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HIV *gag* DNA is Present in Neural Progenitors Harvested by Laser Capture Microdissection from Archival Pediatric Brain

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ABSTRACT

Background: HIV-1 infection of the CNS in infants and children is associated with impaired brain growth, developmental delays and deficits in memory and language development. Neural progenitor cells are critical in pediatric brains for neurogenesis and support of maturing neurons. We had shown previously in cell culture that human, nestin-positive neural progenitors are permissive for HIV-1 infection, and have presented *in situ* hybridization evidence of HIV-1 infection in a nestin-positive periventricular cell identified in archival pediatric brain from a 4 year old female with severe neuroAIDS. We now report HIV-1 *gag* sequences in DNA extracted from nestin-positive neural progenitors excised by laser capture microdissection from paraffin-embedded pediatric hippocampal tissue.

Methods: Nestin-positive neural progenitors were identified in formalin-fixed, paraffin-embedded archival hippocampal and periventricular tissue from 3 HIV-infected pediatric patients and 2 uninfected control infants by tyramide signal-amplified fluorescence immunohistochemistry. After cell capture by laser microdissection, DNA was extracted, PCR amplified with primers directed against a 115 base pair segment of *gag*, visualized by gel electrophoresis, and sequenced. Results were displayed and identified using a basic local alignment search tool (BLAST).

Results: The HIV-1 *gag* sequence was identified in DNA extracted from nestin-positive cells dissected from the hippocampus of a 19 month old vertically infected female. No such sequences were identified in negative control cells from culture or archived human infant hippocampus, nor in cells harvested from the other two HIV-1 infected children studied to date.

INTRODUCTION

HIV-1 infection of the central nervous system (CNS) in infants and children is associated with diminished brain growth, developmental delays and cognitive deficits, especially in the areas of language development and memory. The underlying molecular mechanisms by which HIV-1 impairs global neural development in general, or development in specific regions of the brain, in pediatric patients infected *in utero* or at birth (vertical infection) are incompletely understood. Because direct infection of neurons appears to be a rare event, other explanations for neuronal loss and poor brain growth have been suggested, including neuroinflammation and neurotoxicity from viral products, HIV-associated inflammatory molecules, and / or impaired neural repair mechanisms.

Neural progenitor cells are critical for neurogenesis and support of maturing neurons, and appear to have an important role in both human brain growth and repair after neural insult. How neural progenitor functions might be impaired in the developing human brain infected with HIV-1is not known, and an important question is whether or not these cells can be directly infected with HIV-1 *in vivo*.

Multipotential neural progenitors express the 220 kDa class VI intermediate filament nestin, which can be visualized within the cell cytoplasm by immunocytochemistry using an antibody developed in this laboratory. As published previously, new virus is produced in nestin-positive, multi-potential, human fetal neural progenitors transfected with the HIV-1 plasmid pNL4-3. To determine the relevance of this *in vitro* finding to pediatric neuroAIDS, we examined hippocampal and periventricular tissue from HIV-1 infected infants and children for evidence of HIV-1 DNA within laser-captured cells identified as nestin-positive by immunohistochemistry.

MATERIALS and METHODS

Cells: T-lymphocytes (A3.01), either uninfected or infected with HIV-1 IIIB, as well as T-lymphocytes modified to contain noninfectious HIV DNA (8E5 cell line) were grown as suspensions in RPMI media supplemented with 10% fetal calf serum, 2mM L-glutamine, 100IU/ml penicillin/streptomycin, and 50 µg/ml gentamicin. Cells were fixed in 4% paraformaldehyde, then counted and cytospun onto silane-treated glass slides at a density of $7x10^5$ cells per slide prior to immunocytochemistry.

Tissue: Archived paraffin-embedded pediatric brain tissue was obtained from the Children's National Medical Center, Washington, D.C. (Table 1). Four to six micron thick slices cut from the periventricular region, hippocampus, and cerebellum of pediatric patients diagnosed as HIV-1 positive (N=3) and control patients not identified as HIV-positive (N=2) were adhered to silanized glass slides, deparaffinized in 100% xylene and rehydrated through graded ethanol solutions, followed by microwave antigen retrieval in 10X Antigen Retrieval Citra Plus (BioGenex) diluted 1:10 in glycerol, and a 60 minute incubation in 3% hydrogen peroxide to block endogenous tissue peroxidases.

Materials and Methods (cont.)

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Immunocytochemistry: Cells adhered to silane-treated glass slides were blocked with 1% BSA and 1% goat serum (2% serum), permeabilized with 0.2% Triton X-100, then incubated x50 minutes at room temperature (RT) with a primary monoclonal anti-P24 antibody (1:100) (Zeptometrix) in 2% serum, followed by a 30 minute RT incubation in a biotinylated goat anti-mouse antibody (1:200) (Vector), a 5 minute RT incubation with Vectastain ABC Reagent (Vector), and then a 5 minute exposure to 7mg/ml DAB (Sigma). Nuclei were counterstained with methyl green (Vector), and cells examined on a Zeiss Axiovert microscope using AxioVision Software.

Immunohistochemistry: To visualize nestin-positive cells, tissue was blocked in 3% bovine serum albumen (BSA) and 2% normal goat serum in 1X PBS (5% serum), permeabilized in Triton 0.6% X-100 x60 minutes, then incubated overnight at 4-6ºC in rabbit polyclonal anti-human nestin primary antibody diluted 1:5000 in 5% serum. Immunofluorescence detection of the primary antibody was achieved by 45 minutes incubation with a secondary goat anti-rabbit horseradish peroxidase conjugate (1:200), followed by a 5-6 minute incubation with Alexa-Fluor 568 labeled tyramide (1:200) diluted in 0.0015% hydrogen peroxide amplification buffer (Molecular Probes, TSA kit). Nuclei were counterstained with bisbenzamide (1:500) (Biosciences) and the tissue was examined on a Zeiss Axiovert fluorescence microscope using AxioVision Software.

Laser capture microdissection and DNA extraction: Nestin-positive cells from archival tissues, HIV-1 T-lymphocytes (A3.01 IIIb-infected and 8E5), and uninfected T-lymphocytes (A3.01) were identified and laser catapulted (Zeiss PALM Microlaser Technologies, Germany)) into the caps of 50µl autoclave-sterilized PCR tubes containing 20 µl of lysis buffer (PicoPure DNA extraction kit, Arcturas Bioscience) and centrifuged briefly. An additional 30µl of proteinase-K buffer was added to each cell pellet After additional centrifugation, cells were incubated at 65ºC x ≥ 3 hours for cultured cells or ≥ 16 hours for cells from tissue, then centrifuged briefly, heated to 95ºC x10 minutes, and stored at 4ºC pending PCR amplification. Caps were moved into place but no cells captured in order to obtain "buffer only" negative control samples processed in the same manner as specimen tubes. Cells were harvested in batches of 20-30 cells per tube.

PCR and sequencing: Amplification reactions were run on a GeneAmp PCR System 2400 (Perkin-Elmer). DNA integrity in lysates was confirmed by comparison with positive control template after glyceraldehyde-3 phosphate dehydrogenase (GAPDH) amplification using primers previously described[6] with 2 µl of lysate in a 50 µl reaction with HotStar Taq DNA polymerase Master Mix (Qiagen). PCR parameters for GAPDH amplification after enzyme activation (95ºC x 15 minutes) were: 40 cycles; 95ºC x 30 sec, 55ºC x 30 sec, 72ºC x 1 min, followed by a final 10 minutes at 72ºC. PCR for HIV-1 *gag* was performed using 20 µl of lysate or control DNA (GeneAmplimer HIV-1 Control Reagents, Applied Biosystems). Primers SK38/SK39 (Figure1), HIV-1 negative human placental DNA, HIV-1 positive plasmid DNA (10^3 copies/ µl) or 20 µl of lysate were amplified in a 100 µl reaction with HotStar Taq Master Mix after enzyme activation (95ºC x 15 min). PCR parameters were: 40 cycles; 95ºC x 1 min, 55ºC x 1 min, followed by 7 minutes at 72ºC. PCR products were separated in 1.2 - 2% agarose ethidium bromide gels and photographed. DNA products from the HIV-1 PCR reaction were purified (MinElute PCR Purification kit, Qiagen) and reacted on a PE 9700 thermal cycler using BDT 3.1and the BDT cycling profile. At the conclusion of cycling, SDS was added to a concentration of 10% and the samples were heated to 95ºC x 5 min. The reaction was then purified over superfine Sephadex G-50 and dried. They were then re-suspended in formamide and analyzed on a Applied Biosystems 3100. Results were displayed (Four Peaks, Mekentosji.com) and searched against a reference database [BLAST, National Center for Biotechnology Information(NCBI), National Library of Medicine, National Institutes of Health].

Table 1.

Case	Age at Death	Gender	HIV status	Tissue examined	Pathology
1	19 months	Female	Vertically infected	hippocampus	microcephaly
2	4.5 months	Male	Vertically infected	periventricular region temporal lobe	glial nodules
3	15 months	Male	Vertically infected	periventricular region temporal lobe hippocampus	gliosis and calcifications of basal ganglia
4	26 weeks GA @ delivery: 16 days	Female	Not diagnosed as infected or exposed	hippocampus	hemorrhage
5	40 weeks GA @ delivery: 3 days	Male	Not diagnosed as infected or exposed	hippocampus	gliosis and subdural h emorrhage

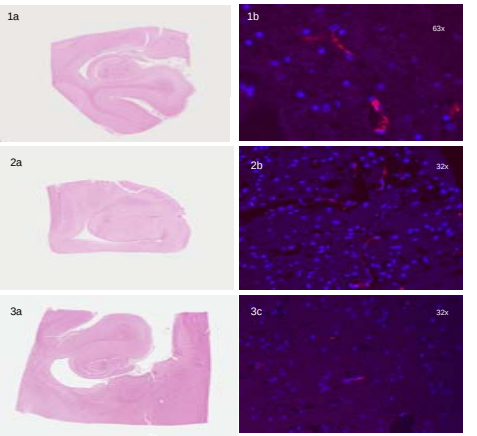
Paraffin-embedded, formalin fixed tissue was obtained from the Children's National Medical Center Department of Pathology archives from 3 HIV+ vertically infected pediatric patients with rapid disease progression, and 2 neonates not diagnosed as HIV-1 infected or exposed. Time from death to autopsy, when available upon review of the medical record, was within 3 days postmortem.

Figure 1.

Primers: Primers based upon the North American Consensus sequence for *gag* resulted in a 115 base pair PCR product from positive control plasmid DNA (GeneAmplimer Applied Biosystems)

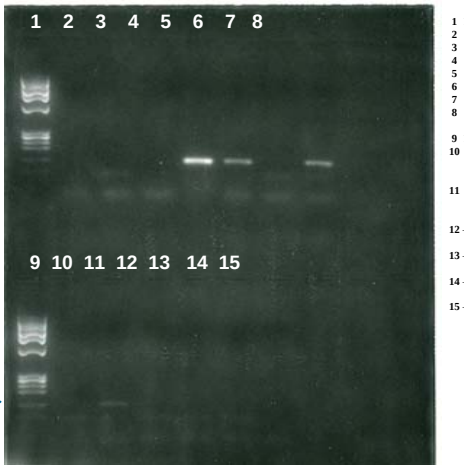
RESULTS

Figure 2.



Immunohistochemical detection of nestin-positive cells in HIV-1 infected pediatric cases 1-3. In this figure, images 1a-3a are the hematoxylin and eosin stained slides from the archived tissue blocks of patient cases 1-3, respectively. Four to six micron thick slices cut from archived brain shown in 1a-3a were adhered to silanized glass slides, deparaffinized in 100% xylene and rehydrated, followed by microwave antigen retrieval and incubation in 3% hydrogen peroxide. Immunofluorescence detection of nestin-positive cells was accomplished after overnight incubation at 4-6ºC in rabbit polyclonal anti-human nestin primary antibody[3] diluted 1:5000 in 5% serum, followed by a secondary goat anti-rabbit horseradish peroxidase conjugate (1:200), and a 5-6 minute incubation with Alexa-Fluor 568 labeled tyramide (1:200) diluted in 0.0015% hydrogen peroxide amplification buffer (Molecular Probes, TSA kit). Nuclei were counterstained with bisbenzamide (1:500) (Biosciences) and the tissue was examined on a Zeiss Axiovert fluorescence microscope using AxioVision Software.

Figure 3.



1 100 base pair ladder
2 Blank
3 Digest buffer
4 Negative control DNA
5 Positive control DNA
6 IIIb$^+$ A3.01 lymphocytes
7 IIIb$^-$ A3.01 lymphocytes
8 8E5 cells

9 100 base pair ladder
10 Negative control tissue nestin$^+$ cells (case 4)

11 **HIV DNA positive**, nestin$^+$ cells (case 1)

12 – Nestin-positive, HIV-negative cells (case 3)
13 – Nestin-positive, HIV-negative cells (case 3)
14 – Nestin-positive, HIV-negative cells (case 3)
15 – Nestin-positive, HIV-negative cells (case 1)

Detection of HIV-1 DNA by PCR amplification: Gel electrophoresis in 2% agarose with ethidium bromide was used to separate and visualize the PCR products of DNA extracted from control DNA, cells and tissue. Nestin-positive cells microdissected from pediatric hippocampal and periventricular tissue were collected in batches of 20-30 cells per sample tube. A 115 base pair product is seen for positive control DNA, positive control cells and in one 25-cell batch of nestin-positive hippocampal cells from a 19 month old vertically infected female with neuroAIDS (Case 1), but not in lanes containing negative control material.

Figure 4.

Sequencing and BLAST results: Lane 11 and control sample PCR products (see above) were reacted on a PE9700 thermal cycler using BDT 3.1 and the BDT cycling profile, then purified, dried, re-suspended, and analyzed on an Applied Biosystems 3100. Results were displayed (Four Peaks, Mekentosji) and searched against a reference database (BLAST, NCBI). Figure 4 shows: the chromatographic display of sequencing results for lane 11 product (a), alignment data (b,d) , and representative statistics (c).

INTERPRETATION/CONCLUSIONS

- The HIV-1 *gag* sequence was identified in DNA extracted from nestin-positive cells dissected from the hippocampus of a 19 month old vertically infected female. No such sequences were identified in negative control cells from culture or archived uninfected human infant hippocampus, nor in cells harvested from the other two HIV-1 infected children studied to date.
- This finding adds to published *in vitro*, and previously presented pediatric periventricular tissue *in situ* hybridization data suggesting that neural progenitor cells may be a target for HIV-1.
- The clinical implications of HIV-1 infection of hippocampal and periventricular nestin-positive neural progenitors remain to be determined.
- Given the critical role neural progenitors play in brain development and response to injury, it is conceivable that the presence of HIV-1 in even limited numbers of progenitor cells might contribute to the diminished brain growth, developmental delays, and cognitive, language and memory impairments seen in HIV-1 infected infants,children and adolescents.