysate fraction from striatal derived hybrid monoclonal cells, Soc. Neurosci. Abstr., in press).

CONCLUSIONS

Parkinson's disease, which is associated with motor dysfunctions, is a major debilitating disorder affecting well over a million individuals in the United States alone. While great strides have been made in the control of the motor disabilities associated with the disease, therapy remains somewhat limited both in extent and duration. Given that Parkinson's disease is a degenerative disorder involving loss of the dopaminergic neurons of the nigrostriatal projection, considerable interest has focused on the possible therapeutic role of trophic agents. Our recent discovery of low molecular weight polypeptide fractions, with stimulatory and inhibitory dopaminergic activities, and the ability to prevent dopaminergic cell loss in the absence of target cells may represent a new approach to therapy of the disease. Of particular significance is the possible administration of such agents by routes which do not necessarily involve CNS invasive

techniques. The primary focus of the current research is the purification, identification and synthesis of these polypeptides which is in progress. The availability of purified polypeptides of known chemical structure with dopaminergic stimulatory or inhibitory activity will permit examination of their potential for prevention or reversal of the motor deficits produced by loss of the nigrostriatal dopaminergic projection in experimental animal models as a prelude to their use in human Parkinson's disease.

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APPENDIX

1. Heller, A., Gross, M., Hessefort, S., Bubula, N. and Won, L., Dopaminergic stimulatory peptides from immortalized striatal cells, Ann. N.Y. Acad. Sci. 991: 339-341, 2003.

2. Won, L., Bubula, N., Hessefort, S., Gross, M. and Heller, A., Enhanced survival of primary murine dopaminergic neurons induced by a partially purified cell lysate fraction from mouse-derived striatal hybrid monoclonal cells, Neuroscience Letters 2003, in press.

3. Won, L., Bubula, N., Hessefort, S., Gross, M. and Heller, A. Enhanced survival of dopaminergic neurons induced by a partially purified cell lysate fraction from striatal derived hybrid monoclonal cells, Soc. Neurosci. Abstr. 2003, in press).

Dopaminergic Stimulatory Polypeptides from Immortalized Striatal Cells

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KEYWORDS: **dopamine; serotonin; trophic factor; cell lines; reaggregate culture; mesencephalon; corpus striatum; nigrostriatal projection**

Somatic cell fusion has been utilized as an approach to the immortalization of neurons for the purpose of obtaining monoclonal cell lines expressing neurotrophic factors.[1] Such fusions have permitted the generation of monoclonal hybrid cells derived from neurons of the nigrostriatal projection expressing specific transmitter phenotypes.[1–3] The cells include a striatal cell line (X61) that served as a source of possible trophic agents[3] and a mesencephalic cell line (MN9D) expressing a dopaminergic (DA) phenotype that was used as a test object.[2]

Cell lysates of the striatal X61 line have been shown to contain factors that have a stimulatory effect on both immortalized DA hybrid cells and primary DA neurons.[4] The DA stimulatory activity resides in a low-molecular-weight polypeptide fraction of less than 5 kDa.

The effect of this polypeptide fraction was examined on primary neurons using the three-dimensional reaggregate system that permits culture of mesencephalic DA and serotonergic (5-HT) cells in the presence or absence of appropriate target cells.[1] In the absence of target cells (mesencephalic-tectal aggregates), no axonal arbors are formed and most monoaminergic neurons disappear, presumably secondary to cell death. Some neurons survive and form fairly large processes, which appear to be dendritic in character and make autotypic connections with other DA neurons. We have already reported on the effect of the crude X61 stimulatory factor on DA neurons in such cultures in terms of increased DA content.[4] Here we describe the effect of the partially purified stimulatory factor on the morphology of both DA and 5-HT neurons by means of immunocytochemical methods.

A partially purified preparation (UF4) from X61 cell lysate was added (20 μL/mL) to the aggregate culture medium from day 1 to day 15 of culture. Aggregate sections were examined for DA neurons using an antibody against tyrosine hydroxylase, and

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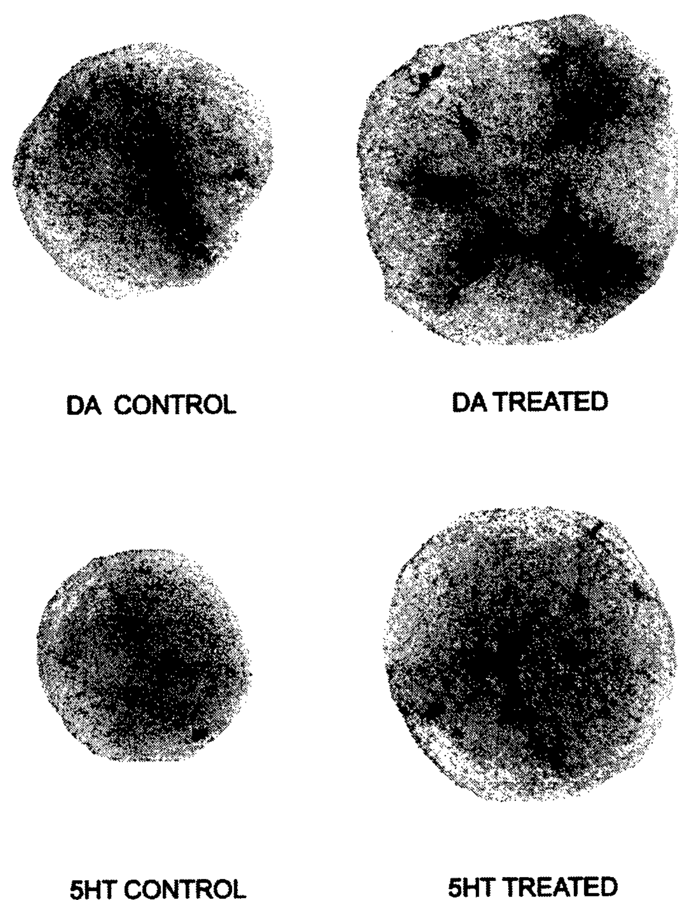

FIGURE 1. Immunocytochemical visualization of monoaminergic neurons in mesencephalic-tectal aggregates following treatment with UF4, a partially purified fraction obtained from the lysate of an immortalized clonal hybrid cell (X61) derived from embryonic murine corpus striatum.

for 5-HT neurons by an antibody against 5-HT. The results of this experiment are shown in FIGURE 1. With UF4 treatment, substantial numbers of densely stained DA neurons with extensive processes are observed. By contrast, while DA neurons are present in the untreated controls, such dense groupings of heavily stained cells with extensive processes are, at best, extremely rare. UF4-treated aggregates also contain 5-HT neurons and axons that are more densely stained than the cells observed in the untreated controls. Neurochemical analysis of the aggregates revealed a 30% increase in aggregate DA ($P < 0.001$) and a 52% increase in aggregate 5-HT follow-

ing treatment with the UF4 partially purified preparation. Homovanillic acid, a major metabolite of DA, was increased by 75% ($P < 0.001$) in the media from UF4-treated aggregates.

A low-molecular-weight polypeptide fraction, obtained from lysate of immortalized monoclonal cells derived from the striatum, is, therefore, capable of increasing DA levels of both a monoclonal cell line (MN9D) and primary DA and 5-HT neurons in three-dimensional reaggregate culture. In addition, the polypeptide fraction increases the immunocytochemical staining of both cell bodies and processes of the monoaminergic neurons. Purification and sequencing of the active polypeptides will permit assessment of their efficacy in the reversal of the motor dysfunction that occurs secondary to degeneration of the DA nigrostriatal projection.

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[Neuroscience Letters, in press]

Enhanced Survival of Primary Murine Dopaminergic Neurons Induced by a Partially Purified Cell Lysate Fraction From Mouse-Derived Striatal Hybrid Monoclonal Cells

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Abstract

Lysates of X61, a striatal derived cell line, and a partially purified preparation from the lysate (UF4) contain a factor(s) capable of increasing the dopamine content of a mesencephalic derived dopaminergic cell line (MN9D) and of cultures containing primary dopaminergic neurons. Treatment of cultures containing dopaminergic primary neurons grown in the absence of target cells over a two week period with X61 lysate or UF4 resulted in an elevation of dopamine levels of the cultures and of media HVA as well as a 2.0-fold (UF4) to 2.9-fold (X61 lysate) increase in the density of dopaminergic neurons in treated cultures. The results suggest that the activity factor derived from X61 is capable of preventing dopaminergic cell loss which occurs in the absence of dopaminergic target cells of the corpus striatum.

Key Words: dopamine, nigrostriatal, trophic factor, Parkinson's disease, cell lines, MN9D, reaggregate culture

We have previously reported that the lysate of an immortalized, monoclonal cell line (X61) derived from the striatum is capable of increasing the dopamine content of a mesencephalic monoclonal hybrid cell line derived from the mesencephalon expressing a dopaminergic phenotype (MN9D) [3]. This lysate also increases the dopamine content of three-dimensional reaggregate cultures containing primary dopaminergic neurons [3]. The reaggregate cultures consist of fetal mesencephalon co-cultured with optic tectum, an area of brain which does not receive a dopaminergic innervation. Under these circumstances, fewer dopaminergic neurons are observed, possibly secondary to cell death, as compared to the quantitative survival of such cells in reaggregate cultures containing dopaminergic striatal target cells [2]. The present experiment was conducted to determine whether the rise in dopamine levels in the reaggregate cultures seen with exposure to X61 cell lysate or with a partially purified, ultrafiltrate fraction of the lysate (UF4) [4] was associated with an increase in the survival of dopaminergic neurons in the absence of target cells.

Reaggregate cultures were prepared from embryonic day 14 C57BL/6 mouse brains by dissecting the mesencephalic tegmentum (containing developing dopaminergic neurons) and the tectum (a region to which dopaminergic neurons do not project)(see [6] for method details). The tissues were dissociated into single cell suspensions and counted using a hemacytometer.

3.25 million mesencephalic cells were dispensed along with 6.5 million tectal cells into 25 ml Erlenmeyer flasks containing 3.5 ml of initial culture medium composed of Basal Medium Eagle's, 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin (5,000 units penicillin - 5,000 μg streptomycin) and 0.025% (w/v) deoxyribonuclease I. The flasks were prepared and placed into a rotatory incubator where reaggregates formed over the course of 24 hrs. After the first 24 hrs, the medium was removed and replaced with fresh medium containing horse serum instead of fetal bovine serum. At this time, the reaggregates were treated with X61 cell lysate (12.5 μl/ml; n = 5 flasks), UF4 (20 μl/ml; n = 6 flasks) or with appropriate volumes of phosphate buffered saline (PBS) vehicle (12.5 μl/ml for X61 cell lysate (n = 5) or 20 μl/ml for UF4 (n = 6) treatment). The culture media were changed every 2 days and fresh amounts of X61 cell lysate, UF4 or PBS were added. After 8 days in culture, reaggregates from the flasks for a given treatment group (X61, UF4 or PBS) were combined and redistributed to 5 or 6 experimental flasks. The pooling of reaggregates and their re-distribution into experimental flasks reduces the variance among flasks subjected to the same experimental treatment [6]. The cultures were maintained in this manner for 15 days at which time reaggregates and media were collected and biochemical and histological examination were conducted. For this experiment, culture media were prepared using sera which had been previously dialyzed to remove endogenous serotonin [6].

X61 cell lysate was prepared by sonicating X61 cells in PBS at room temperature. Purification of the active material from the lysate was facilitated by the finding that the dopaminergic stimulatory activity can be largely converted from a high molecular weight protein fraction to a much lower molecular weight protein fraction when the lysate is allowed to incubate at room temperature for up to 2 days. Amicon YM-5 membranes (5,000 Da mol. wt. cut-off) were utilized to separate a low molecular weight stimulatory fraction (UF4) from the X61 lysate. After sonication, the lysate was concentrated by pressure filtration and allowed to "autodigest" at room temperature overnight. The next morning, the lysate was diluted with PBS to its initial volume and re-concentrated by pressure filtration. After an additional overnight "autodigestion" and re-dilution on day 2, the lysate was re-concentrated once more, generating the UF4 ultrafiltrate fraction. The conversion of the activity from high to lower molecular weight fractions, in all likelihood, represents an enzymatic breakdown of higher molecular weight material. Active lower molecular weight fractions are stable at room temperature despite the finding that the crude cell lysate activity is labile to boiling [3]. The heat lability may represent a precipitation or coagulation of protein entrapping the activity. Dopaminergic stimulatory activity (i.e., the ability to increase cellular dopamine content) of the ultrafiltrates obtained from

each daily filtration was assessed as previously described [3] using a mesencephalic-derived, dopaminergic cell line, MN9D. Of the ultrafiltrates obtained from each daily filtration, the UF4 ultrafiltrate (following 48 hr incubation at room temperature) was the most stimulatory and, therefore, was the preparation used to treat the reaggregate cultures described below. The stimulatory activity in UF4 is approximately equal to that of the initial X61 lysate, and UF4 is approximately 60-fold purer based on absorbance at 230 nm.

Samples of culture media and reaggregates were collected at 15 days from each flask for HPLC analysis of dopamine (DA) and homovanillic acid (HVA) levels. Protein content of the reaggregates was determined spectrophotometrically [12].

The density of dopaminergic cells in the reaggregates, was visualized using immunocytochemical methods and estimated based on the procedure previously described by Heller et al. [6] and validated by computer simulation methods [8]. The cultures were fixed with 4% paraformaldehyde, embedded in gelatin and sectioned (50 μm) with a vibratome. Tyrosine hydroxylase (TH) immunocytochemistry was performed on free-floating tissue sections using standard peroxidase anti-peroxidase techniques. Briefly, the number of TH-

positive cells from a given flask was estimated by counting the cells from a random selection of 30 sections. A digital image was captured of each of the 30 reaggregate sections used for counting wtih a Nikon Coolpix 995 digital camera. The area (in pixels) of each reaggregate section was obtained using Adobe Photoshop 7.0. The pixel area from each reaggregate section was converted to square microns and multiplied by 50 (section thickness) to obtain the sectional volume. The density of TH cells was obtained by dividing the number of TH cells counted by the sum of sectional volumes from the 30 reaggregate sections.

In the case of reaggregates treated with X61 cell lysate or the UF4 ultrafiltrate, it was apparent from gross inspection that many of the reaggregate sections contained considerably more TH-positive neurons than the corresponding PBS controls (shown for UF4 treatment in Fig. 1). The results of the quantitative cell counting and neurochemical analysis of reaggregate tissue and media are presented in Table 1.

It is clear from this study that the X61 cell lysate as well as the UF4 ultrafiltrate are capable of preventing a loss of dopaminergic neurons in the absence of dopaminergic target cells in reaggregate culture. The neurochemical changes observed most probably are a result of the increased density of

neurons expressing a dopaminergic phenotype in the treated reaggregate cultures. While dopamine is only increased by 25 to 30%, this may be a reflection of the immaturity of the neuronal population of the reaggregates. In addition, since no target cells were present in mesencephalic-tectal reaggregates of the current study, axonal process formation is, at best minimal, limiting dopamine storage [10]. The dopaminergic neurons within the mesencephalic-tectal reaggregates are, however, clearly neurochemically active and release dopamine as evidenced by the 1.8- (treatment with UF4) to 3.9-fold (treatment with X61 cell lysate) increase in media content of the dopamine metabolite, HVA, which provides an estimate of released transmitter (Table 1).

Anatomic loss of dopaminergic neurons of the nigrostriatal projection is a central feature of Parkinson's disease (for review see [9]). It is clear that one cause of such degeneration is a loss of contact of the mesencephalic dopaminergic neurons with their striatal targets as evidenced by the fact that lesions which transect the projection of dopaminergic neurons to the striatum result in a loss of cellular components of the substantia nigra [11].

It is a reasonable assumption that in the absence of striatal target cells (mesencephalic-tectal reaggregates) there is an actual loss of the dopaminergic

neurons as occurs *in vivo* following various insults, but the possibility that the cells are present, but no longer express a dopaminergic phenotype cannot be excluded. In either case, there are clearly fewer neurons expressing a dopaminergic phenotype in mesencephalic-tectal as compared to mesencephalic-striatal cultures. The three-dimensional reaggregate system, therefore, provides a reasonable model system for the examination of agents capable of preventing dopaminergic cell loss following either loss or separation from dopaminergic target cells.

X61 cell lysate or partially purified fractions from this material (i.e., UF4 ultrafiltrate) contain an activity capable of increasing the dopamine content of dopaminergic MN9D monoclonal hybrid cells or of three-dimensional reaggregates containing primary dopaminergic neurons in the absence of targets [3]. As demonstrated here, it would appear that this latter effect represents an actual increase (2.0 to 2.9-fold) in the density of dopaminergic neurons surviving in the cultures. The results obtained with the X61-derived dopaminergic stimulatory factor(s) are quite similar to a previous experiment in which we demonstrated that the addition of fetal striatal membrane preparations to mesencephalic-tectal reaggregates resulted in an increased survival of dopaminergic neurons, in some cases almost to the extent of the survival achieved with striatal cells [5]. The result suggested that

"dopaminergic survival factors" associated with membranes were present in the striatum. We did not pursue this finding since the reaggregate system was much too lengthy an assay procedure for the purposes of purification of the cellular derived activity. Instead, we turned our attention to the use of somatic cell hybridization methods to produce the current monoclonal hybrid dopaminergic and striatal cells [1, 7, 13]. The basic hypothesis was that the monoclonal striatal cells, such as X61, would produce dopaminergic "trophic" factors, and the monoclonal dopaminergic cells, MN9D, would serve as a test object for such factors. The data provided here would appear to justify that approach.

The cell-saving activity present in X61 cell lysate is obviously of considerable interest. We have previously demonstrated that the X61 lysate does not contain a significant number of the known trophic factors for dopaminergic neurons [3]. The UF4 ultrafiltrate factor(s) represents dopaminergic stimulatory activity which is less than 5,000 Da. The activity in UF4, capable of increasing MN9D dopamine content, has now been purified some 50,000-fold by gel filtration and charcoal treatment (unpublished observations) and will be tested with regard to its ability to prevent dopaminergic cell loss in the absence of target cells in three-dimensional reaggregate culture as seen with ultrafiltrate (UF4) preparations.

The exact chemical nature and mechanism of action of the X61 cell lysate activity is still under investigation. Demonstration and concentration of the activity was made possible by the availability of an appropriate test object, the dopaminergic MN9D cell. The effect of the lysate activity on such monoclonal cells is to increase dopamine content. As demonstrated here, the biological effect of the partially purified lysate activity includes the ability to prevent the loss of primary dopaminergic neurons which occurs in the absence of target cells. The lysate activity has obvious utility in the investigation and treatment of Parkinson's disease in terms of elevation of transmitter level in surviving cells, prevention of progressive cell loss and as an adjunct to dopaminergic cell survival in fetal transplantation.

Acknowledgments

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Table 1

Effect of X61-derived dopaminergic stimulatory factor(s) on survival of dopaminergic neurons

	TH Cell Density	Reaggregate DA	Media HVA
Ultrafiltrate Treatment			
UF4	563±131/mm^3*	10.4±0.02***	92.9±1.0***
PBS	196±48/mm^3	7.9±0.22	53.1±0.1
Cell Lysate Treatment			
X61 Cell Lysate	839±85/mm^3**	8.5±0.1***	185.0±20.3***
PBS	415±99/mm^3	6.4±0.3	47.5±1.7

Values given are the mean ± SEM of n = 5 for the UF4/PBS and n = 6 for X61 cell lysate/PBS treatment. Reaggregate dopamine (DA) expressed as ng/mg protein. Media homovanillic acid (HVA) expressed as ng/ml of media/mg reaggregate protein. *Significantly different than PBS control $p<0.025$. **Significantly different than PBS control $p<0.01$. *** Significantly different than PBS control $p<0.001$.

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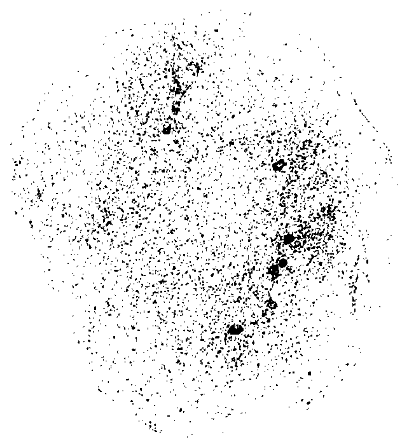
Figure Legend

Figure 1: Sections of mesencephalic-tectal reaggregates with dopaminergic neurons visualized by tyrosine hydroxylase (TH) immunocytochemistry. Microscopic examination of section A (PBS-treated reaggregate) showed that it contained 11 TH labeled cells while section B (UF4-treated reaggregate) contained 35 such cells. Scale bar: 100 μm.

A



B

[Society for Neuroscience Abstract, November 2003]

ENHANCED SURVIVAL OF DOPAMINERGIC NEURONS INDUCED BY A PARTIALLY PURIFIED CELL LYSATE FRACTION FROM STRIATAL DERIVED HYBRID MONOCLONAL CELLS. L. Won, N. Bubula, S. Hessefort, M. Gross and A. Heller. Dept. of Neurobiol., Pharmacol. & Physiol., and Dept. of Pathol., The University of Chicago.

We have previously reported that the lysate of an immortalized, monoclonal cell line (X61) derived from embryonic murine corpus striatum is capable of increasing the dopamine (DA) content of a mesencephalic monoclonal hybrid cell line expressing a DA phenotype (MN9D) as well as three-dimensional reaggregate cultures containing primary DA neurons. The present experiment was conducted to determine whether the rise in DA levels in the reaggregate cultures seen with exposure to X61 cell lysate or with a partially purified, low molecular weight (<5 kDa) ultrafiltrate fraction of the lysate (UF4) is due to an increase in the survival of DA neurons. Treatment of mesencephalic-tectal reaggregate cultures containing DA primary neurons grown in the absence of target cells over a two week period with X61 lysate or UF4 ultrafiltrate resulted in significant elevations of reaggregate DA levels (+32%) and of the DA metabolite, homovanillic acid, in the media (1.8 to 3.9-fold). Quantitation of DA cells, visualized using tyrosine hydroxylase immunocytochemistry, in the reaggregates showed an increase in the density of DA neurons in X61 lysate (2.9-fold) and UF4 (2.0-fold) treated cultures. The results demonstrate that X61 cell lysate or partially purified fractions from this material (i.e., UF4) contain a factor(s) capable of preventing DA cell loss which occurs in the absence of target cells. Such a factor(s) may have utility in the investigation and treatment of Parkinson's disease in terms of elevation of transmitter level in surviving cells, prevention of progressive cell loss and as an adjunct to DA cell survival in fetal transplantation. Supported by DAMD 17-01-1-0819 and the Falk Medical Research Trust.