Neurodegeneration of Somatostatin-immunoreactive Neurons in HIV Encephalitis

LAURA FOX, BS, MICHAEL ALFORD, BA, CRIS ACHIM, MD, PHD, MARGARET MALLORY, BS, AND ELIEZER MASLIAH, MD

Abstract. Recent studies have suggested that neuronal populations that contain glutamate receptors are vulnerable to damage mediated by the human immunodeficiency virus 1 (HIV-1). Somatostatin-immunoreactive neurons contain, among other elements, glutamate receptors, and might therefore be susceptible to HIV-mediated damage. In order to test this hypothesis, we compared patterns of somatostatin immunoreactivity in the cortex and subcortex of autopsied AIDS cases with and without HIV encephalitis (HIVE). Somatostatin immunoreactivity in the frontal cortex interneurons, hippocampal pyramidal and nonpyramidal cells, and globus pallidus was significantly reduced in HIVE. Radioimmunoassay demonstrated a comparable decrease in somatostatin levels in the neocortex of HIVE cases. The decrease in somatostatin immunoreactivity in the neocortex was inversely correlated with the severity of HIVE and global cognitive performance, but not with the extent of the astrogial reaction. These findings indicate that somatostatin-immunoreactive neurons in the cortex are susceptible to damage mediated by HIV and that deficient functioning of this neuronal population might contribute to the cognitive dysfunction observed in AIDS patients.

Key Words: HIV encephalitis; interneurons; neurodegeneration; somatostatin.

INTRODUCTION

Previous studies have shown that diverse neuronal populations are differentially susceptible to damage mediated by the human immunodeficiency virus 1 (HIV-1) (1, 2). Damage to neuronal populations appears in many forms, such as pyramidal neurons bearing glutamate receptors and showing low levels of calcium-binding proteins (1, 3), and interneurons expressing receptors for cytokines (2, 4) and high levels of calcium-binding proteins (1). Therefore, differences in the relative levels of glutamate receptors (3, 5), calcium binding proteins such as calbindin and parvalbumin (PV), and cytokine receptors across different neuronal populations may determine their selective vulnerability to distinct HIV-induced neurotoxins during the course of HIV encephalitis (HIVE) (2, 3, 6).

In the neocortex of patients with HIVE there is a significant loss of pyramidal neurons (7–11), as well as of their dendritic arbor (12, 13). These neuronal populations express microtubule-associated protein 2 (MAP2), calbindin, neurofilaments (1), and glutamate receptors (15), but not PV (14). In contrast, in the hippocampus of patients with HIVE, pyramidal neurons are relatively spared (16, 17); however, there is a significant loss of PV-immunoreactive interneurons in the CA3 region (14). The later neuronal populations express cytokine receptors (4, 18). Moreover, in the basal ganglia of patients with HIVE, there is significant loss of large spiny neurons that express MAP2 (2) and contain glutamate receptors (19); however, neurons that express calbindin are relatively spared (2). Studies in experimental models of neurodegeneration have also corroborated the concept that individual HIV-associated neurotoxins selectively affect specific neuronal populations in a pattern similar to the one observed in the brains of AIDS patients. For example, overexpression of gp120 results in significant damage to MAP2-immunoreactive pyramidal neurons in the neocortex (20), while PV-immunoreactive interneurons are spared (21). In contrast, transgenic mice that express interleukin 6 (IL6) display widespread loss of PV-immunoreactive interneurons in the hippocampus (21, 22). These regional differences in the patterns of selective damage to neurons in patients with HIV, as well as in transgenic mice, suggest that in the neocortex neurons are more vulnerable to direct damage mediated by HIV1-derived factors, while in the hippocampus and basal ganglia cytokines might play a significant role (2).

Other neuronal populations that could be selectively affected in HIVE are the somatostatin-immunoreactive neurons (23), because these neurons contain glutamate receptors (24); also, in Rhesus monkeys infected with the simian immunodeficiency virus (SIV), the mRNA levels for somatostatin are altered (25). Somatostatin-14, made from the precursor somatostatin-28, is believed to function by inhibiting growth hormone release, and also plays a role in sensory and cognitive pathways (26, 27). In the neocortex, somatostatin is produced by interneurons (28) that also contain calbindin and gammaaminobutyric acid (GABA) (24, 29, 30). In order to determine if somatostatin-expressing neurons are sensitive to damage mediated by HIV, we examined the patterns of immunoreactivity of these neurons in the neocortex, basal ganglia, and hippocampus in cases representing varying degrees of HIVE.

From the University of California, San Diego, Departments of Neurosciences (LF, MA, MM, EM) and Pathology (EM), La Jolla, CA 92039-0624 and the University of Pittsburgh, Division of Neuropathology (CA), Pittsburgh, PA 15213.

Correspondence to: Dr Eliezer Masliah, Department of Neurosciences, University of California, San Diego, La Jolla, CA 92039-0624.

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MATERIALS AND METHODS

Samples and Immunohistopathological Staging

A total of 31 cases clinically and neurobehaviorally characterized at the San Diego HIV Neurobehavioral Research Center (HNRC) were used in the present study. Global cognitive performance was assessed, as previously described, by the Heaton Global Scale (31). On average, cases were usually last tested within 18 months prior to death. Exclusion criteria included history of non-HIV related neurological disorders or medical disorders affecting the central nervous system (CNS), schizophrenia, and active substance abuse. Cases were also excluded if significant CNS opportunistic infections were discovered at autopsy. Tissue blocks from the frontal cortex (Brodmann’s areas 45 and 46), hippocampus (at the level of the lateral geniculate body), and basal ganglia (at the level of anterior commissure) were fixed overnight in 2% paraformaldehyde and serially sectioned on 40 μm with the Vibratome 2000 (Technical Products International, St. Louis, MO) for subsequent immunocytochemical analysis. Additional formalin-fixed tissue blocks from the frontal cortex, basal ganglia, thalamus, hippocampus, midbrain and cerebellum were stained with hematoxylin and eosin for routine histopathological examination. As previously described (12), sections were immunolabeled with the monoclonal antibody against the HIV gp41 envelope protein (anti-gp41, Genetics Systems, Seattle, WA) to score the severity of HIVE, and with the polyclonal anti-glial fibrillary acidic protein (GFAP) antibody (Biogenex, San Ramon, CA) to score the severity of gliosis. Cases were divided as follows: (a) controls, without immunocytochemically detectable gp41 (summary score of neocortical gray, cerebral white and deep gray matter = 0); (b) mild HIVE, with minimal numbers of gp41 immunoreactive cells (summary score = 1); (c) moderate HIVE, with occasional gp41 immunostaining (summary score = 2–3); and (d) severe HIVE, with abundant gp41-positive cells (summary score = 4–6) (12).

Immunocytochemistry and Densitometry

Blind-coded 40-μm-thick sections from the frontal cortex, hippocampus and basal ganglia of all cases were immunolabeled with an antibody against somatostatin (rabbit polyclonal, 1:500, DAKO, Carpinteria, CA). This polyclonal antibody was prepared by immunizing rabbits with synthetic cyclic (1–14) somatostatin conjugated to bovine thyroglobulin. Briefly, as previously described (14), sections were blocked with 20% normal goat serum/10% bovine serum albumin, followed by an overnight incubation at 4°C with the primary antibody. The free floating sections were then washed and incubated with biotinylated goat anti-rabbit IgG, followed by Avidin D-horseradish peroxidase (ABC Elite, Vector Laboratories, Burlingame, CA) and reacted with diaminobenzidine tetrahydrochloride (DAB) containing 0.001% hydrogen peroxide. Control experiments verifying the antibody specificity included immunolabeling of paraformaldehyde-fixed vibratome sections from the pancreas, as well as adsorption of the primary antibody with its corresponding peptide.

In order to assess the relative levels of somatostatin immunostaining, blind-coded sections were analyzed with the Quantimet 570C equipped with a microdensitometer; as previously described (32). For each case, approximately 10 fields per section were analyzed with a 40× NA 1.25 objective (field area = 0.16 mm²), each field containing 1 to 2 somatostatin-immunoreactive neurons. For each case, 3 serial sections were analyzed and the individual optical density per neuron was averaged and expressed as a mean. Additional determinations of dendritic and synaptic content were performed as previously described by laser scanning confocal microscopy/image analysis in sections immunolabeled with anti-synaptophysin (presynaptic terminal marker) and anti-MAP2 (dendritic marker) monoclonal antibodies (12). All the experiments were repeated at least twice to assess the reproducibility of the results.

Somatostatin Radioimmunoassay

Levels of somatostatin in the frontal cortex were determined, as previously described by radioimmunoassay (RIA) (33). Briefly, extracts of human brain samples from 4 HIV-seropositive cases (without HIVE) and 4 cases with HIVE were prepared by placing 100 mg of frozen tissue into boiling 2M acetic acid. After cooling, samples were homogenized, centrifuged and freeze-dried. Samples were resuspended in PBS-BSA (0.1%) and at least 3 dilutions of each sample were assayed in duplicate to insure that at least 2 measurements were made on the linear portion of the standard curve. The assay employed an equilibrium system in which the rabbit polyclonal antibody against somatostatin (DAKO, 1:1000), 125I-Tyr°-somatostatin (New England Nuclear, 2200 Ci/m mole) and sample or standard curve were incubated for 48 hours (h) at 4°C. Free and bound 125I-Tyr°-somatostatin was separated by adsorption of free tracer on dextran-coated charcoal. Standard displacement curves were generated by addition of various amounts of synthetic somatostatin (Fig. 1a).

Statistical Analysis

After quantitative analysis was completed, the code was broken and cases were assigned to a HIVE category (12). Differences among the groups were tested using one factor ANOVA with post-hoc Dunnet’s or Tukey Kramer. Comparison between control and HIV groups were performed by the two-tailed Student’s t-test. To assess the relationship between somatostatin immunoreactivity, viral burden, and behavioral performance, Pearson product-moment correlation and the (r) value were calculated with simple linear regression analysis with the StatView II program (Abacus Concepts). All results were expressed as mean ± SEM.

RESULTS

Somatostatin Immunoreactivity in the Neocortex in HIV

Of the 31 cases analyzed, 8 displayed severe HIVE, 8 displayed moderate HIVE, 6 displayed mild HIVE, and 9 were considered seropositive controls (without HIVE). Consistent with previous studies (23, 34), in the control neocortex, antibodies against somatostatin immunolabeled 2 populations of neurons (Fig. 2). The most intense immunolabeling was observed associated with fusiform multipolar interneurons in layers 3 to 6 (Fig. 2a, b), and in neurons of the subcortical white matter (Fig. 2c). In
Fig. 1. Determination of somatostatin levels by radioimmunoassay. (a) Competitive binding of labeled somatostatin was specifically displaced by excess unlabeled somatostatin. (b) In HIVE there was a significant decrease in the levels of somatostatin in the frontal cortex (** = p < 0.01, by two-tailed unpaired Student's t-test).

Fig. 2. Patterns of somatostatin immunoreactivity in the frontal cortex. In the control sections, somatostatin antibody immunostained interneurons and their processes (arrowheads) in lamina 2 (a), 4 (b) and in the white matter (c). In AD, there was a decrease in somatostatin immunoreactivity in interneurons in lamina 2 (d), 4 (e) and in the white matter (f). Furthermore, the neuritic processes were short and fragmented (arrows). Bar = 20 μm.

contrast, the pyramidal neurons in layers 2 to 3 and 5 were mildly labeled. Further corroboration of the antibody specificity was done by immunolabeling sections from the pancreas. These experiments showed strong anti-somatostatin immunoreactivity associated with a subpopulation of islet cells (Fig. 3a). Preadsorption of the antibody with the peptide completely blocked the islet cell immunostaining (Fig. 3b).

Computer-aided microdensitometrical analysis of somatostatin-immunoreactivity revealed a significant 15% decrease in the levels of immunostaining in the interneurons of moderate and severe HIVE cases (one-way ANOVA, post-hoc Dunnet's test, p < 0.05) compared with controls (Figs. 2d, 4a). Furthermore, the somatostatin immunoreactive neurons in the HIVE cases displayed a poorly ramified neuritic arbor and on occasion the dendritic processes appeared to be fragmented (Fig. 2d–f). Overall, the decrease in somatostatin immunoreactivity per neuron was observed in the absence of loss of relative numbers of somatostatin-immunoreactive neurons per field. Additional determinations of levels of somatostatin in the brain were performed by RIA. This study showed a significant decrease in the levels of somatostatin in the frontal cortex of AIDS patients with
Fig. 3. Somatostatin antibody specificity. (a) In control experiments where pancreatic sections were immunolabeled with anti-somatostatin antibody, strong immunoreactivity was observed in subpopulation of islet cells. (b) Control experiments where anti-somatostatin antibody was preincubated with synthetic somatostatin peptide (1-14) completely abolished the islet staining.

Fig. 4. Computer-aided microdensitometrical analysis of somatostatin-immunoreactivity in the brain. (a) Somatostatin-immunoreactivity in the frontal cortex was decreased by 15% in moderate and severe HIVE cases (one-way ANOVA, post-hoc Dunnet's test, p < 0.05) compared with controls. (b, c) On average, HIVE cases showed a 35% decrease in somatostatin immunoreactivity in pyramidal neurons and interneurons when compared with controls (one-way ANOVA, post-hoc Dunnet's, p < 0.05). Loss of somatostatin immunoreactivity in pyramidal and nonpyramidal neurons was more prominent in mild and moderate HIVE cases than in the severe HIVE group. (d) Cases with severe HIVE displayed a 30% decrease in levels of somatostatin immunoreactivity in the globus pallidus when compared with controls (one-way ANOVA, post-hoc Dunnet's). (e) No statistically significant differences were observed in the levels of somatostatin immunoreactivity in the putamen of HIVE cases.

HIVE (Fig. 1b). In order to determine the relationship between viral burden and loss of somatostatin immunoreactivity, simple linear regression analysis was performed. Statistically significant correlations were found between the severity of the viral burden and decrease in somatostatin immunoreactivity ($r = -0.519$, $p = 0.003$) (Fig. 5a). Furthermore, levels of somatostatin immunoreactivity were correlated with the integrity of the synapto-dendritic complexity assessed by MAP2 ($r = 0.421$, $p = 0.02$) (Fig. 5b) and synaptophysin ($r = 0.463$, $p = 0.01$) immunoreactivity (Fig. 5c). However, no significant correlations were observed when compared to levels of...
Fig. 5. Linear regression analysis between somatostatin immunoreactivity and neuronal damage markers. (a) Statistically significant correlations were found between the severity of the viral burden and decrease in somatostatin immunoreactivity. (b, c) Levels of somatostatin immunoreactivity were correlated with the integrity of the synapo-dendritic complexity assessed by MAP2 and synaptophysin immunoreactivity. (d) No significant correlations were observed when compared with levels of glial fibrillary acidic protein (GFAP) immunoreactivity. (e) Deficient cognitive performance, as assessed by the Heaton Score, was inversely correlated with decreased levels of somatostatin immunoreactivity in the frontal cortex.

Glial fibrillary acidic protein (GFAP) immunoreactivity was significantly increased in the frontal cortex (inset). In order to determine the relationship between cognitive deficits and somatostatin immunoreactivity levels, linear regression analysis was performed. This study showed an inverse correlation between Heaton global score and levels of somatostatin immunoreactivity in the frontal cortex ($r = -0.494, p = 0.0268$) (Fig. 5d).

In the control hippocampal sections, anti-somatostatin immunostained pyramidal neurons in CA1-2 with moderate intensity (Fig. 6a). Interneurons located in areas...
CA1-2 (Fig. 6b) and CA3-4 (Fig. 6c) were strongly immunolabeled. Somatostatin-immunoreactive interneurons were more prominent in CA1 and CA2, compared with CA3 and CA4. Somatostatin antibody also strongly immunostained the neuropil of the molecular layer and the polymorph layer. HIVE cases showed an average decrease of 35% in somatostatin immunoreactivity in pyramidal neurons (Fig. 4b) and interneurons (Fig. 4c), when compared with controls (one-way ANOVA, post-hoc Dunnett’s, p < 0.05). Loss of somatostatin immunoreactivity in pyramidal (Fig. 6d) and nonpyramidal neurons (Fig. 6e, f) was more prominent in mild and moderate HIVE cases than in the severe HIVE group (Fig. 4b, c). Levels of somatostatin immunoreactivity in the hippocampus were not significantly correlated with GFAP immunostaining (not shown).

Patterns of Somatostatin Immunoreactivity in the Basal Ganglia

In the globus pallidus of the control cases, mild somatostatin immunolabeling was identified in woolly fibers and neuronal cell bodies associated with them (Fig. 7a). In the putamen of the control cases, intense somatostatin immunoreactivity was associated with medium-sized multipolar neurons (Fig. 7b, c). Cases with severe HIVE displayed a 30% decrease in levels of somatostatin immunoreactivity in the globus pallidus when compared with controls (one-way ANOVA, post-hoc Dunnett’s, p < 0.05) (Figs. 4d, 7d). No statistically significant differences were observed in the levels of somatostatin immunoreactivity in the putamen of HIVE cases (Fig. 4b).

However, the somatostatin-immunoreactive neurons in the putamen of cases severely affected with HIVE displayed a fragmentation and atrophy of their processes (Fig. 7e, f). No statistically significant correlations were observed between levels of somatostatin immunoreactivity in the basal ganglia and gliosis (not shown).

**DISCUSSION**

The present study showed that in the CNS of patients with AIDS, somatostatin immunoreactivity was associated with nonpyramidal and, to a lesser extent, with pyramidal neurons in the neocortex and hippocampus, and with nonpyramidal cells in the basal ganglia. This is consistent with previous studies showing that somatostatin-immunoreactive neurons are distributed in all parts of the cerebral cortex, as well as in the hippocampus and certain regions of the basal ganglia including caudate, putamen, and subthalamic nucleus (23, 28, 34–36). The nonpyramidal somatostatin-immunoreactive neurons have been shown to contain neuropeptide Y, calbindin, and GABA throughout the cortex and striatum (29, 30, 37). Furthermore, these neurons contain glutamate receptors (24), suggesting that they may be vulnerable to the excitotoxic damage mediated by HIV-1 (5, 38).

In the present study, decreased levels of somatostatin immunoreactivity were found in the frontal cortex and hippocampus of HIVE cases in the absence of overt loss of somatostatin-immunoreactive neurons. Radioimmunoassay confirmed that the decreased immunolabeling observed by immunocytochemistry was actually associated with decreased somatostatin levels within the cortex.
Fig. 7. Patterns of somatostatin immunoreactivity in basal ganglia. (a) In the globus pallidus of the control cases, mild somatostatin immunolabelling was identified in woolly fibers and neuronal cell bodies associated with them. (b, c) In the putamen of the control cases, intense somatostatin immunoreactivity was associated with medium-sized neurons. (d) Cases with severe HIVE displayed decreased somatostatin immunoreactivity in the globus pallidus. (e, f) Somatostatin-immunoreactive neurons in the putamen of cases severely affected with HIVE displayed a fragmentation and atrophy of their processes (arrowheads). Bar = 20 μm.

of patients with HIVE. This decrease in somatostatin immunoreactivity could be: (a) associated with impaired somatostatin synthesis by neurons in HIVE; (b) associated with decreased somatostatin mRNA transcription or message stability; and/or (c) secondary to the overall ongoing neurodegenerative process. The fact that neurons showing decreased somatostatin immunoreactivity also presented poorly branched and fragmented neuritic processes with shrunken cell bodies suggests that the altered somatostatin immunolabeling might be associated with direct damage to these neurons. However, since for the present study somatostatin mRNA levels or message stability were not determined, it is difficult to assess if the impaired somatostatin synthesis might be related to transcriptional or translational alterations. In this regard, recent studies in SIV-infected monkeys have shown an early increase in somatostatin mRNA expression in the frontal cortex (25). Taken together, these data suggest that cortical dysfunction might be related to altered patterns of activity in cortical afferents and/or neuronal susceptibility to locally generated mediators in response to the viral infection of the brain (25). The apparent discrepancy between levels of somatostatin immunoreactivity in HIVE and mRNA in SIV-infected monkeys might be related to differences in cellular response among species. Moreover, DaCunha et al (25) reported increased mRNA somatostatin levels in early stages of SIV infection, while in our study the more widespread damage to somatostatin neurons was observed in later stages of the disease.

The present study showed that decreased somatostatin immunoreactivity in the neocortex correlated with increased levels of HIV-gp41-immunoreactivity. However, in the hippocampus and basal ganglia, the decrease in somatostatin immunoreactivity did not directly correlate with levels of HIV-gp41-immunoreactivity. This indicates that neocortical somatostatnergic neurons are more susceptible to the neurotoxic effects of viral products, while in the hippocampus and basal ganglia, other factors, such as cytokines, might be involved. Studies have shown that in HIV-positive children, there is a trend toward decreased serum somatostatin (39), which supports the hypothesis that HIV plays a role in the injury of somatostatin-producing cells. Previous studies (40) have shown that T cells synthesize somatostatin and that this peptide inhibits HIV replication in CD8+ cells, but enhances replication in CD4+ infected cells (41). There are many possible mechanisms by which HIV could mediate damage to somatostatin-producing neurons. Among them, HIV-potentiated excitotoxicity (38, 42) might play a role, since somatostatin-positive neurons contain glutamate receptors (24). Furthermore, quinolinic acid and other mediators that are abnormally produced in the brain during HIV infection might play a role, since it has been shown that they can alter somatostatin gene expression (43). Another possible mechanism by which HIV might promote injury could include dysregulation of growth factors involved in maintenance of somatostatin neurons (2). In this regard, previous studies have shown that somatostatin neurons

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are dependent on brain-derived neurotrophic factor (BDNF) for release and expression of the peptide, and depleted levels of BDNF in the neocortex result in depleted levels of somatostatin neurons (44, 45). Thus, it is possible that HIV-1 alters levels of BDNF expression, which instead of enhancing survival, causes altered levels of somatostatin production and release, leading to the decrease in somatostatin immunoreactivity observed in the brains of patients with HIV.

Altered levels of somatostatin in HIV have contributed to the cognitive alterations seen in AIDS patients. For the present study, decreased somatostatin immunoreactivity correlated with poor global cognitive performance. Supporting the possibility that altered somatostatin levels have an effect on the altered behavioral performance, studies in SIV-infected monkeys have shown that dysregulation of somatostatin mRNA in the frontal cortex was associated with motor impairment (25). Somatostatin has been shown to promote memory enhancement and cyssteamime, which depletes somatostatin, induces memory deficits in experimental rats (26, 27). It has been shown that depletion of somatostatin causes deficits in passive avoidance and spatial memories of the rats (27). In the frontal cortex, aged rats have been shown to contain an increase in levels of somatostatin and a decrease in the number of somatostatin receptors when compared with young rats, which may be correlated with the memory failure observed in aged rats (26). It has also been shown that intracerebroventricularly administered somatostatin significantly ameliorated these memory impairments (27). In conclusion, this study supports the possibility that HIV might play a role in mediating damage to somatostatin neurons and this, in turn, may contribute to the motor and cognitive deficits in patients with AIDS.

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