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E2F1 localizes predominantly to neuronal cytoplasm and fails to induce expression of its transcriptional targets in Human Immunodeficiency Virus-induced neuronal damage

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Abstract

As human immunodeficiency virus (HIV) does not induce neuronal damage by direct infection, the mechanisms of neuronal damage or loss in HIV associated dementia (HAD) remain unclear. We have shown previously that immunoreactivity of transcription factor, E2F1, increases in neurons, localizing predominantly to the cytoplasm, in HIV-associated pathologies. Here we confirm that E2F1 localization is predominantly cytoplasmic in primary post-mitotic neurons in vitro and cortical neurons in vivo. To determine whether E2F1 contributes to neuronal death in HAD via transactivation of target promoters, we assessed the mRNA and protein levels of several classical E2F1 transcriptional targets implicated in cell cycle progression and apoptosis in an in vitro model of HIV-induced neurotoxicity and in cortical autopsy tissue from patients infected with HIV. By qPCR, we show that mRNA levels of E2F1 transcriptional targets implicated in cell cycle progression (E2F1, cyclin A, proliferating cell nuclear antigen (PCNA), and dihydrofolate reductase (DHFR)) and apoptosis (caspases 3, 8, 9 and p19^{ARF}) remain unchanged in an in vitro model of HIV-induced neurotoxicity. Further, we show that protein levels of p19^{ARF}, Cyclin A, and PCNA are not altered in vitro or in the cortex of patients with HAD. We propose that the predominantly cytoplasmic localization of E2F1 in neurons may account for the lack of E2F1 target transactivation in neurons responding to HIV-induced neurotoxicity.

Keywords

E2F1; HIV-associated dementia; transcription factor

Human immunodeficiency virus (HIV)-associated dementia (HAD) is a common neurological disorder associated with HIV infection. Pathologic studies of the brains of patients with HAD suggest an inflammatory mechanism in the progression of this disease, as evidenced by astrogliosis, microgliosis, and perivascular macrophage infiltration (1–3). Although neuronal death, dendritic loss and synaptic loss are features of HAD, there is little evidence of direct HIV infection of neurons. Instead, neuronal dysfunction and death likely result from the release of various neurotoxic factors from activated macrophages and microglia, such as reactive oxygen species and excitatory amino acids (4–6). Correlative

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evidence suggests that neuronal damage in HAD may result from several mechanisms, including decreased neuronal autophagy (7), NMDA receptor activation (8,9), abnormal CDK5 kinase activity (10), activation of the p38 mitogen-activated protein kinase (MAPK) cascade (11), caspase activation, inhibition of the nuclear factor- κ B survival pathway via glycogen synthase kinase-3 β activation (12), and/or aberrant cell cycle regulation involving E2F1 (13,14).

E2F1 is a member of the E2F family of transcription factors, which play a pivotal role in cell differentiation, proliferation, and apoptosis through transcriptional regulation. In non-neuronal cells, E2F1 is predominantly nuclear and its transcriptional activity is regulated by Retinoblastoma protein (Rb). Among the categories of genes regulated by E2F1 are those necessary for RNA and DNA synthesis, such as dihydrofolate reductase (DHFR) and proliferating cell nuclear antigen (PCNA) and those necessary for cell cycle progression, such as Cyclin A (15) and E2F1 itself (16). In addition, E2F1 regulates genes involved in apoptosis, including p19^{ARF}, an initiator of p53-dependent apoptosis (17), as well as other apoptotic genes that are independent of the p53 pathway, including APAF1, BID, and caspases 2, 3, 7, 8, and 9 (18). Finally, E2F1 has been shown to induce cell death independently of gene transactivation through inhibition of the anti-apoptotic signaling of the NF- κ B pathway (19) or through induction of the calcium-activated, cysteine protease, calpain (20).

Using in vitro models of neurodegeneration, several studies have demonstrated that E2F1 contributes to neuronal damage and death (21–27). These studies speculate that E2F1 mediates neuronal death via activation of its transcriptional targets (28). However, several reports have observed cytoplasmic localization of E2F1 in postmitotic neurons of patients with neurodegenerative diseases including HIV encephalitis, SIV encephalitis, Alzheimer Disease, Parkinson Disease, Huntington Disease, and amyotrophic lateral sclerosis (13,28–31). In these diseases, E2F1 immunoreactivity and/or protein levels were also reported to increase (see review (30)). To assess whether E2F1 induces cell death in a transcription-dependent manner in neurons in HIV-induced neurotoxicity despite a predominantly cytoplasmic localization (13,20,29), we determined the localization of E2F1 in primary cortical neurons and in neurons from human cortex and assayed the expression of several classic E2F1 targets in an in vitro model of HIV-induced neurotoxicity and in cortical autopsy tissue from 14 HIV-positive individuals.

As an in vitro model of HIV-induced neurotoxicity, we used a previously well-described model in which supernatants from HIV-infected primary monocyte-derived macrophages (HIVMDM) are used to treat primary rat cortical neurons (8). Primary rat cortical cultures were prepared from embryonic day 17 Sprague–Dawley rat pups. Cells were plated at a density of 2×10^6 cells per 60 mm dish pre-coated with poly-L-lysine (Peptides International, Louisville, KY, USA) and maintained in neurobasal media (Invitrogen) with B27 supplement (Invitrogen) at 37°C and 5% CO₂. Cultures were utilized at 21 days in vitro (DIV) unless indicated otherwise.

For western blotting, fresh frozen tissue samples from midfrontal cortex of HIV-infected patients suffering from neurocognitive impairments (n = 13) and control patients with normal neurocognitive status (n = 5) were obtained from the tissue banks of National NeuroAIDS Tissue Consortium (33). Tissue was homogenized on ice in Lysis Buffer [50 mmol/L Tris–HCl (pH 7.5), 0.5 mol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 2 mmol/L EGTA, 2 mmol/L EDTA, 5 mmol/L NaF, 0.1 mmol/L phenyl-methane-sulphonylfluoride, 1 μ g/mL leupeptin, 1 mmol/L DTT, and protease inhibitor cocktail]. Membranes were incubated overnight at 4°C with primary antibodies against p19^{ARF} (Abcam, MA, USA), PCNA, DHFR and Cyclin A (Santa Cruz, CA, USA).

Quantitative real-time polymerase chain reaction (Q-PCR) was performed as described previously (10). Expression levels of E2F1, p19^{ARF}, PCNA, caspases 3, 8, 9 and the internal controls of 18S or TATA box binding protein (TBP) were quantified using the LightCycler Amplification Kit SYBR Green I (Roche Applied Science, Indianapolis, IN, USA). Results were normalized to either 18S or TBP RNA levels and were reported using arbitrary units. The primer sequences are available upon request.

Immunofluorescent staining was described previously (34). The tyramide amplification system (New England Biolabs, USA) was used to detect E2F1. DNA was visualized by DAPI staining. Neurons were marked by staining for the neuronal marker, MAP2. The dilutions used were as follows: E2F1 (1:50, Santa Cruz, CA, USA), DAPI (5mM, Molecular Probes, CA, USA), MAP2 (1:200, Covance, USA). Slides were mounted and analyzed by laser confocal microscopy.

The MAP2 cell-based ELISA assay used in the current studies was described previously (10). Relative changes in the intensity of the fluorescent MAP2 signal were used to estimate neuronal damage. Values were expressed as mean $\pm$ SEM. Data with multiple categories was analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test using Prism software (GraphPad Software, San Diego, CA, USA). Values of $p < 0.05$ were considered significant.

E2F1 is well-characterized as a transcription factor that functions to control cell cycle progression. Its nuclear activity is regulated primarily by its interaction with DP1, pRB, and Cyclin A:cdk2. However, in 3-week-old rat cortical neurons left untreated (Fig 1, top row), we observed E2F1 (green) predominantly in the neuronal cytoplasm; a compartment inconsistent with its defined role as a transcription factor. In the mid-frontal cortices of uninfected, normal brains, we observed the same pattern of localization (Fig 1, bottom row). It is worth noting that the predominantly cytoplasmic localization is not altered by the ages of rat cortical cultures nor by different neurotoxic treatments, such as mock-infected MDM (mock MDM) and HIVMDM. Moreover, the cytoplasmic localization of E2F1 in cortical brain tissue of HIV-infected individuals is not dependent on the neurocognitive status of the patient (data not shown). Taken together, these data suggest that, in neurons in vitro and in the brains of HIV-infected individuals, E2F1 is predominantly localized in a subcellular compartment that is inconsistent with its well-characterized role as a transcriptional activator.

Previous studies (8,10) have demonstrated that the NMDA receptor, at least partially, mediates neuronal death in our HIVMDM induced neurotoxicity model. As NMDAR levels peak in primary neurons at 21 days in culture, we use primary rat cortical neurons at this age for our studies. Using this model, we observed 50–80% neuronal death depending on the dilutions of HIVMDM (Fig 2A). For our analysis of E2F1 target transactivation, to capture a point in which neurons were in the process of undergoing cell death, we used a dilution of HIV MDM that resulted in 50% neuronal loss at 20 hours.

To determine if HIVMDM-induced neuronal death is mediated by activation of classical E2F target genes, we assessed mRNA levels of select E2F target genes from each of the following categories: cell cycle progression, RNA and DNA synthesis, or apoptosis. E2F1, an indicator of G1 to S progression, and cyclin A, a regulator of S phase exit, were assessed in our model of HIV-induced toxicity as examples of E2F1 targets that regulate cell cycle progression. To determine if E2F1 is upregulating genes whose products are necessary for DNA and RNA synthesis, we assessed PCNA and DHFR, respectively. Finally, E2F1 has been shown to induce apoptosis by transactivation of several pro-apoptotic gene products which act in distinct apoptotic cascades. Thus, we assessed the mRNA levels of targets from

each of these pathways (intrinsic apoptosis – caspase 9, extrinsic apoptosis – caspase 8, effector caspase – caspase 3, and p53-dependent apoptosis – p19^{ARF}) in neurons responding to HIVMDM. Three week-old primary cortical cultures were treated with mock MDM or HIVMDM or were left untreated. As NMDA activation partially mediates neuronal death in this model, we also included a set of cultures pretreated with MK801, an NMDA receptor antagonist, to determine if any observed changes in E2F1 target genes were NMDA-dependent. By Q-PCR, we found that the mRNA levels of all analyzed target genes were similar in neurons treated with HIVMDM, mock MDM, MK801-pretreatment, and in cultures left untreated (Fig 2B–I). When we selectively compared the protein levels of DHFR, PCNA, Cyclin A, and p19^{ARF} by immunoblot, we were again unable to detect a difference in the expression of these targets across different treatments (Fig 3A). Our Q-PCR results are consistent with our previous observation that neither caspase 3 protein levels nor cleaved-caspase 3 are altered by HIVMDM treatment (10).

Consistent with our findings in vitro, we did not observe differences in expression of the E2F1 targets, PCNA, Cyclin A, and p19^{ARF}, at the protein level in cortical tissue from patients with HAD compared with cognitively normal, HIV-infected cortex (Fig 3B, C). We also did not see significant differences in these same target genes in cortex from HIV-infected patients compared with that from uninfected patients. However, we did observe an increase in DHFR in cortical tissue of HIV-infected patients (Fig 3B, C). These data suggest that any role for E2F1 in this model of neurodegeneration would likely occur via a transcription-independent mechanism.

E2F1 is upregulated in neurons subjected to apoptotic stimuli (19,35) and is elevated in neurons of patients with HIV (13), Alzheimer disease (28), Down syndrome (36), Parkinson disease (37), Huntington disease (31), amyotrophic lateral sclerosis (38) and in an animal model of HIV encephalitis, SIV encephalitis (SIVE) (29). In addition, neurons cultured from mice null for E2F1 are at least partially protected from diverse insults (25,37,39). While transcription-dependent E2F1-induced apoptosis has been implicated in several in vitro models of neuronal death (37,40), the role of E2F1 transactivation in neuronal death was not explicitly demonstrated (41–46). Surprisingly, in our in vitro model of HIV-induced neurotoxicity and in cortical tissues from HIV-infected individuals, we did not observe a change in typical E2F1 target genes (Fig 2B–I and Fig 3), including those implicated in apoptosis. Interestingly, we have also shown here that E2F1 is predominantly cytoplasmic in 3-week-old primary rat cortical cultures. Considering that we have found that E2F1 predominantly resides in the neuronal cytosol independent of culture treatment (Untreated, Mock MDM or HIVMDM), our results suggest that the main function of E2F1 in post-mitotic neurons may not be to regulate its nuclear targets.

E2F1 is able to induce cell death via several distinct pathways. Transcription-dependent E2F1-induced apoptosis can be both dependent on the p53 tumor suppressor protein or independent of p53. In p53-dependent apoptosis, E2F1 transactivates p19^{ARF}, leading to stabilization of p53 by blocking MDM2-mediated p53 degradation. E2F1 can also induce apoptosis by transactivation of caspase proteins directly. Our results indicate that HIVMDM does not induce increases in p19^{ARF} or caspases at the mRNA or protein levels (Fig 2B–I, Fig 3). Further, HIVMDM treatment does not induce caspase 3 protein levels or cleavage of caspase 3 in primary rat cortical neurons, as we have reported previously (10). Among all of the classical targets of E2F1 analyzed here, the protein level of DHFR is the only one to increase in the cortex of HIV-infected patients compared with that of uninfected patients, although it does not change in the in vitro model of HIV-induced neurotoxicity. DHFR has been implicated in the onset and progression of several neurodegenerative disorders and its turnover can be affected by oxidative conditions (47). Therefore, it is possible that the observed alteration in DHFR protein levels in cortical tissue from HIV-infected patients are

independent of E2F1 transcriptional regulation. Our data from the in vitro model of HIV-induced neurotoxicity and from cortical tissue from HIV-infected patients suggests that E2F1 is not acting via a classical, transcription-dependent mechanism in this disease.

The predominant cytoplasmic localization of E2F1 in postmitotic neurons is highly interesting. Other transcription factors have been reported as being abnormally localized in the cytoplasmic compartment of neurons under pathological conditions (see review (30)); however, we also observe cytoplasmic E2F1 in neurons in vitro that are not exposed to stress, as well as in neurons from autopsy tissue of cognitively normal control cortex. Interestingly, levels of nuclear E2F1 have been reported to be down-regulated during the differentiation of keratinocytes (48), and our unpublished data also reveal a similar finding during the differentiation of neuronal cell lines (data not shown). These data suggest that nuclear E2F1 may be decreased as cells permanently exit the cell cycle and undergo differentiation. In both keratinocytes and primary mouse neurons, E2F1 has been shown to shuttle between the nucleus and the cytoplasm in a CrmA-dependent manner (48–50). Based on these observations, it would be interesting to investigate the relationship between nuclear E2F1 and the differentiation process to determine whether a change of predominant E2F1 localization to the cytoplasm triggers differentiation or vice versa. Furthermore, shuttling and localization of E2F1 are affected by its nuclear localization signal, its nuclear export signal, and its binding partners, such as Cyclin A and pRb. While we have previously detected pRb in the nuclear compartment of primary neurons (13), we have been unable to co-immunoprecipitate pRb and E2F1 in postmitotic neurons (unpublished data). Further investigation is required to determine the role that these factors play in the cytoplasmic localization and shuttling of E2F1 in postmitotic neurons.

To summarize, our data suggest that the two groups of classical targets of E2F1, genes associated with cell cycle progression, such as Cyclin A, PCNA, DHFR, and genes associated with apoptosis, such as p19^{ARF}, and caspases 3, 8, and 9, are not affected in HIV-induced neurotoxicity which may be explained by the predominantly cytoplasmic localization of E2F1 in postmitotic neurons that we show here. Further studies are needed to understand the role of cytoplasmic E2F1 in normal neurons and in neurons responding to neurodegenerative stimuli, such as those seen in HAD. As such roles for E2F1 are largely unknown, studies to uncover these previously undescribed roles may lead to novel and unique therapeutic opportunities.

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Figure 1. E2F1 is predominantly cytoplasmic in neurons

In 3-week-old rat cortical neurons (top panel) and cortical tissues from individuals (bottom panel), immunofluorescent staining for E2F1 (green) is predominantly cytoplasmic in neurons (MAP2, cytoplasmic neuronal marker, red); the nuclei are stained blue (DAPI). Co-localization of E2F1 and MAP2 appears yellow. Bar = 10 μ m

Figure 2. E2F1 targets are not induced in HIVMDM-treated primary rat cortical neurons

A) HIVMDM supernatants induce cortical neuron death in a dose-dependent manner. 3-week-old rat cortical cultures treated for 20 h with 3 increasing dilutions of HIVMDM resulted in a significant decrease in relative MAP2 fluorescence when assessed by a MAP2, cell-based ELISA, whereas untreated cultures or treatment with Mock-infected MDM supernatant (Mock MDM) did not result in a detectable loss of MAP2 fluorescence. Values are mean $\pm$ SEM, $n = 6$, one-way ANOVA, Newman-Keuls, *** $p < 0.001$ compared with their respective Mock MDM-treated cultures. B – I) E2F1 target genes were not altered at the mRNA level in HIVMDM-treated rat cortical neurons. Using Q-PCR, mRNA levels of E2F1 target genes involved in cell cycle progression (E2F1 (B) and Cyclin A (C)), in RNA

and DNA synthesis (DHFR (D) and PCNA (E)), and those involved in apoptosis (p19^{ARF} (F), caspase 3 (G), caspase 8 (H) and caspase 9 (I)) were not altered by HIVMDM, Mock MDM, or MK801 treatment.

Figure 3. E2F1 target genes were not altered at the protein level in the in vitro model of HIV-induced neurotoxicity or in the cortex of patients with HAD

A) As detected by immunoblotting, protein levels of PCNA, Cyclin A, DHFR and p19^{ARF} were unchanged in HIVMDM, Mock MDM, and MK801 treatments. B) Protein levels of PCNA and Cyclin A were not significantly different in the frontal cortex of HIV-infected cases compared with HIV negative cases, nor with 13 cases with neurocognitive impairment as compared with 5 cognitively normal cases, one of which was also infected with HIV. Bands were quantified and normalized to actin. Protein levels of DHFR were significantly increased in HIV-infected patients and in neurocognitive impairment cases, as compared with uninfected and with neurocognitively normal cases, respectively. C) PCNA levels were not significantly different ($p>0.05$); similar results were observed for Cyclin A and p19^{ARF}

(data not shown). Statistical analysis of DHFR showed significant increases in HIV-infected individuals and in those with neurocognitive impairment, ** $p < 0.01$.