Long-term HIV-1 infection of neural progenitor populations

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Background: HIV can reside in the brain for many years. While astrocytes are known to tolerate long-term HIV infection, the potential of other neural cell types to harbour HIV is unclear.

Objective: To investigate whether HIV can persist in neural progenitor cell populations.

Design: A multipotent human neural stem cell line (HNSC.100) was used to compare HIV infection in neural progenitor and astrocyte cell populations.

Methods: Expression of cellular genes/proteins was analysed by real-time reverse transcriptase PCR, Western blot, immunocytochemistry and flow cytometry. Morphological properties of cells were measured by quantitative fluorescent image analysis. Virus release by cells exposed to HIV-1_{IIIB} was monitored by enzyme-linked immunosorbent assay for Gag. Proviral copy numbers were determined by real-time PCR and early HIV transcripts by reverse transcriptase PCR. Rev activity was determined with a fluorescent-based reporter assay.

Results: Progenitor populations differed from astrocyte populations by showing much lower glial fibrillary acidic protein (GFAP) production, higher cell-surface expression of the CXCR4 chemokine receptor, higher Rev activity and distinct cell morphologies. HIV-exposed progenitor cultures released moderate amounts of virus for over 2 months and continued to display cell-associated HIV markers (proviral DNA, early HIV transcripts) during the entire observation period (115 days). Differentiation of HIV-infected progenitor cells to astrocytes was associated with transient activation of virus production. Long-term HIV infection of progenitor populations led to upregulation of GFAP and changes in cell morphology.

Conclusion: These studies suggest that neural progenitor populations can contribute to the reservoir for HIV in the brain and undergo changes as a consequence of HIV persistence.

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Introduction

Numerous findings support early entry of HIV into the central nervous system in the course of HIV infection and indicate that HIV can persist there for many years [1,2]. Although HIV replication is most readily apparent in brain macrophages and microglial cells, cell-associated HIV markers have also been identified in other cell types in the brain, particularly astrocytes. Numerous cell culture studies confirm that astrocytes are targets for

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HIV and demonstrate that astrocytic populations sustain chronic HIV infection. In general, virus production is severely restricted in infected astrocytes and apparent mainly in the acute phase. However, quiescently infected astrocytes can be stimulated to release HIV transiently in response to extracellular stimuli. Together these studies indicate that astrocytes are cellular sites of HIV persistence and reservoirs for replication-competent virus in the brain (reviewed by Kramer-Hammerle *et al.* [3] and Brack-Werner [4]).

Various studies have identified differences in virus–cell interactions between astrocytes and other HIV target cells. For example, astrocytes characteristically display only very weak activity of the HIV Rev transactivating factor compared with other cell types [5]. Since Rev is crucial for HIV replication [6], weak Rev activity in astrocytes can be expected to have a major impact on limiting HIV production in astrocytes.

Little information is available regarding the capacity of HIV to persist in other neural cell types. Neural stem cell populations have been shown to be susceptible to HIV [7], indicating that they are potential HIV targets. Neural stem cells are defined as clonogenic, self-renewing cells that are multipotent, that is capable of generating neurons, oligodendrocytes and astrocytes [8,9]. They also give rise to neural progenitor cells, which still divide but may commit to more restricted lineages. Neural stem cells and progenitor cells occur in fetal and adult brains and in the latter can be activated and mobilized in response to various pathogenic insults [10]. Glial progenitors make up over 4% of the cell population of the adult white matter [11] and likely provide cellular replacement of glial populations throughout life [12,13].

HNSC.100 is a monoclonal, human cell line that displays the defining criteria of neural stem/progenitor cells [14]. HNSC.100 cells can be expanded indefinitely as progenitor cells in the presence of epidermal growth factor and basic fibroblast growth factor and can be induced to differentiate by withdrawal of these mitogens. Here the HNSC.100 cell line is used to explore whether HIV can establish a chronic infection in neural progenitor cell populations and to examine the potential effects of chronic infection on these cells.

Materials and methods

Cells

Establishment and properties of the HNSC.100 cell line have been described in detail [14,15]. Briefly, the HNSC.100 cell line was derived from primary human fetal neural stem cells infected with a retroviral vector expressing v-*myc* and shown to be clonal, nontransformed and multipotential. All cultures of the HNSC.100 line were carried out on poly-D-lysine coated plastic dishes (BD Bioscience, Heidelberg, Germany), in a basal medium consisting of DMEM:F12, 0.5% fetal calf serum, 1% bovine serum albumin, 1% PenStrep, N2 supplement and 5 mmol/l Hepes buffer. The basal medium was supplemented with 20 ng/ml epidermal growth factor and 20 ng/ml basic fibroblast growth factor (i.e. proliferation medium) for culture of self-renewing HNSC.100 progenitor cell populations (i.e. HNSC-progenitor populations). HNSC.100-derived astrocytes (i.e. HNSC-astrocyte populations) were generated and cultured in basal medium supplemented with 100 ng/ml ciliary neurotrophic factor instead of basic fibroblast growth factor and epidermal growth factor (i.e. astrocyte-differentiation medium) for at least 1 week. Basic fibroblast growth factor, epidermal growth factor and ciliary neurotrophic factor were purchased from Tebu-Bio (Offenbach, Germany); all other cell culture reagents were from Life Technologies (Karlsruhe, Germany).

Human astrocytoma cell lines 85HG66 and U138MG and the stably HIV-1-infected cell lines KE37/1-IIIB (T-lymphoma) and TH4-7-5 (astrocytoma) were cultured as described [5,16].

Antibodies, immunocytochemistry and Western blot assays

The following primary antibodies were used: rabbit polyclonal antiserum against glial fibrillary acidic protein (GFAP) (DAKO Diagnostics, Hamburg), 1 : 500 for immunocytochemistry, 1 : 5000 for Western blot and 1 : 50 for flow cytometry; rabbit polyclonal antiserum against human nestin [17], 1 : 3000; mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (Chemicon Europe, Hofheim, Germany), 1 : 10 000; mouse monoclonal antibody against betaIII-tubulin (Chemicon), 1 : 1000; mouse monoclonal antibody against 2′, 3′-cyclic nucleotide 3′-phosphodiesterase, 1 : 1000; mouse monoclonal antibody against tubulin (Chemicon), 1 : 200; allophycocyanin (APC)-conjugated mouse monoclonal antibody against CD4, CXCR4 or CCR5 (BD Pharmingen, Heidelberg, Germany), 1 : 50. Secondary goat antirabbit or anti-mouse antibodies used were conjugated with horseradish peroxidase (1 : 10 000), Cy3 (1 : 800 for immunocytochemistry; 1 : 100 for flow cytometry) (Dianova, Hamburg, Germany) or Alexa488 (1 : 500; Molecular Probes; Invitrogen, Karlsruhe, Germany). APC-conjugated mouse IgG_{2a}, κ antibodies (1 : 50) (BD Pharmingen) were used as isotype control.

For immunocytochemistry, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% TritonX-100 and blocked with 2% bovine serum albumin.

Western blot assays were performed as described [18]. The intensities of bands on X-ray films were quantified by densitometry using NIH Image J software [19].

Flow cytometry analysis

For staining of intracellular GFAP, 1×10^6 cells were fixed with 2% paraformaldehyde for 15 min, washed and permeabilized with 1% saponin in phosphate-buffered saline (PBS) for 10 min. After incubating in blocking solution (1% fetal calf serum, 0.1% saponin in PBS) for 20 min, cells were incubated with primary anti-GFAP antibodies for 1 h and subsequently stained with secondary Cy3-conjugated antibodies for 30 min and washed. A sample was incubated without primary antibody as control for unspecific staining.

For staining of cell-surface CD4, CCR5 or CXCR4, 1×10^6 cells were fixed, washed with PBS and incubated with the appropriate APC-conjugated antibodies for 1 h. Unspecific staining was evaluated with isotype control antibodies.

Cells were analysed with a FACScalibur flow cytometer (Becton Dickinson, San Jose, California, USA), using channel FL-3 (Cy3) or FL-4 (APC).

Analysis of cell morphology

Cells were grown in 96-well plates and fixed with 4% paraformaldehyde. Nuclei were labeled with Hoechst 33342 (1 : 1000; Chemicon), cytoskeletal structures by immunfluorescence with antibodies against tubulin and Alexa488-conjugated secondary antibodies and by binding of rhodamine-conjugated phalloidin (Chemicon) to F-actin filaments. Images were acquired with a Zeiss Axiovert 200M research microscope (Carl Zeiss, Goettingen, Germany) using a 10× Plan-Neofluar objective, filter sets for Hoechst 33342 (360/40; FT 400; BP 460/50), Alexa488 (475/40; FT 500; BP 530/50) and rhodamine (545/25; FT 570; BP 605/70). Images were analysed using the Morphology Analyst BioApplication of the ASSAY-builder module (powered by Cellomics) of Software AxioVision 4.6 (Carl Zeiss Imaging Solutions, Hallbergmoos, Germany). Following the manufacturer's protocol, nuclei, microtubules and actin were used to detect cells and measure various morphological parameters automatically (including areas of cell bodies and cell processes) for at least 400 individual cells of multiple, randomly selected fields in different wells.

Rev activity assay

Rev activity was determined essentially as described previously [18,20]. Rev-dependent expression of the red fluorescent reporter protein (RFP) was assessed by transfecting cells with plasmid mixtures containing 1.5 μg pLRed(INS)$_2$R [18], 0.2 μg pL3Tat [5] and 0.2 μg pCsRevsg143 [5] or pBsRev [5]. Mixtures with pBsRev also had 0.1 μg of pFRED143 [21] added. Plasmid mixtures for assessment of basal reporter expression contained instead of functional Rev plasmid either pFRED143 or a plasmid expressing defective Rev (pCsRevNES(A)$_4$sg143, pCsRevΔARMsg143 [22]).

The percentage of the transfected cell population expressing the RFP reporter was determined by flow cytometry. Rev activity signifies the increase (fold) in the percentage of RFP-producing cells in the presence of Rev relative to basal reporter production.

HIV infection experiments

HNSC.100 cells were seeded at a density of 5×10^5 per well in six-well plates, incubated for 24 h with 2 ml cell-free culture supernatant (p24, 1266 ng/ml) of KE37/1-IIIB HIV-producer cells, washed twice with PBS and culture continued in fresh medium.

Quantification of release of Gag by HIV-infected cultures

Cells were harvested from HIV-infected cultures and duplicate samples reseeded at a density of either 1×10^5 or 5×10^5 per well in six-well plates and cultured in 2 ml medium for 24 h. Supernatants were centrifuged at $200 \times g$ for 5 min to remove cells, and Triton X-100 added to a final concentration of 0.5%. Gag (p24) was determined by enzyme-linked immunosorbent assay (Beckmann Coulter, Krefeld, Germany) according to the manufacturer's instructions.

Quantitative and qualitative PCR

Quantitative real-time PCR was performed with the Roche LightCycler 2.0 System, using LightCycler FastStart DNA Master SYBR Green I-Kit and standard LightCycler protocol (Roche Diagnostics, Mannheim, Germany).

For absolute quantification of HIV proviral copies, genomic DNA was extracted using Qiagen DNA MiniKit (Qiagen, Hilden, Germany). DNA from TH4-7-5 astrocytoma cells, which contain a single copy of HIV-1 proviral DNA [16], was used to generate external standard curves for the ß-globin reference gene and for the HIV-1 target DNA: 30 ng genomic TH4-7-5-DNA was set to 10 000 genome and 5000 provirus equivalents. Primers are described in Kabamba-Mukadi *et al.* [23].

Total RNA was extracted by RNAzol method or with Qiagen RNeasy Mini Kit; samples were subjected to DNAse digestion (Promega, Mannheim, Germany) and first strand complementary DNA was generated from 1 μg total RNA with M-MLV reverse transcriptase RNase H Minus, Point Mutant, followed by an RNase H digestion step (Promega).

For relative quantification of *gfap* RNA, real-time PCR reactions were performed with the following primers: forward 5′-GCTTCCTGGAACAGCAAAACAAGGC-3′, reverse 5′-GTCTATAGGCAGCCAGGTTGTTC-3′. RNA-polymerase II transcripts were analysed as internal standard, using primers described in Radonic *et al.* [24]. The fold change in *gfap* expression in HIV infected HNSC-progenitor populations was calculated by the

$2^{-\Delta\Delta C}$T method [25], with values normalized to the RNA-polymerase II transcripts and relative to *gfap* expression in uninfected HNSC-progenitor populations.

Multiply spliced HIV-1 transcripts were detected by reverse transcriptase PCR as described previously [26], using primers BSS and SJ4.7A and GoTAQ Polymerase (Promega).

Statistical analysis

Statistical analysis was carried out with GraphPadPRISM 4.0 (GraphPad Software, San Diego, California, USA). The Mann–Whitney U test was used to determine the significance of differences between two data sets (two-tail *P* value).

Results

Characterization of progenitor and astrocyte populations

Self-renewing HNSC-progenitor cell populations were analysed for production of various proteins commonly used as neural cell markers (Fig. 1a). Western blot analysis of whole-cell lysates of HNSC-progenitor cells showed abundant production of nestin, a frequently used marker for neural progenitor cells that is also produced by astrocytes [27]. Levels of the astrocyte marker GFAP in HNSC-progenitor cells were very low. The neuronal marker β-tubulin III [28] and the oligodendrocyte marker 2′, 3′-cyclic nucleotide 3′-phosphodiesterase [29] were not detected (not shown).

To investigate the capacity of HNSC-progenitor populations to generate astrocytes, the production of GFAP and the morphological properties of cells were compared in HNSC-populations cultured in astrocyte-differentiation medium and cultured in proliferation medium. HNSC-populations cultured in astrocyte-differentiation medium strongly increased production of GFAP (Fig. 1a) and showed intense GFAP immunoreactivity on a single-cell level (Fig. 1b). Flow cytometry confirmed increased GFAP antigen (Fig. 1c). To quantify changes in cell morphology between HNSC-progenitor and HNSC-astrocyte populations, cells were visualized by fluorescent labelling of nuclei and cytoskeletal structures and morphological parameters were measured by automated image analysis (Fig. 1d). Culture of HNSC-populations in astrocyte-differentiation medium resulted in a significant decrease of median cell body size and an increase in median process area compared with the HNSC-progenitor populations.

Weak Rev activity is a characteristic feature of cultured human astrocytes [5]. A previously described fluorescence-based Rev-reporter assay [18,20,30] was used to compare the activity of Rev in HNSC-progenitor and HNSC-astrocyte populations. HNSC-progenitor populations showed higher Rev activity than the HNSC-astrocyte populations (Fig. 2). Low Rev activity in the HNSC-astrocyte population was comparable to Rev activity in cultures of the astrocyte cell lines U138MG and 85HG66, further confirming astrocyte-typical behaviour of HNSC-astrocyte populations.

These results demonstrate that HNSC-progenitor populations are capable of highly efficient generation of astrocytes under the culture conditions used in this study.

HIV-1 persistence in HNSC-populations

Cell-surface expression of HIV-1 receptor/coreceptor molecules

HNSC-populations were examined for cell-surface expression of the HIV receptor CD4 and the chemokine receptors CCR5 and CXCR4, which are preferentially used by different HIV strains as coreceptors [31].

Neither HNSC-progenitor cells nor HNSC-astrocyte cells showed cell-surface staining of CD4 or CCR5 molecules in flow cytometry analysis (Fig. 3a). In contrast, both populations showed cell-surface expression of CXCR4, the HNSC-progenitor populations expressing higher levels than the HNSC-astrocyte populations.

Early phase of HIV-1 infection

HNSC-progenitor and HNSC-astrocyte populations were exposed to HIV-1_{IIIB}, which is a CXCR4-using HIV-1 strain previously employed to address HIV-1 infection of astrocytes or neural stem cells [7,16]. HIV-exposed cultures were subjected to at least three rounds of trypsinization to eliminate input virus. HIV replication was monitored by quantifying release of Gag antigen since this is a sensitive parameter [32] frequently used in past studies to investigate HIV infection of brain cells [3,4,7]. Nine days after exposure to HIV-1, both HNSC-progenitor and HNSC-astrocyte cultures released Gag (Fig. 3b), demonstrating HIV infection. At 13 and 17 days after infection, Gag release was detected only for HNSC-progenitor cultures, not for HNSC-astrocyte cultures.

Quantitative real-time PCR analysis of HIV proviral DNA in HNSC-progenitor and HNSC-astrocyte populations 28 days after HIV infection demonstrated similar numbers of HIV proviral copies in both populations (Fig. 3c).

Long-term HIV-1 infection

Prolonged monitoring revealed that HIV-infected HNSC-progenitor populations continued to release detectable amounts of Gag antigen for over 60 days (Fig. 4a). While Gag release subsequently declined to very low or undetectable levels, HNSC-progenitor cultures continued to bear cell-associated markers of HIV infection at later time points: multiply spliced, early HIV transcripts that encode the regulatory factors Tat, Rev and Nef were detected in progenitor populations 80 days after infection (Fig. 4b). Furthermore, quantification of HIV DNA copy

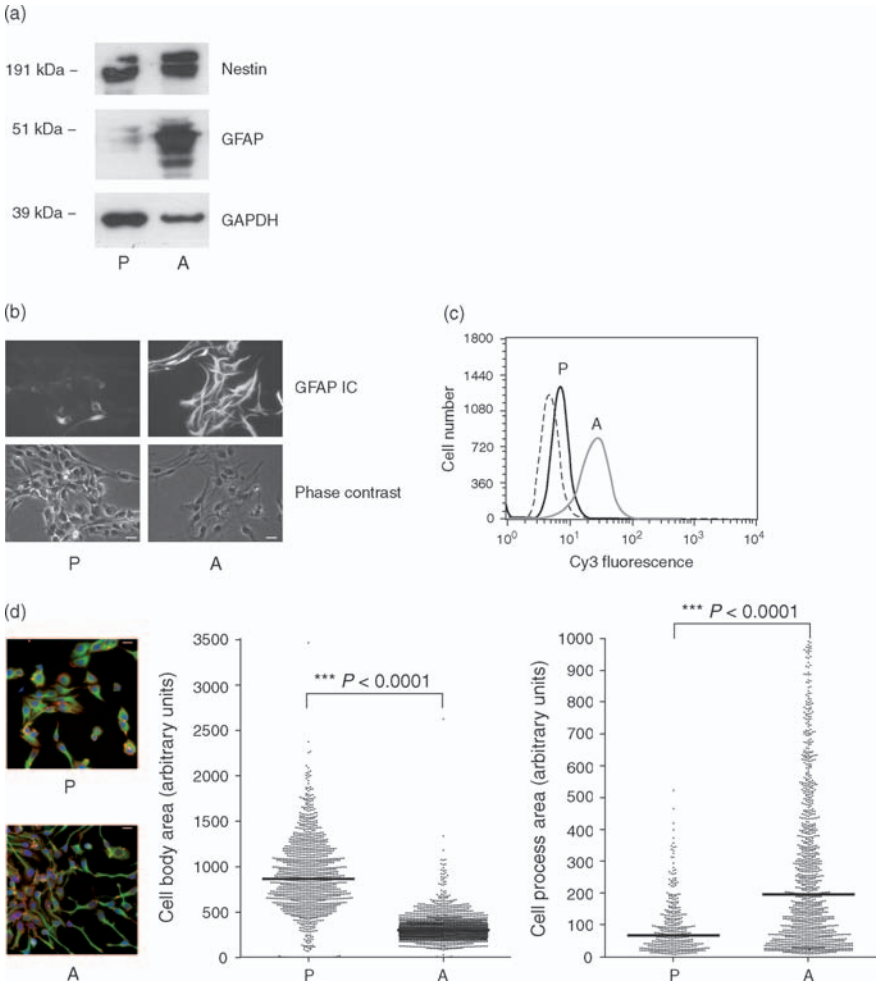

Fig. 1. Analysis of expression of the astrocyte marker glial fibrillary acidic protein (GFAP) and of morphological properties of HNSC-progenitor and HNSC-astrocyte populations. (a) Western blot analysis of GFAP production in whole-cell lysates of HNSC-progenitor (P) populations and HNSC populations cultured in astrocyte-differentiation medium for 10 days (A). The blot was analysed by densitometry and the intensity value of the GFAP band normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control protein in the same lane. Relative GFAP production was 0.22 (P) and 2.46 (A). Both populations expressed nestin, which is known to be expressed by progenitor cells as well as astrocytes [27]. (b) Single-cell analysis of GFAP production. Cells cultured in astrocyte-differentiation medium for 7 days (A) showed strongly increased immunoreactivity for GFAP antigen compared with P populations. Scale bars: 20 μm. (c) Flow cytometry histogram profiles of GFAP production are shown for populations cultured in astrocyte-differentiation medium for 10 days (grey line, A), and P populations (black line, P). Cells only incubated with the secondary antibody were analysed as control (dotted line). The median fluorescence intensity and the percentage of GFAP-positive cells (both corrected for background) were 10.6 (15%) for P and 25.3 (94%) for A. (d) Morphological properties of cells in P and A populations visualized by fluorescent labelling of nuclei (blue) and cytoskeletal structures (microtubules, green; F-actin filaments, red). Representative images show phenotypes of cells in both populations (Scale bars: 20 μm). The scatter plots show values measured by quantitative automated image analysis for the areas of bodies and processes of cells ($n > 400$). Median values (indicated by horizontal lines) of cell body areas/cell process areas are 865/66 for P and 302/195 for A. Cells in P populations had significantly larger bodies and smaller processes than cells in A populations.

numbers confirmed the presence of HIV proviral DNA at 96 and 115 days after infection (Fig. 4c). Interestingly, the number of proviral copies declined by only approximately a half during prolonged culture of HIV-infected HNSC-progenitor populations, indicating that HIV DNA can persist in these progenitor cells for long periods.

To investigate the influence of astrogenesis on HIV production, samples of HIV-infected HNSC-progenitor cultures taken at 35 days after infection were transferred to astrocyte-differentiation medium, in which they were cultured for the remaining observation period. Infected HNSC-cultures responded to the changed culture conditions by transiently increasing Gag production (Fig. 4a). Gag production subsequently decreased progressively and at 70 days post-infection was similarly low in the astrocyte populations derived from HIV-infected progenitors as in the HNSC-progenitor

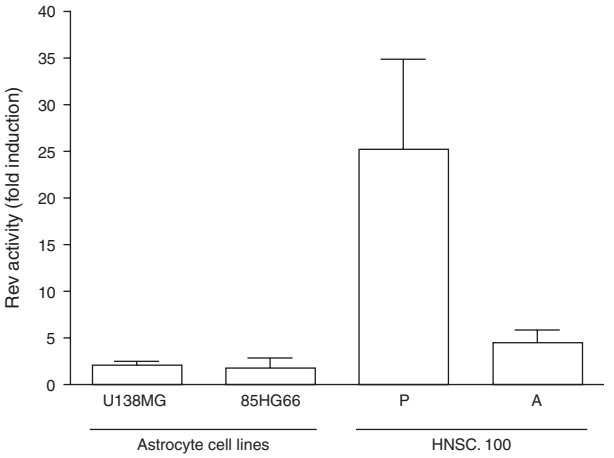

Fig. 2. Activity of the HIV Rev regulatory factor in HNSC-progenitor (P) and HNSC-astrocyte (A) populations. Rev is more active in P populations than in A populations (differentiation period 7 days), the latter displaying similarly low Rev activity as seen in cultures of the astrocyte cell lines U138MG and 85HG66. Rev activity was determined as described in the Materials and methods. Bars represent means of least three independent transfection experiments. Error bars represent ±SD.

populations continuously cultured in proliferation medium (Fig. 4a). Detection of early HIV transcripts confirmed the presence of HIV in the astrocyte populations at 80 days post-infection (i.e. after 45 days of culture in astrocyte-differentiation medium) (Fig. 4b).

These results demonstrate that HNSC-progenitor populations can harbour HIV for long periods. During long-term infection, they show prolonged release of moderate amounts of HIV, and transiently increase HIV production after transfer to astrocyte-differentiation medium.

Influence of long-term HIV infection on biological features of HNSC-populations

To investigate whether HIV persistence has the potential to cause biological changes in neural progenitor cell populations, GFAP production, CXCR4 cell-surface expression and cell morphologies were compared in HIV-infected and uninfected HNSC-progenitor cell populations.

Western blot analysis showed elevated production of GFAP in HIV-infected HNSC-progenitor populations compared with uninfected populations, with GFAP increasing over time (Fig. 5a). Flow cytometric analysis

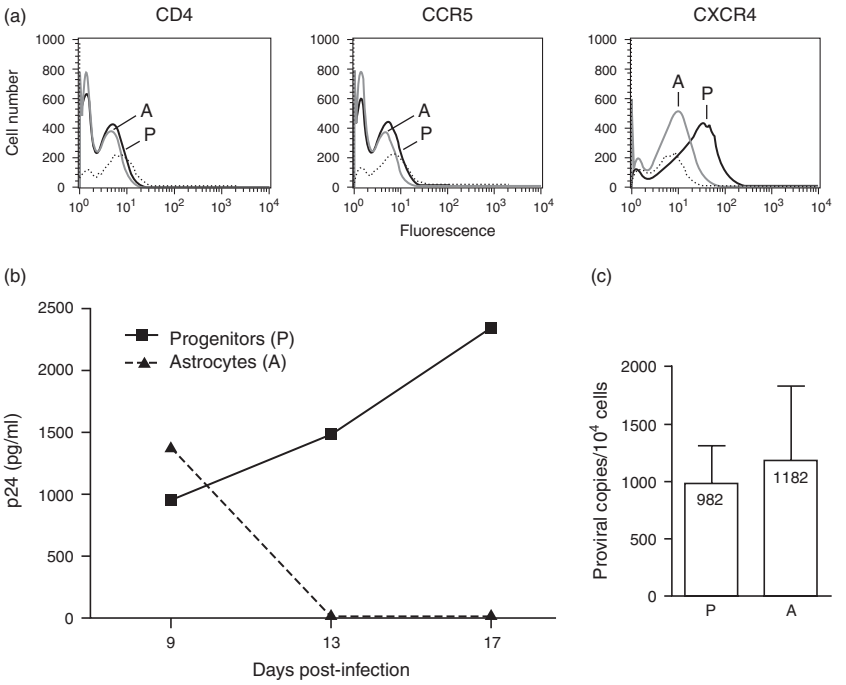

Fig. 3. Early phase of HIV infection of HNSC-progenitor (P) and HNSC-astrocyte (differentiation period 10 days) (A) populations. (a) Flow cytometry analysis of cell-surface expression of CD4, CCR5 and CXCR4 in P (black line) and A (grey line) populations. The profile of the isotype control is shown with a dotted line. Populations were negative for cell-surface CD4 and CCR5 but positive for CXCR4. The median fluorescence intensity and the percentage of CXCR4-positive cells (corrected for the isotype control) were 37 (60%) for P and 22 (13%) for A populations. (b) Monitoring of Gag release by P and A cultures exposed to HIV-1_{IIIB}. At the indicated time points, cells were reseeded and culture supernatants were analysed for Gag (p24) antigen as described in the Materials and methods. The graph shows the mean values of p24 released by 5×10^5 reseeded cells. (c) Quantification of HIV-1 proviral DNA copy numbers by real-time PCR analysis 28 days after exposure of P and A populations to HIV. Bars represent mean copy number per 10 000 cells, error bar represent the SD from three analyses.

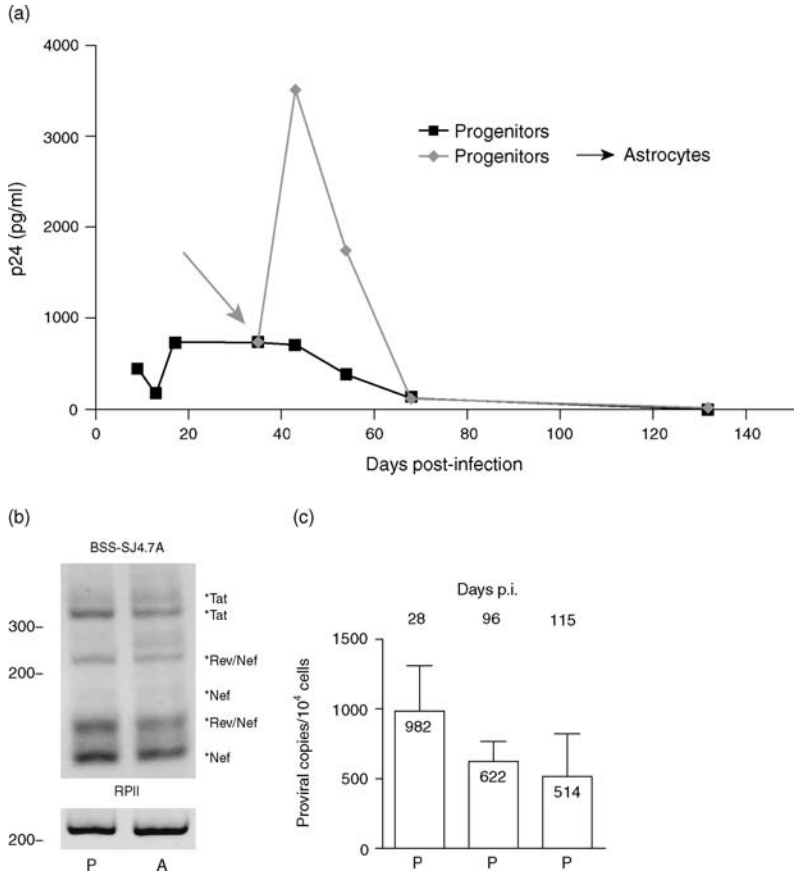

Fig. 4. Long-term monitoring of HIV infection by HNSC cultures exposed to HIV as progenitor cells. (a) Gag (p24) release was monitored for 132 days in samples of HIV-exposed HNSC-populations either continuously cultured in proliferation medium (i.e. progenitors, P) or transferred to astrocyte-differentiation medium at 35 days post-infection (p.i.; arrow) and maintained as astrocytes for the remaining observation period (progenitors → astrocytes). Gag release was measured as described in the Materials and methods. The graph shows the mean values of p24 released by 1×10^5 reseeded cells. (b) Detection of multiply spliced HIV transcripts encoding Tat, Rev and Nef in HNSC cultures. HIV-exposed progenitor populations (P) and astrocyte populations derived from HIV-exposed progenitors (A) were analysed by reverse transcriptase PCR at 80 days p.i., using a primer pair (BSS-SJ4.7A) that specifically amplified multiply spliced HIV transcripts. The product sizes of the bands and their coding capacities are as follows: 399 bp (Tat), 325 bp (Tat), 192 bp (Rev/Nef), 176 bp (Nef), 148/142 bp (Rev/Nef) and 126 bp (Nef). A segment of RNA-polymerase II transcripts (RPII) was amplified as control. (c) Quantification of HIV-1 proviral DNA copy numbers by real-time PCR analysis of P populations at the indicated time points after exposure to HIV. Bars represent mean copy number per 10 000 cells, error bars represent the SD from three analyses.

confirmed upregulated production of GFAP in the HIV-infected HNSC-progenitor population (65 days after infection, data not shown). Quantitative real-time RT-PCR demonstrated elevated levels of *gfap* RNA, which increased over time (Fig. 5b). These results indicate that long-term HIV-infection of HNSC-populations is associated with upregulation of *gfap* and increased GFAP protein concentrations. CXCR4 cell-surface expression profiles were similar in the HIV-infected (143 days after infection) and uninfected HNSC-progenitor cell populations (Fig. 5c). Quantification of morphological properties showed that the median cell body and cell process areas of long-term HIV-infected progenitor populations (141 days after infection) differed significantly from those of astrocytes derived from these HIV-infected progenitors (differentiated for 10 days) and from uninfected progenitor populations (Fig. 5d). These results demonstrate that long-term HIV infection leads to changes in morphological properties of HNSC-progenitor populations. However, these changes are less pronounced than those associated with differentiation of progenitor cells to astrocytes.

Discussion

During chronic HIV infection, the brain provides a sanctuary for HIV, potentially fostering the virus for decades. In this study we asked whether neural progenitor cells have the potential to act as HIV reservoirs.

As an experimental model for monitoring long-term effects in neural progenitor cell populations, we used the

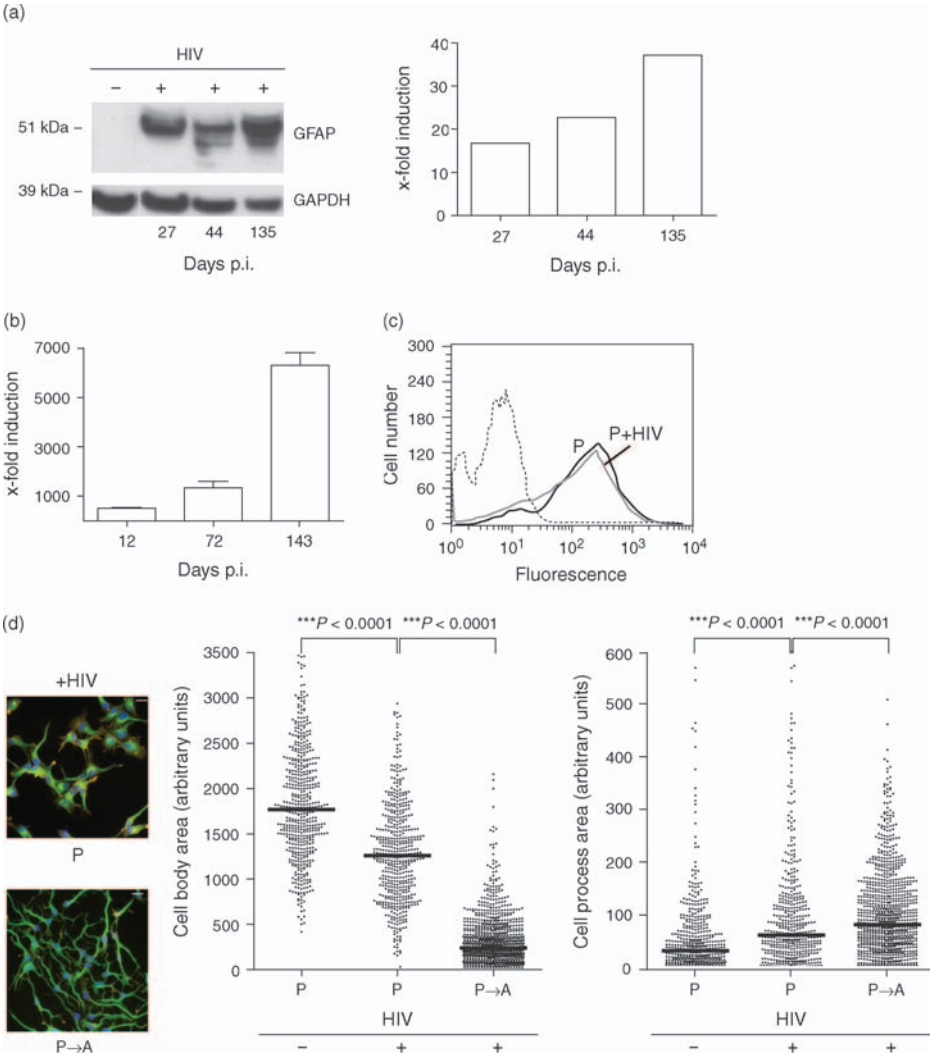

Fig. 5. Influence of prolonged HIV infection on biological features of HNSC-progenitor populations (P). (a) Upregulation of GFAP protein was analysed by Western blot in whole-cell lysates at 27, 44 and 135 days post-infection (p.i.) in HIV-infected P cultures and in uninfected P populations. The intensities of bands in the blot were quantified by densitometry and intensity values of GFAP normalized to the control protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The graph shows the fold-increase of GFAP production in HIV-infected progenitor cultures relative to uninfected progenitor populations. (b) Upregulated expression of *gfap* in HIV-infected P cultures was quantified by real-time PCR analysis using RNA-polymerase II (RP II) expression as an internal control in P populations at 12, 72 and 143 days p.i. and in uninfected P populations. The graph shows the fold-increase of *gfap* expression in HIV-infected progenitor cultures relative to uninfected progenitor populations. (c) Cell-surface expression of CXCR4 by HIV-infected and uninfected HNSC populations. Flow cytometry profiles are shown of cell-surface expression of CXCR4 by HIV-infected P populations at 143 days p.i. (grey line; P+HIV), by uninfected P populations (black line; P) and of the isotype control (dotted line). The median fluorescence intensity and the percentage of CXCR4-positive cells (corrected for the isotype control) were 153 (80%) for P+HIV and 180 (84%) for P. (d) Analysis of morphological properties of cells in HIV-infected HNSC-populations. Representative images show the morphologic phenotypes of HIV-infected P populations at 141 days p.i. and of populations of astrocytes derived from HIV-infected P cultures (P → A) (differentiation period 10 days) (scale bars: 20 μm). The scatter plot shows values measured for the areas of bodies and processes of cells ($n > 500$). Median values (indicated by horizontal lines) of cell body areas/cell process areas are 1771/32 for P, 1262/61 for P+HIV and 241/81 for P→A(+HIV). The medians of cell body and cell process areas of HIV-infected P populations differed significantly from those of astrocytes derived from HIV-infected P populations and from uninfected P populations.

self-renewing, multipotent human neural stem cell line HNSC.100 [14], which can be cultured indefinitely in the presence of mitogens. Astrocytes were generated from HNSC.100 cells by adding the astrogenesis-promoting cytokine ciliary neurotrophic factor to the culture medium in lieu of mitogens [33,34]. HNSC-populations cultured in astrocyte-differentiation medium for at least 7 days differed from HNSC-progenitor populations by

markedly increased GFAP production (Fig. 1), diminished cell-surface expression of CXCR4 (Fig. 3) and altered cell morphologies (Fig. 1). In addition they showed diminished Rev activity and rapidly ceased to produce virus after exposure to HIV, which are hallmarks of interactions of astrocytes with HIV [3,4,35]. Together these results confirm reliable functional and phenotypical distinctions between HNSC-progenitor and HNSC-astrocyte populations.

A previous report demonstrated that HIV can invade cells in neural progenitor populations [7]. The objective of our study was to investigate whether HIV can establish long-term infection in neural progenitor populations. We identified HIV proviral sequences in HNSC-progenitor populations up to 115 days after virus exposure, and HIV-exposed HNSC-progenitor populations released moderate amounts of virus for over 60 days and still expressed transcripts encoding Nef, Tat and Rev at a later time point. These results indicate persistence and sustained production of functional HIV in HNSC-progenitor cell populations. Thus, our study provides evidence for the capacity of human neural progenitor cells to generate HIV reservoirs.

In various reservoirs, including astrocytes, HIV can be activated by extracellular cues [36,37] (see the review by Blankson *et al.* [38]). Here we found that differentiation of HIV-infected HNSC-progenitor populations to astrocytes was associated with a transient activation of HIV production. HNSC cultures exposed to HIV after differentiation to astrocytes showed the HIV-restriction phenotype typical for astrocytes (Fig. 3). These results suggest a dynamic model in which the changes in gene expression patterns of cells during differentiation to astrocytes induce both HIV-stimulatory as well as HIV-restrictive mechanisms. Initially, stimulatory mechanisms would outweigh restrictive mechanisms, whereas, later, restrictive mechanisms would prevail. The molecular mechanisms underlying this model are bound to be multiple and complex. In a first attempt to identify potential HIV-stimulatory signalling pathways induced during astrogenesis, we investigated Nf-κB, a known inducer of HIV production in latently infected reservoirs [39,40]. Preliminary data indicate increased nuclear levels of Nf-κB in HNSC cells after initiation of astrocyte differentiation (data not shown).

In this study we also provide evidence that persistence of HIV can influence biological properties of HNSC-progenitor populations by showing upregulated production of GFAP and phenotypical changes in HIV-infected neural progenitor populations (Fig. 5). Upregulated production of GFAP is a hallmark of reactive astrocytes [41,42] and occurs in response to HIV infection [43], suggesting similarities in the responses of neural progenitor cells and more mature astrocytes to HIV. The upregulation of GFAP by HIV may be direct or indirect and could involve Tat and/or Nef, which both have been reported to upregulate GFAP in astrocytes [44,45].

Despite upregulated GFAP production, HIV-infected HNSC-progenitor populations still differed from HNSC-derived astrocytes. They showed less-pronounced changes in cell morphology than those associated with differentiation of HNSC-progenitor populations (Figs 1d and 5d). Furthermore, HIV-infected HNSC-progenitor populations expressed similar levels of cell-surface CXCR4 as uninfected progenitor populations (Fig. 5c), whereas HNSC-derived astrocyte populations had reduced CXCR4 expression (Fig. 3a). Glial cells are derived by maturation of neural stem cells through various stages of development (reviewed by Liu and Rao [12]). Our results suggest that HIV infection may promote advancement of progenitors along the glial lineage to a developmental stage that is more advanced than that of the progenitors but still less mature than that of HNSC-derived astrocytes.

This cell culture study supports the hypothesis that HIV can persist in neural progenitor populations, which can release the virus in different amounts depending on the extracellular environment and can undergo changes in response to HIV persistence. Owing to the manifold inherent difficulties of investigating HIV persistence in primary human brain tissues, it is still unclear at this stage to what extent this hypothesis reflects the in-vivo situation. However, evidence supporting HIV-1 infection of neural progenitor cells *in vivo* comes from a recent study identifying HIV-1 sequences in nestin-positive cells in brain tissues of children [46]. The widespread implications of HIV infection of neural progenitor populations for central nervous system disease in both adults and children (reviewed by Schwartz and Major [47]) underscore the necessity for further investigation of this important issue. Expanded cell culture studies based on the results reported here will be an invaluable aid in unravelling molecular mechanisms of HIV–cell interactions in neural progenitor populations, assessing the pathogenic potential of HIV-infected neural progenitor populations and devising appropriate therapeutic strategies.

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