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Gene expression in TUNEL-positive neurons in human immunodeficiency virus–infected brain

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Human immunodeficiency virus (HIV) infection of the central nervous system (CNS) results in neuronal damage and apoptosis, and both *in vitro* models and pathological studies suggest that a variety of neurotoxins released by HIV-infected and -activated macrophages/microglia selectively damage susceptible subsets of neurons. Confirmation of *in vitro* findings of mechanisms of neurodegeneration and neuronal cell dysfunction *in vivo* has been approached through detailed pathological analysis of regional structural damage, immunohistochemical detection of selected antigens in damaged cells, and, more recently, analysis of gene expression in whole tissue blocks or pooled populations (hundreds/thousands) of microdissected cells. Recently developed techniques of gene expression analysis through antisense mRNA amplification (aRNA) at the single-cell level may offer the potential to study pathways of neuronal cell death and to determine patterns of coordinated gene expression that may more specifically identify susceptible neuronal subclasses *in vivo*. Utilizing this unique technique, the authors have demonstrated, for the first time, RNA amplification and gene expression profiling in individual deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL)-reactive neurons microdissected from fixed, archival human brain tissue. RNA amplification was successful in >80% of TUNEL-positive neurons, and quantitative aRNA/cDNA hybridization slot-blot analysis demonstrated similar levels of actin RNA but significant differences in caspase-2 RNA expression between TUNEL-reactive and -nonreactive neurons. Reliable quantitative comparisons were achieved with modest numbers of sampled neurons (∼10). These studies suggest that analysis of coordinated gene expression in individual damaged neurons *in vivo* can be reliably used to identify neuronal subclasses that express certain susceptibility- or survival-promoting genes that may be targeted for more specific neuroprotective strategies against HIV. *Journal of NeuroVirology* (2004) **10(suppl. 1),** 102–107.

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Introduction

Neuronal loss and damage are common in human immunodeficiency virus (HIV) infection of the central nervous system (CNS), and magnetic resonance spectroscopy studies suggest that such loss begins long before overt clinical signs of acquired immunodeficiency syndrome (AIDS) dementia complex (ADC) appear. Pathological studies have demonstrated that neuronal apoptosis is common, although regionally variable, in CNS HIV infection, even in the brains of individuals with mild neurological dysfunction (Adle-Biassette *et al*, 1999; Petito and Roberts, 1995; An *et al*, 1996; Gelbard *et al*, 1995). This suggests that development of ADC is a chronic, progressive neurodegenerative process of repeated or continual neuronal damage that ultimately ends in apoptosis in a significant number of neurons. The mechanisms and pathways of neuronal apoptosis

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in this process are unclear, partly because of difficulties in manipulating and studying such pathways in neurons *in vitro* and *in vivo*. Pathological studies suggest that certain classes of neurons are selectively vulnerable to damage by HIV *in vivo* (Wiley *et al*, 1991; Masliah *et al*, 1992). Studies using *in vitro* neuronal model systems, including primary human and rodent mixed neuronal/glial cultures, neuronal cell lines, and organotypic brain slice preparations clearly demonstrate that HIV infection of macrophages results in release of cellular and viral neurotoxins that induce neuronal damage and apoptosis (reviewed in Kaul *et al*, 2001; Kolson, 2002; Gendelman *et al*, 1997). These processes often involve neuronal *N*-methyl-D-aspartate (NMDA) receptor overactivation and mitochondrial-mediated apoptosis cascades (intrinsic apoptosis pathway). Alternatively, activation of death receptors (e.g., tumor necrosis factor (TNF)-α, Fas, TRAIL) on neurons may activate the extrinsic (death receptor) apoptosis pathway.

Several pathological studies have supported the notion of "selective neuronal vulnerability" within HIV-infected brain, and whether or not a neuron or other cell within the CNS is damaged or ultimately triggered to enter the apoptosis cascade likely depends upon the cell's expression of specific receptors and components of cell survival and death pathways (Pettmann and Henderson,1998; Wiley *et al*, 1991; Masliah *et al*, 1992). Major unanswered questions regarding the pathogenesis of neuronal damage and apoptosis in HIV-infected brain include (1) Which cell death pathways are activated in damaged neurons *in vivo*? and (2) Why are certain classes of neuronal selectively vulnerable? Immunohistochemical labeling and *in situ* hybridization techniques are sensitive and specific for identification of expressed genes in individual cells, but are limited to the concurrent identification of only one, or at most several, genes of interest in an individual cell. Furthermore, such labeling techniques are difficult to apply to cells labeled by the deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL). In contrast, techniques based upon the detection of simultaneously expressed mRNAs in individual cells allow analysis of coordinated gene expression that may identify cells coexpressing specific receptors, signaling molecules, or other genes that may determine susceptibility.

To develop an approach to identify susceptible neuronal subclasses and potential pathways of neuronal cell death *in vivo*, we have utilized recently developed techniques of single-cell microdissection and antisense mRNA (aRNA) amplification to attempt to analyze gene expression in TUNEL-positive neurons in HIV-infected brain. We have demonstrated, for the first time, RNA amplification and gene expression profiling in individual TUNEL-reactive neurons microdissected from fixed, archival human brain tissue. RNA amplification was successful in >80% of TUNEL-positive neurons, and reverse Northern slot-blot hybridization to custom cDNA arrays demonstrated robust gene expression, including actin and caspase-2 in both TUNEL-positive and -negative cells. Quantitative analysis of 15 individual cells (8 TUNEL-positive and 7 TUNEL-negative) indicated statistically significant differences in RNA expression for caspase-2, suggesting that this technique will be useful for discriminating gene expression in populations of apoptotic and nonapoptotic cells in archival tissue specimens. Furthermore, because we prepare our own custom cDNA arrays, newly identified candidate genes modulating neuronal death and survival can be easily incorporated into the *in vivo* expression analysis. Analysis of coordinated gene expression in apoptotic and nonapoptotic neurons may identify neuronal subclasses that express certain susceptibility- or survival-promoting genes that may be targeted for more specific neuroprotective strategies against HIV.

Results

Both TUNEL-reactive and nonreactive cells (11 reactive, 11 nonreactive) within the basal ganglia of the brain of an individual dying with AIDS dementia were microdissected for analysis. We restricted our dissections of both cell types to those cells demonstrating a neuronal morphology: pyramidal shape, with at least one elongated cellular process, and an identifiable nucleus (O'Dell *et al*, 1998, 2000). Cells with intense nuclear TUNEL staining and little or moderate cytoplasmic staining were selected (Figure 1, *black arrows*), as were nonreactive cells within the same microscopic field. Individual dissected cells (Figure 2A) were subjected to two rounds of aRNA amplification (antisense, amplified RNA) as described in Materials and Methods, yielding

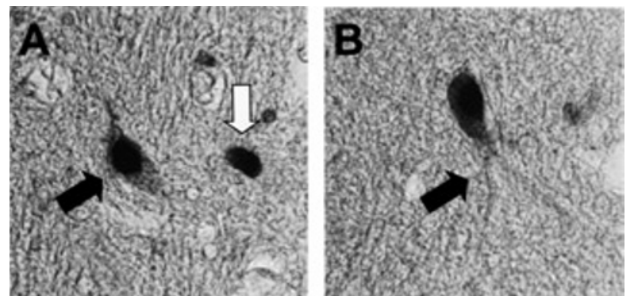

Figure 1 Selection of TUNEL-positive neurons in fixed brain tissue. TUNEL was performed on brain sections derived from the basal ganglia of an individual dying with ADC, and cells were selected for microdissection based on demonstration of darkly stained nucleus, lightly stained cytoplasm and one or more neuritic projections. **A** shows one selected cell (*black arrow*), and one cell not selected (*white arrow*) because of lack of an obvious projection and cytoplasm. **B** shows a selected cell with a double projection.

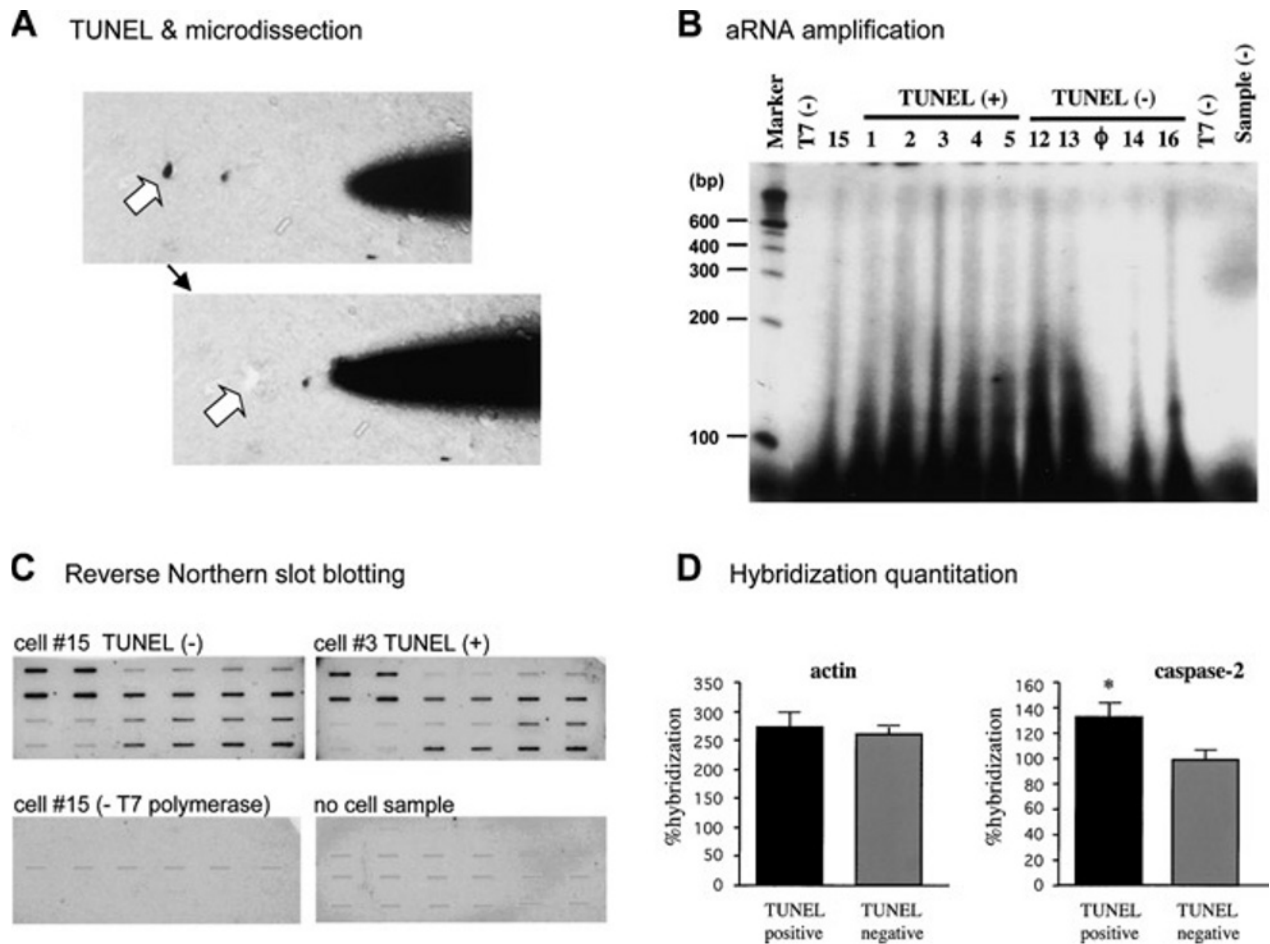

Figure 2 RNA amplification and gene expression in microdissected TUNEL-positive and -negative neurons in ADC brain. A section from fixed, paraffin-embedded basal ganglia was subjected to TUNEL, followed by microdissection of pyramidal-shaped TUNEL-positive and -negative cells (**A**). ^{32}P-labeled anti-sense RNA probes were synthesized by two successive rounds of oligo-dT priming, reverse transcription, and RNA synthesis. Probe size was assessed on a polyacrylamide gel (**B**), which shows aRNA from five individual TUNEL-positive cells (numbers 1 to 5) and five TUNEL-negative cells (numbers 12 to 16). Control lane T7 (−) received products of probe synthesis from cell number 15 performed without T7 RNA polymerase, and control lane Sample (−) received products of probe synthesis performed without cellular cDNAs added. "ϕ" indicates an unloaded lane. aRNA probes were then hybridized to custom-designed hybridization membranes containing selected genes of interest. **C** shows hybridization of aRNA probe from one TUNEL-negative cell (cell number 15, *top left*) and one TUNEL-positive cell (cell number 3, *top right*). Bottom left shows lack of hybridization when probe synthesis for cell number 15 was attempted without T7 RNA polymerase, and the bottom right panel shows similar failure of hybridization when probe synthesis was attempted without cellular cDNA reverse transcription products added to the reaction. **D** shows quantitaive comparisons of mean aRNA/cDNA hybridization signal intensities for eight TUNEL-positive and seven TUNEL-negative neurons.

^{32}P-labeled aRNA probes, which typically display a size distribution up to 1 kb (Figure 2B) (Eberwine *et al*, 1992). No RNA was detected when T7 RNA polymerase was excluded from the amplification mix used to synthesize the probe [lane T7 (−)], or when reverse-transcription cDNA product was excluded from the probe amplification mix [lane Sample (−)].

These antisense probes, representing specific mRNAs present in the cell, were then utilized for reverse Northern (slot) blotting (Figure 2C) against a cDNA array made with linearized plasmid cDNAs that were ultraviolet (UV)-crosslinked to nylon membranes. Figure 2C demonstrates representative hybridization slot blots from two of the dissected cells (no. 15, TUNEL-negative; no. 3, TUNEL-positive), with duplicate slots representing each cDNA. Strong hybridization signals were seen with aRNA probes from 8 of 11 apoptotic cells, and 7 of 11 nonapoptotic cells, which are represented in the top panels (*top left*, apoptotic; *top right*, nonapoptotic). In separate hybridizations, expression of neurofilament RNA was demonstrated, thus confirming the neuronal phenotype of the cells (not shown). As expected, no hybridization was seen when aRNA probe synthesis was attempted without T7 RNA polymerase (*lower left panel*) or without reverse-transcription cDNA product (*lower right panel*). Figure 2D represents the mean relative hybridization signal intensities calculated for the eight TUNEL-positive and seven TUNEL-negative neurons. Each TUNEL-positive/negative pairing thus

represents the average hybridization signal for the specified gene from eight and seven individual blots, respectively. Although no differences in actin expression was seen between TUNEL-positive and -negative neurons, a statistically significant difference in caspase-2 RNA expression was seen, with higher expression in the TUNEL-positive neurons ($P < .05$).

Discussion

We have demonstrated the feasibility of comparative analysis of gene expression at the single-cell level in TUNEL-positive neurons in archival, formalin-fixed paraffin-embedded human brain tissue, through the application of mRNA amplification techniques (White *et al*, 2001; Crino and Eberwine, 1996; Crino *et al*, 1996). Our results showed consistent expression of mRNA in TUNEL-positive neurons, and the detection of statistically significantly higher levels of caspase-2 mRNA in these neurons suggests that these techniques are sufficiently sensitive and reproducible to detect even modest differences in gene expression between dying and normal-appearing cells *in vivo*. The biological significance of the differences in caspase-2 RNA expression between TUNEL-positive and -negative neurons requires further investigation, but these encouraging results suggest transcriptional up-regulation of this caspase in dying neurons in the basal ganglia in ADC.

The amplification of mRNA from individual cells is a powerful tool for rapid expression screening of relatively large numbers of genes for which cDNA clones are widely available. This technique is effective at the single-cell level because of the incorporation of the T7 RNA polymerase promoter into the oligo-dT primer used for initiation of reverse transcription, and its subsequent incorporation into the cDNA product (Eberwine *et al*, 1992, 1995). The amplification of aRNA is thus governed by the linear amplification kinetics of T7 RNA polymerase, allowing for quantitative representation of mRNA species present in the cell (Eberwine *et al*, 1992; Crino *et al*, 2001). Accordingly, the ability to analyze expression in dying neurons should allow identification of patterns of coordinated gene expression that define susceptible neuronal subclasses and pathways of death and survival activated in specific disease states (e.g., ADC), and allow the screening of newly identified genes that may modulate these processes. Although not previously applied to the study of TUNEL-positive neurons in archival human brain tissue, these techniques have been utilized to demonstrate amplification of mRNAs in single, fixed TUNEL-positive neurons dissected within 24 h postmortem from rat brain tissue (O'Dell *et al*, 1998, 2000). Our study differs in profiling TUNEL-positive neurons derived from archival human brain tissue that has been stored for prolonged periods (greater than 1 year) after processing (National NeuroAIDS Tissue Consortium; http://www.hivbrainbanks.org).

Our experience indicates expected success rates of >80% in obtaining aRNA/cDNA hybridizations with material derived from TUNEL-positive neurons selected carefully on the basis of identifiable morphological features of pyramidal shape, one or more cellular processes, and well-defined nuclear and cytoplasmic areas, along with neurofilament RNA expression. Although the apparent morphological integrity of these TUNEL-positive neurons does not meet criteria for apoptosis (nuclear pyknosis with or without blebbing, shrunken cytoplasm), the TUNEL reactivity indicates DNA damage that likely heralds an apoptotic, or possibly, necrotic cell death (O'Dell *et al*, 2000; Gold *et al*, 1993). Nonetheless, this technique clearly allows identification of gene expression in damaged neurons, likely in intermediate stages of cell death pathways, and as such it is clearly not limited solely to the study of neuronal apoptosis. Our data indicate that sampling modest numbers of neurons can detect highly statistically signficant differences in RNA expression between TUNEL-positive and -negative cells, which supports the feasibility of interregional analysis within an individual brain and comparisons among different brains through single-cell analysis.

Although *in vitro* models of neuronal death have proved invaluable in studying pathways of apoptosis, mechanisms of cell injury, effector molecules and receptors mediating these processes, *in vivo* analysis and validation of these models are critical. Numerous *in vitro* models of AIDS neuropathogenesis have demonstrated that cellular products released from HIV-infected macrophages as well as viral proteins (gp120, Tat, Vpr) can induce neuronal apoptosis that may be mediated largely through the intrinsic mitochondrial-associated pathway (Chen *et al*, 2002; Kaul *et al*, 2001). Several of these studies have confirmed specific activation of neuronal cell surface receptors (NMDA, CXCR4) or signaling pathways (p38 mitogen-activated protein kinase (MAPK), nitric oxide synthase) that may offer specific targets for neuroprotective strategies (Kaul *et al*, 2001). Indeed, several large multicenter clinical trials for neuroprotection against AIDS dementia have been developed, based largely upon such models (memantine, an NMDA receptor antagonist; selegiline, a monoamine oxidase inhibitor and antioxidant). However, whether such agents or as yet otherwise untested agents can be expected to provide full, partial, or no protection in HIV-infected brain will depend on identifying whether similar mechanisms and effector molecules/receptors are operational *in vivo*. The analysis of gene expression at the single-cell level in brain tissue offers a powerful means for studying such processes in dying/damaged neurons and other bystander cells (glia, endothelia) that can confirm and extend the range of therapeutic targets for the rational

design of clinical neuroprotection trials in degenerative diseases such as ADC.

Materials and methods

The detailed protocols for RNA amplifcation, reverse Northern hybridization, and quantitative array analysis are described in references (Eberwine *et al*, 1992; Crino *et al*, 1996). Brain specimens were provided by the National NeuroAIDS Tissue Consortium (http://www.hivbrainbanks.org). Formalin-fixed paraffin-embedded brain slices (7 to 10 μ) were deparaffinized and subjected to TUNEL (TdT FragEL kit; Oncogene, Boston, MA). For initiation of *in situ* reverse transcription (IST) of poly-A+ mRNA, sections were incubated with an oligo-dT(11–24) primer coupled to a T7 RNA polymerase promoter sequence (oligo-dT-T7), and cDNA was synthesized on the section with Avian myeloblastosis virus reverse transcriptase (AMV RT, Seikagaku America).

After IST, individual neurons were microdissected from the surrounding neuropil with a micromanipulator (Eppendorf), and single neurons were aspirated into separate Eppendorf tubes (Figure 2A). Double-stranded template cDNA was then synthesized (T4 DNA polymerase I) from the cell, all within the tube. mRNA was amplified (amplified, antisense RNA; aRNA) from the double-stranded cDNA template with T7 RNA polymerase (2000 U/reaction; Epicentre Technologies). The resulting “first-round” aRNA is amplified ~2000-fold over starting material (Eberwine *et al*, 1992). First-round aRNA then served as a template for cDNA synthesis. aRNA was again reverse-transcribed with AMV RT. cDNA generated from this aRNA was made double-stranded, and then used as template for a second round aRNA amplification. In this round, ^{32}P-CTP was incorporated, and radiolabeled aRNA (total amplification $\sim 1 \times 10^6$ [Eberwine *et al*, 1992]) was used as a probe for reverse Northern (slot) blotting using a custom cDNA array. We prepared the cDNA arrays by adhering linearized plasmid cDNAs via UV-crosslinking to nylon membranes in a slot-blot array (Hybond, Amersham, Piscataway, NJ).

The hybridization intensity of aRNA-cDNA hybridization was determined by densitometric analysis of the slot-blot image (ImageQuant 5.0 software). Nonspecific hybridization to pBS plasmid cDNA was subtracted from the signal intensity for each aRNA/cDNA hybridization. Because of linear kinetics of aRNA amplification using T7 RNA polymerase, hybridization intensity reflects the relative abundance of mRNAs in single cells (Eberwine *et al*, 1992; Crino *et al*, 2001). The relative abundance of each aRNA (% hybridization) was calculated by determining the average hybridization signal intensity above background for all slots, and then expressing individual signals as a percentage of this average (Crino *et al*, 2001). Differences in relative abundance were determined by a one-way analysis of variance (ANOVA) and Fisher’s post hoc test, comparing each signal in TUNEL-positive and TUNEL-negative neurons ($P < .05$, significance).

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