Single-Cell Molecular Biology

Implications for the Diagnosis and Treatment of Neurological Disease

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The normal functioning of the central nervous system (CNS) requires complex interactions among numerous biological components. The pathophysiology of perturbations in this system is as complex as that of neurological disease. Many methods exist to examine the biological output of dysfunctional cells from a diseased system (eg, immunohistochemical analysis, electrophysiology, and microdialysis), with one goal being to understand the mechanisms of cell death. This understanding may allow the design of therapeutic strategies to prevent cell death and ensuing behavioral abnormalities. Analysis of messenger RNA (mRNA) levels for various genes in CNS tissue may enhance understanding of neurological disease, since cells differ in the complement and abundance of genes they express. One popular method for detecting changes in gene expression is the Northern blot technique, in which total RNA from a sample is extracted and the RNA molecules are separated by size on a denaturing gel and transferred or “blotted” onto nylon membranes that are then probed with radiolabeled DNA for subsequent autoradiographic detection of gene expression.

While this approach may yield information about the level of expression for a particular gene, the lack of cellular specificity is problematic for 2 reasons. First, the Northern blot technique calls for the use of brain tissue homogenates, which contain a heterogeneous population of cells (eg, neurons, glia, and macrophages), thus making conclusions about cellular specificity of the detected changes in gene expression impossible to determine. Second, in a diseases system, not all cells are necessarily affected by the disease process. Frequently, cells damaged by disease in a region may be surrounded by healthy cells. For example, in Alzheimer disease (AD), cell death of cholinergic neurons in the nucleus basalis of Meynert located in the basal forebrain is a consistent pathological feature of this disease. Neurodegeneration is also prevalent in the entorhinal cortex, subiculum, and hippocampus of patients with AD. Within the hippocampus, however, the CA1 pyramidal cells are particularly vulnerable to cell death, while the CA2 and CA3 sectors as well as the granule neurons in the dentate gyrus of the hippocampus are relatively resistant to degeneration in AD.1 Cerebellar Purkinje cells and subcortical structures are also not as affected in AD. Furthermore, the distribution of neuritic plaques and neurofibrillary tangles—2 hallmark pathological features of AD—is also confined to specific brain areas, with plaques being highly concentrated in the temporal neocortex, while tangle-bearing neurons are more often found in the entorhinal cortex, subiculum, and CA1 hippocampus.2 Therefore, to examine the gene expression changes characteristic of dysfunctional or selectively vulnerable cells, a method that permits analysis at the single-cell level is warranted.

Techniques that currently allow visualization of genes in single cells include in situ hybridization and polymerase chain reaction (PCR). While these techniques are useful for visualization of specific genes in individual cells, analysis of multiple genes is difficult. This is an important point to consider because it is likely the coordinate expression of many genes, rather than the expression level of a single gene, that determines the physiology of an...
individual cell. A method to detect numerous mRNA abundances within individual abnormal cells will provide valuable information about changes in gene expression that may contribute to the development of CNS disease. Furthermore, understanding the temporal changes in the pattern of gene expression at the single-cell level is important for discovering the molecular mechanisms underlying the progression and severity of the disease. For instance, in AD, the CA1 pyramidal cells are among the first cells to degenerate, with cells in other regions becoming prone to degeneration and associated AD pathology as the disease progresses.

### METHODS

The amplified antisense mRNA (aRNA) technique allows the simultaneous detection and quantification of multiple changes in gene expression in single cells. Single cells in vitro or from live or fixed-slice preparations are amenable to single-cell aRNA amplification. The latter approach allows additional phenotypic characterization of cell type prior to single-cell aRNA amplification. With this technique, cells are first immunohistochemically labeled, permitting not only an evaluation of cell type but also potentially an evaluation of at least 1 particular protein product of an mRNA of interest. Recently, we have adapted the aRNA method to immunohistochemically analyze cells labeled with terminal transferase-mediated-dUTP nick-end labeling (TUNEL). A marker for fragmented DNA, TUNEL has been widely used to characterize damaged cells or apoptosis (in combination with the appropriate morphological criteria) in many neurological diseases. Programmed cell death, or apoptosis, is a form of “cell suicide” controlled by regulatory genes. Although a normal process during development of the CNS, apoptosis also occurs during many neurological diseases and may represent a second type of cell death (in addition to necrosis) contributing to neurological dysfunction. Apoptotic cells have been detected using the TUNEL method in the hippocampus of patients with AD. Apoptosis is also a component of cell death in Huntington disease and Parkinson disease. Thus, the ability to characterize cells by TUNEL, coupled with aRNA amplification, may lead to the identification of genes that function either to initiate or prevent apoptosis.

The first step in the aRNA amplification process is identification of the cell type to be analyzed, either by electrophysiological recording or immunolabeling. The poly (adenylic acid) tail of the mRNAs present in the tissue sections or group of cultured cells are then hybridized with a 25-nucleotide (oligo dT primer) with an attached T7 RNA polymerase promoter site. The mRNA is copied into complementary DNA (cDNA) through the action of reverse transcriptase (in situ transcription). Individual cells are then microscopically identified by immunohistochemical or TUNEL staining and aspirated into glass electrodes (Figure 1).

The second strand of the cDNA is then made, with each double-stranded cDNA now containing a functional T7 RNA polymerase promoter site. After dialysis to remove free deoxynucleotide triphosphates, the aRNA is made using T7 RNA polymerase, which binds to the functional promoter site, and a reporter nucleotide such as radiolabeled 32-phosphorous-ctosine triphosphate is incorporated for visualization of the amplified product.

Often the first round of aRNA generated from single cells does not contain enough material for use as a hybridization probe to detect RNAs. Hybridization occurs when 2 complementary nucleic acid strands anneal and are detected, usually with 1 of the strands being labeled. Therefore, a second round of amplification is performed, following which the aRNA that is made is more than a millionfold amplified over the original cellular mRNA abundances. Radiolabeled aRNA can then be used as a hybridization probe on reverse Northern blots containing an array of candidate cDNAs. Unlike Northern blots in which RNA is isolated and then probed with cDNA, reverse Northern blots contain immobilized cDNAs that are probed with the aRNA generated from single cells. Alternatively, the aRNA can be used

![Figure 1](image-url). Top, Photomicrograph of a cell (arrowhead) from the white matter near the cortical region of an adult rat. This cell tested positive using terminal transferase-mediated-dUTP nick-end labeling (TUNEL). Bottom, The same section (arrowhead) after aspiration of the cell. Note that adjacent TUNEL-positive cells remain intact. In both panels, sections are viewed uncoverslipped (original magnification ×100).

![Figure 2](image-url). Schematic of the amplified antisense RNA (aRNA) amplification procedure, including possible uses for aRNA. mRNA indicates messenger RNA; cDNA, complementary DNA; and PCR, polymerase chain reaction.
for PCR—a method for amplifying specific regions of DNA—when at least part of the DNA sequence is known. Amplification of mRNA can also be accomplished by PCR after copying of the mRNA into cDNA by reverse transcriptase. The aRNA generated from single cells can also be used for differential display (a PCR-based technique for analyzing changes in gene expression between samples, which also facilitates novel gene discovery), library screening, or library generation.

Following hybridization of the aRNA probe to candidate genes arrayed on a blot, the blots are then apposed to film. The resulting images are called expression profiles. As illustrated in Figure 2, a slice preparation is immunostained for detection of a particular protein or fragmented DNA by TUNEL. After the mRNA population is copied into cDNA by the action of the enzyme reverse transcriptase, the cDNA is removed. The cDNA is made double stranded with subsequent addition of T7 RNA polymerase and the aRNA is made. This labeled aRNA is used as a probe to screen an array of candidate genes. An example of the types of results one may obtain from an expression profile from a single cell includes an observation of a high relative intensity for the mRNA of a pro–apoptotic protein in the cell and a low relative expression for the mRNA of a high relative intensity for the pro-apoptotic protein) in the cell. By such comparisons of the relative hybridization intensities of radiolabeled aRNA for various genes among cells affected by a disease state and normal cells, one can gain information about changes in gene expression that may contribute to cellular dysfunction and/or cell death.

With the development of microarrays (filters or slides containing thousands of cDNAs), the potential exists for simultaneously examining the relative levels of thousands of mRNAs in single cells. An important aspect of aRNA amplification is that relative levels of mRNAs can be obtained by comparing the abundance of a particular mRNA with the abundance of a predetermined mRNA on the blot. This normalization procedure limits the sample to sample variation that could occur by absolute quantitation. One potential limitation of aRNA amplification that uses an oligo-dT-T7 promoter primer is a 3’-end bias for the amplified products. The oligo-dT-T7 primer is a chain of 25 thymidine bases that is extended at its 5’ end with a T7 RNA polymerase promoter site. Because this primer anneals to the 3’ end of the poly(adenylic acid) tail present in the untranslated region of mRNAs, the cDNA that is copied and the aRNA that is eventually made may have a 3’-end bias. The aRNA size can be greater than 2 kilobases long, suggesting that some full-length sequences may be amplified. The aRNA amplification method provides a way to evaluate simultaneously the expression of multiple genes within a set of phenotypically identified cells. Thus, aRNA amplification offers several advantages relative to other gene expression techniques to evaluate the mRNA complexity of individual cells.

APPLICATION TO NEUROLOGICAL DISEASE

A potentially important application of the current technology is to advance our understanding of the mechanisms contributing to the pathophysiology of neurological disease. For example, one report found that giant cells in cortical tubers from postmortem adult patients with tuberous sclerosis exhibited expression of several embryonic markers, including the mRNA for the cytoskeletal element nestin—an intermediate filament found in neuronal progenitor cells—that was not detected by other methods. Such data suggest that giant cells result from either a failure to terminally differentiate or a de-differentiation, providing further rationale for analysis of the mRNA complement present in a subtype of specifically identified cells. Frequently in neurological disease, a subpopulation of cells is affected by the disease process while others are not. In AD, some cells become tangle-bearing neurons while others do not. Furthermore, the recent discovery of RNA in senile plaques and tangles of patients with AD poses the question of what changes in gene expression may contribute to the formation of these pathological structures. Single-cell aRNA amplification can be used to compare the expression profile of tangle-bearing neurons or plaques with that of nonaffected cells in AD to understand the possible role of mRNA level changes in the development of plaques or tangles in AD.

In recent years, many reports have documented apoptosis in neural tissue from many neurological diseases and/or insults, including AD, Huntington disease, Parkinson disease, amyotrophic lateral sclerosis, stroke, and traumatic brain injury. The aRNA amplification method coupled with TUNEL-positive cells may now permit an analysis of the expression profiles of cells exhibiting DNA fragmentation.

Comparing expression profiles of normal vs damaged cells in a particular CNS disease can have important diagnostic and therapeutic implications. The first step in this process involves generating expression profiles from both affected and nonaffected cells in a diseased specimen. Next, an examination of the differentially expressed genes between these cellular populations can lead to the generation of hypotheses about which mRNAs or set of mRNAs and their consequent proteins may be biologically relevant to the disease process. The ultimate goal would be early detection of these mRNA or protein differences in individuals prior to the development of the CNS disease in hopes of early therapeutic intervention.

Several potential applications exist, such as detecting the proteins in body fluids and/or diagnostic mRNA profiles in the lymphocytes of individuals. Single-cell expression profiling can potentially be used to discover and evaluate therapeutic interventions for neurological disease. If the abundance of selected mRNAs is different in diseased vs nondiseased cells as shown by expression profiling, a therapeutic intervention (transcript-aided drug design or TADD) based on the changes in mRNA levels may be designed. For example, if a certain mRNA (X) is found to be increased in abundance in individual cells from...
AD specimens, it may be beneficial to block the translation of (X) through antisense oligonucleotides or pharmacological manipulation. The challenge is to decipher which changes in mRNA abundances are biologically relevant to the disease process under study as well as which ones can be experimentally manipulated. The administered agent may then compensate for changes or alter the abundance of particular mRNAs in cells damaged by disease. Alternatively, the selected drug could operate by restoring the proteins encoded by the mRNAs that are affected. Another example might be that the mRNA component of a signal transduction pathway be altered, so perhaps an effective drug would target genes that are downstream in the biological pathway of the particular protein whose mRNA abundance was altered. While any of these avenues of drug intervention is possible, the ideal end result would be a restoration of cellular function that would translate into an improvement in the patient’s clinical status.

Expression profiling can also help our understanding of the function of known genes in disease states in the hope of rationally creating animal models of disease. The information provided by expression profiles can lead to the design of knockout or transgenic mice to increase our knowledge of the consequences of altering mRNA levels using this technology. For instance, if an mRNA encoding for a pro-apoptotic protein were to be differentially increased in abundance in diseased cells compared with the relative abundance of mRNAs encoding for anti-apoptotic proteins, it would be beneficial to attempt to regulate the expression of this set of genes in mice with the pro-apoptotic gene deletion or other such alterations.

The expression profiles generated from single cells represent the molecular fingerprints of those cells. Differences in the pattern of relative mRNA levels from damaged and normal cells provide information about the molecular pathology of individual cells. The wealth of information gleaned from such data may be used as potential diagnostic or prognostic markers for CNS disease. This technology is currently being used to analyze differences in gene expression from individuals affected by disease in the hope of discovering the pattern of gene expression responsible for the development of the disease. Alternatively, novel genes could be found to be important mediators of the disease process, and these could be sequenced. Thus, the phenotype of a cell from a diseased individual may be linked to the diseased expression profile through aRNA amplification methods. It is hoped that this technology will provide a therapeutic prescription for new strategies to treat the pathophysiology of CNS disease.

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