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Induction of Apoptosis by HIV-1-Infected Monocytic Cells

Kirk Sperber, Prarthana Beuria, Netai Singha, Irwin Gelman, Patricia Cortes, Houchu Chen, and Thomas Kraus

We have previously described a soluble 6000-Da peptide produced by an HIV-1-infected human macrophage cell line, clone 43, which induces apoptosis in T and B cells. We have identified this factor as the novel cDNA clone FL14676485 that encodes for the human hypothetical protein, FLJ21908. The FL14676485 cDNA clone was isolated from a 43HIV λ ZAP Escherichia coli expression library and screened with a panel of rabbit and mouse anti-apoptotic Abs. We transfected the FL14676485 clone into Bosc cells and non-HIV-1-infected 43 cells. Western blot analysis of lysates from the FL14676485-transfected 43 cells and Bosc cells using anti-proapoptotic factor Abs revealed a protein with a molecular mass of 66 kDa corresponding to the size of the full-length gene product of the FL14676485 clone, while Western blot of the supernatant demonstrated a doublet of 46-kDa and 6000-Da peptide that corresponds to our previously described proapoptotic factor. Primary HIV-1infected monocytes also produce the FLJ21908 protein. Supernatants from these transfected cells induced apoptosis in PBMC, CD4+, and CD8+ T and B cells similar to the activity of our previously described proapoptotic factor. PCR analysis of 43 cells and 43 HIV cells revealed a base pair fragment of 420 bp corresponding to the FL14676485 gene product in 43 HIV cells, but not in 43 cells. The FLJ21908 protein induces apoptosis through activation of caspase-9 and caspase-3. We have further demonstrated that the FLJ21908 protein has apoptotic activity in the SH-SY5Y neuronal cell line and can be detected in brain and lymph nodes from HIV-1-infected patients who have AIDS dementia. The FLJ21908 protein may contribute to the apoptosis and dementia observed in AIDS patients. The Journal of Immunology, 2003, 170: 1566–1578.

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3 Abbreviations used in this paper: FasL, Fas ligand; CM, complete medium; GFP, green fluorescent protein; HAD, HIV-1-associated dementia; NMDAR, N-methyl-D-aspartate subtype; PARP, poly(ADP-ribose) polymerase; TPR, tetratricopeptide.
that chronically HIV-1-infected human macrophage hybridomas induce apoptosis in CD4+ and CD8+ T and B cells by multiple mechanisms, including gp120, FasL expression, and the production of a soluble 6000-Da proapoptotic peptide (49). In this study, we identify a novel cDNA clone isolated from the chronically HIV-1-infected human macrophage cell line, 43HIV, that induces apoptosis in T and B cells, and in a neuronal cell line similar to our previously described proapoptotic factor. Furthermore, using specific Abs generated against the novel cDNA clone, we demonstrate that the proapoptotic factor is present in brain and lymphoid tissue from patients that are HIV-1 infected with HAD, but not in non-HIV-1-infected controls, Alzheimer’s patients, and non-HIV-1 encephalitis patients.

Materials and Methods

Monocyte isolation and HIV-1 infection

Mononuclear cells were separated from buffy coats obtained from normal healthy volunteers by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. The cells were washed three times with sterile PBS and resuspended in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS (Life Technologies), 2 mM t-glutamine, and 1% penicillin/streptomycin (Life Technologies), henceforth called complete medium (CM). Freshly isolated PBMC were incubated at 37°C in CM and allowed to adhere for 45 min. Nonadherent cells were removed, and adherent cells were washed with sterile PBS, harvested with a rubber policeman, and stained with monocyte-specific anti-CD14 mAbs to assess the purity of the preparation. Ninety percent of the isolated cells expressed CD14 (45). Monocytes were infected with HIV-1_HIV, as previously described (49). The HIV-1_HIV virus was obtained from the AIDS Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). Dilutions of HIV-1 supernatant standardized to contain reverse transcriptase activity to 80,000 cpm/ml were incubated for 1 h with freshly isolated cells three washes with PBS.

Human macrophage hybridomas, cell lines, and SH-SY5Y cells

Human macrophage hybridomas, cell lines, and cell cultures (obtained by allowing monocytes to adhere to fibroblast feeder layers in tissue culture flasks) with a hypoxanthine-guanine phosphoribosyltransferase-deficient promonocytic line (U937) as previously described (45). We have uniformly infected and characterized the cell lines with different strains of HIV-1 (43HIV) (47–49). Bosc-29 cells obtained from the American Type Culture Collection (Manassas, VA) (51). The SH-SY5Y and THB cells were purchased from the American Type Culture Collection (Manassas, VA) (52).

PBMC, CD4+, and CD8+ T and B cells

PBMC were separated from buffy coats obtained from normal healthy volunteers by Ficoll-Hypaque (Pharmacia) density gradient centrifugation. The cells were washed three times with sterile PBS and resuspended in RPMI 1640 (Life Technologies) supplemented with 10% FCS (Life Technologies), 2 mM t-glutamine, and 1% penicillin/streptomycin (Life Technologies), henceforth called CM (45). We isolated purified CD4+ and CD8+ T and B cell populations by RosetteSep (Stem Cell Technologies, Vancouver, British Columbia, Canada). RosetteSep is a rapid, easy cell separation kit for the isolation of highly purified CD4+ and CD8+ T and B cells from whole blood. Whole blood is added to a RosetteSep cocktail, and cells are cross-linked with tetramer complexes. The cells are then incubated at room temperature, layered over Ficoll-Hypaque, and centrifuged for 20 min, and the enriched CD4+ and CD8+ T and B cells were isolated (53).

Spleen cells

BALB/c mice used for the spleen cultures were purchased from Charles River Laboratories (Wilmington, MA). Spleen cells were taken and treated with 0.17 M Tris-NH4Cl RBC lysis buffer to remove RBC by previously described methods (54). The isolated spleen cells were used in the apoptosis assay.

Acetone precipitation

Acetone precipitation was used to isolate the proapoptotic factor from supernatants from the 43 and 43HIV cell lines. Acetone (Sigma-Aldrich, St. Louis, MO) was chilled in an ice-salt bath to attain a temperature below 0°C. Proteins were fractionated from 43 and 43HIV supernatants by precipitation in 95% (v/v) acetone. The precipitated proteins were collected by centrifugation, and the residual acetone in the precipitates was removed by vacuum centrifugation in a Speed-Vac (Savant, Piscataway, NJ) (49).

Reverse-phase HPLC analysis

Reverse-phase HPLC was performed on the acetone precipitate from the 43 and 43HIV cells using a C18 (4.6 × 250-mm) column. Elution of bound proteins was developed using a linear gradient of 0.1% (v/v) trifluoroacetic acid. A gradient of 60 ml was developed at a flow rate of 1 ml/min. Elution profiles were monitored at an absorbance of 215 nm. Solvent in the protein-containing fractions was removed by vacuum centrifugation in a Speed-Vac (Savant) (49).

Anion exchange chromatography

Anion exchange chromatography was performed with a Mono-Q HR5/5 (5 × 50-mm) column on an FPLC system (Pharmacia). The elution gradient was developed using 20 mM Tris-HCI, pH 7.5 (buffer A), and 1 M NaCl in buffer A (buffer B) at a flow rate of 1 ml/min. Samples were prepared for anion exchange chromatography by exhaustive dialysis in buffer A. The protein elution profile was monitored by absorbance at 280 nm (49).

Generation of Abs

To clone the proapoptotic factor, we generated anti-proapoptotic factor mAbs and polyclonal Abs. Two BALB/c mice immunized with the proapoptotic fraction of the 43HIV cell line, as previously described (55). Ouchterlony immunodiffusion in gels was used to test the fraction containing nitrocellulose paper using PBMC as target cells. For immunoprecipitation, supernatants from the 43 and 43HIV supernatant was fractionated from the supernatants from the 43HIV supernatant containing fractions was removed by vacuum centrifugation in a Speed-Vac (Savant) (49).

Annexin V and intracytoplasmic staining for activated caspase-3

FITC-labeled annexin V, a phospholipid-binding protein of the annexin family (56, 57), was used to measure apoptosis in vitro using annexin V staining. Protein G-purified anti-proapoptotic bodies were isolated from culture supernatants and coupled to cytochrome c Sepharose 4B beads (Pharmacia) by methods established in our laboratory (55). Coupling efficiency was determined by measuring the protein concentration of the remaining Ab solution. Greater than 90% of the Ab was bound to the beads. One milliliter of the Ab-coated beads was rotated overnight (18–20 h) at 4°C with supernatant from 43HIV and Escherichia coli transfected with the FL14676485 gene. After washing, unabsorbed proapoptotic factor activity was monitored by absorbance at 405 nm.

Annexin V and intracytoplasmic staining for activated caspase-3

FITC-labeled annexin V, a phospholipid-binding protein of the annexin family (56, 57), was used to measure apoptosis using a commercially available kit (Coulter, Hialeah, FL). After incubating supernatants containing concentrations of the FLJ21908 protein with PBMC, T cells, and B cells, the cell samples were washed with ice-cold PBS, followed by centrifugation at 500 × g at 4°C. The cells were incubated with Annexin VFITC at
room temperature for 10 min in the dark. In other experiments, propidium iodide (100 µg/ml) was added to assess cell necrosis. The cells were then analyzed by flow cytometry to measure the annexin V+ population, gating on the live cells (49). Cells were also stained intracellularly and then analyzed on a flow cytometer to measure the FITC-labeled Abs directed against activated caspase-3. The cells were then analyzed by flow cytometry to confirm the annexin V+ population, gating on the live cells (49).

λ ZAP EcoRI/XhoI 43HV cDNA Library

A 43HV library was synthesized using the ZAP-cDNA synthesis method (Stratagene, La Jolla, CA) (58). The linker-primer was designed with a GAGA sequence to allow for efficient transfection. The restriction site allows the finished cDNA to be inserted onto a nitrocellulose membrane, blocked with 5% milk in PBS at room temperature for 2 h, and the blot was developed by chemiluminescence using a commercially available ECL kit (DuPont, Wilmington, DE) (50).

Western blot

The 43, 43HIV, Bosc, THB, and SH-SY5Y cell lines used in this study and all other procedures were performed using buffer containing PMSF (100 µM), aprotinin (10 µg/ml), leupeptin (25 µg/ml) and a mixture (50:50) of phenol/chloroform, as described previously (RNAzol, Linnai, TX) (33). Known quantities of RNA were mixed with 1 µg total cellular RNA and reverse transcribed at 37°C for 60 min in 10 µl of buffer containing 10 mM Tris (Sigma-Aldrich), pH 8.3; 50 mM KCl (Sigma-Aldrich); 5 mM MgCl2 (Sigma-Aldrich); 1 mM each of dATP, dCTP, dGTP, and dTTP (Sigma); and 20 U RNase inhibitor (Promega); 0.1 µg oligo(dT)15 (Boehringer Mannheim, Indianapolis, IN); and 50 U murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD). The PCR for FL14676485 was performed using the upstream primer 5′-TTGGCAAACGGGATGAGATAC-3′ complementary to each other and have an XhoI cohesive end. The adaptors are comprised of 9- and 13-mer oligonucleotides that are compatible with each other and have an XhoI cohesive end. The adaptors have the restriction site XhoI cohesive end. The adaptors have the restriction site XhoI cohesive end. The adaptors have the restriction site XhoI cohesive end.

Purification of the 6000d peptide

We purified the 6000-Da peptide from the supernatant of E. coli expressing the FLJ21908 protein. To this end, we concentrated 2 L of bacterial supernatant by lyophilization, resuspended the concentrated material in 100 mM NaCl and 20 mM Tris-HCl, pH 7.5, and loaded it onto a DEAE Sepharose column (Pharmacia), increasing the salt concentration from 100 mM to 1 M NaCl eluted fractions. The isolated fractions were run on a 10% polyacrylamide gel, transferred onto nitrocellulose paper, and Western blotted with the rabbit anti-FLJ21908 Abs. The Western blot-positive fractions were then silver stained to ascertain the purity of the protein separation.

Transfection with FL14676485

The 43 and Bosc cells were transiently transfected with a bacterial plasmid containing the FL14676485 cDNA clone using CaPO4, Superfect (Qiagen, Valencia, CA), or DEAE-dextran (50). The bacteria containing the FL14676485 cDNA clone were cultured, ethanol precipitated, extracted, and then centrifuged and resuspended in TBS. For CaPO4, a DNA/CaCl2/H2O mixture (500 µl) containing 20 µg of expressed FL14676485 plasmid, ssDNA carrier, 438 µl of H2O, and 62 µl 2 M CaCl2 was added to HBSS and incubated with either Bosc cells or 43 cells for 3 h. The 43 and Bosc cells were washed, and the medium was replaced. The cells were harvested at 48–60 h posttransfection. For Superfect (Qiagen), 5 µg of FL14676485 DNA was dissolved in TE buffer, pH 7.4, with medium that contains no serum proteins or antibiotics to a total volume of 150 µl. A total of 30 µl of Superfect (Qiagen) reagent was added to the DNA solution, mixed, and then added to the Bosc and 43 cells for 10 min at 25°C to allow for transfection complex formation. The cells were then washed once with 4 ml of PBS, and then 1 ml of CM was added. The cells were incubated with the transfection complex for 3 h at 37°C. The medium was removed, and the cells were washed in PBS. Fresh CM was added, and the cell supernatants were assayed at 48 and 60 h for apoptotic activity. For the DEAE-dextran method, the resuspended DNA was added to 10 mg/ml of DEAE-dextran (Sigma-Aldrich) and incubated with the 43 and Bosc cells for 4 h at 37°C. After aspirating the DEAE-dextran, the cells were shocked by adding 5 ml of 10% DMSO (Sigma-Aldrich) in PBS for 1 min at room temperature, washed with sterile PBS, and resuspended in CM for 48 h at 37°C. In some experiments, the 43 and Bosc cells were treated with CaPO4 alone, Superfect alone, or DEAE-dextran alone without the FL14676485 cDNA clone, while in other experiments the cells were treated with CaPO4, Superfect (Qiagen), DEAE-dextran, and jellyfish green fluorescent protein (GFP, Promega, Milwaukee, WI) to assess the efficacy of transfection.

Polymerase chain reaction

RNA was extracted from 43 and 43HV using acid phenol:chloroform, as described previously (RNAzol, Linnai, TX) (33). Known quantities of RNA were mixed with 1 µg total cellular RNA and reverse transcribed at 37°C for 60 min in 20 µl of buffer containing 10 mM Tris (Sigma-Aldrich), pH 8.3; 50 mM KCl (Sigma-Aldrich); 5 mM MgCl2 (Sigma-Aldrich); 1 mM each of dATP, dCTP, dGTP, and dTTP (Sigma); and 20 U RNase inhibitor (Promega); 0.1 µg oligo(dT)15 (Boehringer Mannheim, Indianapolis, IN); and 50 U murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD). The PCR for FL14676485 was performed using the upstream primer 5′-TTGGCAAACGGGATGAGATAC-3′, while in other experiments the cells were treated with CaPO4, Superfect (Qiagen), DEAE-dextran, and jellyfish green fluorescent protein (GFP, Promega, Milwaukee, WI) to assess the efficacy of transfection.

Results

Further characterization of the proapoptotic factor

In our previous work, we demonstrated proapoptotic activity from fractionated supernatant from the chronically HIV-1-infected cell line, 43HV (49). Proapoptotic activity could not be precipitated with acetone at a concentration lower than 80% saturation, a characteristic observed with smaller peptides. Results from peptide binding to anion exchange matrices at different pH indicated that the pI of the proapoptotic activity was between 6.5 and 7.0. When active fractions were electrophoresed on a 10% SDS-PAGE gel, a band corresponding to a Mr of 6000 Da was detected. Furthermore, active fractions from 43HV were electrophoresed on a nondenaturing SDS-PAGE gel, and proapoptotic activity was electroeluted from gel slices corresponding to a Mr of less than 10,000. Although we identified two proapoptotic fractions from 43HV in our previous studies, fractions 5 and 6, we investigated only fraction 5 in this study. We further characterized fraction 5 from the 43HV supernatant by reverse-phase HPLC analysis. We compared HPLC elution profiles of fraction 5 and demonstrated that eight peaks were present in fraction 5 from 43HV, but none of the fractions from the HPLC had activity in the apoptosis assay.
measuring annexin V staining in bystander T cells. It is possible that during the isolation procedure, biological activity was lost. We then developed a panel of murine mAbs and rabbit polyclonal Abs by immunizing mice and rabbits with the eight unique subfractions of fraction 5. We first screened by dot-blot reaction and then attempted to block apoptosis induced by crude 43HIV fraction 5. Abs directed against subfractions 2, 5, and 8 of fraction 5 of 43HIV were all capable of blocking apoptosis in unstimulated target T cells (Fig. 1A).

Identification of the proapoptotic factor
Using the panel of Abs directed against subfractions 2, 5, and 8 of fraction 5, we have identified the proapoptotic factor by screening a cDNA library (AZAPII) generated from HIV-1-infected 43 cells.

**FIGURE 1.** FLJ21908 protein. A, Blocking studies. Abs generated against subfractions 2, 5, and 8 blocked apoptosis induced by crude 43HIV supernatant. Abs were added to T cell cultures along with 50% crude 43HIV supernatant for 2 h at 37°C. The cells were then simultaneously stained with annexin V and propidium iodide to assess apoptosis and necrosis. The percentage of annexin V-positive, propidium iodide-negative cells is indicated in the right upper corner of each panel. This is representative of an experiment repeated three times. B, Alpha helical structure of the proapoptotic factor of the hypothetical protein FLJ21908. C, Western blot analysis of the FLJ21908 protein lysate and supernatant from E. coli expressing the FLJ21908 protein were run on a 10% polyacrylamide gel, transferred onto nitrocellulose paper, blotted with the rabbit polyclonal anti-proapoptotic factor Ab, and then developed. This is representative of an experiment repeated five times.
The 43\textsubscript{HIV} cDNA library was expressed in \textit{E. coli}, and in the screening process the expressed proteins were transferred onto nitrocellulose filters. The expressed proteins were lifted off onto nitrocellulose filters. The expressed proteins were transferred onto nitrocellulose filters. The expressed proteins were then transferred onto nitrocellulose membranes and the membranes were blocked with milk proteins (Carnation milk 10% w/v) and subsequently incubated with the rabbit and mouse anti-apoptotic factor Abs (10 μg/ml) or a pre-immune serum/Ab negative control. Ab bound to the expressed proteins was detected by incubation with either goat anti-rabbit IgG or goat anti-mouse IgG conjugated with HRP. Initially, we established the integrity of our library by screening with Abs directed against proteins expressed in 43\textsubscript{HIV} cells (e.g., IL-10, IL-8, IL-6, and HIV viral products). We screened the library first by Western blot with the rabbit polyclonal Abs and then screened the positive clones by Western blotting with the murine mAbs. The plaques that we identified were picked, regrown, rescreened, and expanded, and eventually a pure clone was obtained. The cDNA isolated from this clone was sequenced at the Mount Sinai DNA core sequencing facility and found to be the recently described FL14767485 gene that encodes for the hypothetical protein FLJ21980 (Fig. 1B).

Our initially described proapoptotic factor had a molecular mass of 6000 Da, while the FLJ21908 protein has a molecular mass of 66 kDa (49). To reconcile this discrepancy, we performed Western analysis on lysates and supernatants from \textit{E. coli} expressing the FLJ21908 protein (Fig. 1C). In the lysate, a 66-kDa band was identified in accord with the molecular mass of the full-length FLJ21908 protein, while in the supernatant a doublet of 46-kDa and a 6000-Da band consistent with our previously described factor was identified (Fig. 1C). In our initial characterization of proapoptotic activity from the 43\textsubscript{HIV} supernatant, active proapoptotic fractions were isolated with 95% acetone. Ninety-five percent acetone treatment eliminates proteins with molecular mass less than 10 kDa, including the 46-kDa doublet from the FLJ21908 protein. We purified the 6000-Da peptide from the supernatant of \textit{E. coli} expressing the FLJ21908 protein by lyophilizing and resuspending bacterial supernatant in 100 mM NaCl and 20 mM Tris-HCl, pH 7.5, and loading it onto a DEAE Sepharose column. Fractions were eluted by increasing the salt concentration. The isolated fractions were Western blotted with the rabbit anti-FLJ21908 Abs. The Western blot-positive fractions were then silver stained to ascertain the purity of the protein separation. Fractions 39–49 that were eluted with 1 M NaCl demonstrated a band at 6000 Da consistent with the previously described proapoptotic factor (Fig. 2).

**Biological activity of the FLJ21908 protein**

Because we identified the FLJ21908 protein as the proapoptotic factor, we then wanted to determine whether it had the same apoptotic activity as our initially defined factor (49). We first used annexin V staining of unstimulated PBMC as target cells to determine the biological activity of the FLJ21908 protein. Supernatant from the FLJ21908-expressing \textit{E. coli} induced apoptosis in unstimulated target PBMC (Fig. 3). To ensure that the FLJ21908 protein was our initially defined proapoptotic factor, polyclonal rabbit anti-proapoptotic factor and mouse anti-apoptotic mAbs were used to block the apoptotic activity of the FLJ21908 protein (Fig. 3). The rabbit and murine anti-apoptotic Abs blocked FLJ21908-induced apoptosis, while the isotype-specific (IgG1) irrelevant murine mAbs were ineffective. The previously described proapoptotic factor was also observed to induce apoptosis in unstimulated PBMC (data not shown).

**FIGURE 2.** The FLJ21908 protein induces apoptosis in unstimulated PBMC. Supernatant from \textit{E. coli} and supernatant from \textit{E. coli} containing the FLJ21908 protein were incubated with PBMC for 2 h, and apoptosis was determined by annexin V staining. In blocking experiments, either the rabbit polyclonal or murine anti-apoptotic factor Abs or preimmune rabbit serum or isotype-specific (IgG1) irrelevant murine mAbs were added to supernatant containing the FLJ21908 for 2 h, and apoptosis was evaluated by annexin V staining. The percentage of positively staining cells is indicated in the right upper corner of each panel. This is representative of an experiment repeated five times using PBMC from different donors.
Transfection of the FL14767485 gene into Bosc cells

To further confirm the apoptotic activity of the FLJ21908 protein, we transfected the FL14676485-containing plasmid that encodes the FLJ21908 protein and GFP to assess the efficiency of transfection into a human cell expression system, Bosc cells, and into non-HIV-1-infected 43 cells. Bosc cells are derived from the 243T human embryo kidney cell line and are efficiently transfected with CaPO₄ (51). Mock transfection of the Bosc cells using GFP demonstrated that 90% of the cells were transfected (data not shown). We also used CaPO₄ to transfect the 43 cells with DEAE dextran and Superfect produced similar rates of transfection in the 43 cells (data not shown). After transfection, apoptotic activity was determined in the supernatant from Bosc cells and the 43 cells by annexin V staining using unstimulated PBMC as target cells. A dose-dependent increase in annexin V staining was observed in PBMC incubated with supernatants from the Bosc and 43 cells transfected with FL14767485 gene, but not in Bosc and 43 cells transfected with GFP (Fig. 4A). To demonstrate that the FLJ21908 protein is necessary and sufficient for apoptotic activity, we made an Ab affinity column using the three Abs that blocked apoptosis against subfractions 2, 5, and 8 (Fig. 1A). We passed supernatant from 43HIV and E. coli transfected with the FL14676485 gene over the Ab affinity column and assessed apoptotic activity as determined by intracytoplasmic staining using FITC-labeled Abs directed against activated caspase-3. There was no apoptotic activity from supernatant passed over the Ab column in both 43HIV and E. coli transfected with the FL14676485 gene using PBMC as the target cells (Fig. 4B).

Demonstration of FLJ21908 protein

We next performed Western blot analysis using the supernatant and lysate of the untransfected and transfected 43 and Bosc cells.

FIGURE 3. Purification of the 6000-Da peptide. Two liters of E. coli supernatant containing the FLJ21908 protein were lyophilized and then passed over a DEAE Sepharose column, increasing the concentration of NaCl from 100 mM to 1 M eluted fractions. Only fractions that were Western blot positive were run on a 10% polyacrylamide gel and silver stained. This representative of an experiment was repeated three times.

FIGURE 4. Transfection with FL14676485. A, Supernatant from the FL14676485-transfected Bosc and 43 cells induces apoptosis in target PBMC. Different concentrations (50, 25, 10, and 0%) of supernatant from the FL14676485- and GFP-transfected Bosc and 43 cells were cocultured with freshly isolated PBMC for 2 h and then assessed for apoptosis by annexin V staining. The percentage of positively staining cells is indicated in the right upper corner of each panel. This is representative of an experiment repeated five times. B, Supernatant from 43HIV and E. coli transfected with FL14676485 43 were passed over an anti-apoptotic Ab column, cocultured with freshly isolated PBMC for 2 h, and then assessed for apoptosis by staining with FITC-labeled Abs directed against activated caspase-3. The percentage of positively staining cells is indicated in the right upper corner. This is representative of an experiment repeated three times.

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along with 43\textsubscript{HIV} cells using the polyclonal rabbit anti-proapoptotic factor Abs to determine whether the 6000-Da peptide was being produced (Fig. 5). A protein with a molecular mass of 66 kDa corresponding to the FLJ21908 protein was detected in the lysate of the FL14676485-transfected 43 and Bosc cells and 43\textsubscript{HIV} cells, while a doublet of 46- and a 6-kDa band were found in the supernatant. The 6000-Da molecular mass protein corresponds to our previously described proapoptotic factor. There was no detectable protein in either the lysate or supernatant of the untransfected 43 and Bosc cells (Fig. 5A). We next determined whether RNA for the FLJ21908 protein is constitutively expressed in 43 cells or whether it is induced after HIV-1 infection. We investigated by PCR whether we could demonstrate the presence of RNA for the FL14767485 gene in the uninfected 43 cells and in 43\textsubscript{HIV} cells. Forty-three cells were either left alone in culture or infected with either HIV-1\textsubscript{Lm} or HIV-1\textsubscript{BaL} for 35 days, and RNA was harvested for PCR analysis. Usage of HIV-1\textsubscript{Lm} base pair fragments consistent with the predicted size of the FL14767485 (420 bp) was observed in the 43\textsubscript{HIV} cells, but not in the non-HIV-1-infected 43 cells, demonstrating that HIV-1 infection induces the FLJ21908 protein (Fig. 5B). Actin (661 bp) was the positive control. Similar results were observed with 43 cells infected with HIV-1\textsubscript{BaL} (data not shown). In these experiments, we used uninfected 43 supernatant for the mock infection. We have tried to induce proapoptotic activity from 43 cells and primary monocytes using UV-treated HIV-1, but failed. Direct HIV-1 infection of 43 cells and primary monocytes appears to be necessary for FLJ21908 production because infection with HIV-2 and influenza virus did not induce the production of the FLJ21908 protein (data not shown). We have also used different HIV-1 proteins, including pol, gag, env, rev, tat, and nef, to alter the expression of the FLJ21908 protein in both HIV-1-infected and uninfected 43 cells and monocytes, and again found that there was no effect (data not shown). We were also unable to induce production of the FLJ21908 protein in 43 cells and primary monocytes using different cytokines, including TNF-\alpha, IL-10, IL-12, IFN-\gamma, and GM-CSF. These cytokines also failed to either up-regulate or down-regulate the production of the FLJ21908 protein in 43\textsubscript{HIV} cells and primary HIV-1 BaL-infected monocytes (data not shown). In addition, HIV-1-infected primary T cell and T cell lines did not produce the FLJ21908 protein (data not shown).
FIGURE 6. Apoptotic signaling pathways. A. Treatment with the FLJ21908 protein activates caspase-3 in the THB cell line. THB cells were treated with 50% supernatant from the FL14676485-transfected Bosc cells for 16 h or left untreated and then stained with FITC-labeled Abs directed against activated caspase-3. The percentage of positively staining cells is indicated in the right upper corner of each panel. This is representative of an experiment repeated three times. B. The same THB cells that were used in A were lysed, run on a 10% polyacrylamide gel, transferred onto nitrocellulose paper, incubated with anti-caspase-8 Abs, and then developed. Fas-treated Jurkat cells (BD Bioscience) served as the positive control (PC). This is representative of an experiment repeated three times. C. The same THB cells that were used in A were run on a 10% polyacrylamide gel, transferred onto nitrocellulose paper, incubated with anti-caspase-9 Abs, and then developed. Cytochrome c-treated U937 cells (BD Bioscience) served as the positive control (PC). This is representative of an experiment repeated three times.

not shown). We infected 10 different monocyte preparations with HIV-1<sub>ind</sub> to determine whether the FLJ21908 protein was produced in vivo to validate our findings in the 43 cells. Similar to our previous description of the 6000-Da proapoptotic factor, we found that 3 of 10 monocyte preparations produced the FLJ21908 after HIV-1<sub>ind</sub> treatment. Interestingly, Western blot analysis demonstrated the presence of only the 6000-Da peptide, but not the 46-kDa doublet (Fig. 5B). An important role of the FLJ21908 protein in neuronal apoptosis

Although it is beyond the scope of this study to investigate in detail the mechanisms involved in the activation of 6000-Da pro-apoptotic pathways, it was important to identify some of the pro-apoptotic pathways used by the FLJ21908 protein, we used the THB T cell line. Fas, a 40-kDa transmembrane death receptor, is activated by other caspases, in particular, caspase-8 and caspase-9 (59). Caspase-8 is a 55-kDa cytosolic protein that can activate caspase-3, as well as other caspase cysteine proteases (59). Caspase-8 is produced as a proenzyme (55 kDa) that is proteolytically cleaved into smaller subunits of 40/36 and 23 kDa (59). Caspase-9 is another member of the cysteine protease family that can activate caspase-3 (59). Procaspase-9 (44–48 kDa) when activated is processed into large (37-kDa) and small (10-kDa) subunits and is activated in the presence of cytochrome c (59). To determine the apoptotic signaling pathways used by the FLJ21908 protein, we used the THB T cell line. There was a marked increase in the expression of activated caspase-3 following treatment with 50% supernatant from FL14676485-transfected Bosc cells containing the FLJ21908 protein (Fig. 6A). We next investigated whether the activation of caspase-3 was occurring through caspase-8 or caspase-9. To this end, we performed Western blot analysis for caspase-8 (Fig. 6B) and caspase-9 (Fig. 6C) using lysates from the same THB cells that demonstrated activated caspase-3. There was no activation of caspase-8 in the THB cells, as a 55-kDa doublet consistent with the molecular mass of procaspase-8 was present (Fig. 6B). In the same lysates, however, there was activation of caspase-9, as a 37-kDa band representing an active subunit of caspase-9 was detected (Fig. 6C).

Induction of apoptosis in CD4<sup>+</sup> and CD8<sup>+</sup> cells by the FLJ21908 protein

In our initial studies characterizing the proapoptotic factor, we used human PBMC to demonstrate apoptosis (49). It is possible that the proapoptotic factor might be conserved in other animal species. If the FLJ21908 protein has proapoptotic activity in other species, it would be particularly useful in further studies to determine its biologic significance. To test this hypothesis, we attempted to induce apoptosis in murine splenocytes generously provided by T. Moran (Mount Sinai School of Medicine, Department of Microbiology). Different concentrations of the FLJ21908 protein (50, 25, 10, and 0%) from the supernatants of the FL14676485-transfected Bosc cells containing the FLJ21908 protein was demonstrated for CD4<sup>+</sup>, CD8<sup>+</sup> T cells, and CD8<sup>+</sup> T cells by annexin V staining (Fig. 7).

Demonstration of apoptotic activity in murine splenocytes

Role of the FLJ21908 protein in neuronal apoptosis

Apoptosis of neurons is a prominent feature of AIDS dementia (41). Macrophages play an important role in this process. Macrophages and microglia cells infected with HIV-1 produce neurotoxins that damage neurons by releasing excitotoxins that produce excessive activation of glutamate receptors, primarily of the NMDAR (41). To determine a role for the FLJ21908 protein in neuronal apoptosis, we investigated whether the FLJ21908 protein
induces apoptosis in the neuroblastoma cell line SH-SY5Y. In these experiments, different concentrations of the FLJ21908 protein (50, 25, 10, and 0%) derived from the supernatants of Bosc cells were added to cultures of the neuronal cell line SH-SY5Y, and apoptosis was assessed by the induction of caspase-3 and PARP. Active caspase-3 consists of a heterodimer of 17- and 12-kDa subunits that are derived from the 32-kDa proenzyme. Active caspase-3 proteolytically cleaves and activates other caspases as well as relevant targets in the cytoplasm, e.g., D4-GDI and Bcl-2 and PARP in the nucleus. PARP is a 116-kDa nuclear chromatin-associated enzyme that catalyzes the transfer of ADP-ribose units from NAD$^+$ to a variety of nuclear proteins, including topoisomerases, histones, and PARP itself (59). During apoptosis, PARP is cleaved from its 166-kDa intact form into 85- and 25-kDa fragments. In the SH-SY5Y cultures incubated with supernatant from Bosc cells containing the FLJ21908 protein, the 17-kDa subunit of caspase-3 (60) was demonstrated along with the 85-kDa fragment (61) of PARP (Fig. 9).

Presence of the proapoptotic factor in macrophages in HAD

We next investigated whether we could detect the presence of the FLJ21908 protein in histological sections from patients with HAD. In these experiments, we used normal brain, Alzheimer’s disease, and non-HIV-1 encephalitis as controls. Widespread reactive astrogliosis, myelin pallor, and infiltration predominantly by monocytoid cells, including blood-derived macrophages, resident microglia, and multinucleated giant cells, characterize the neuropathology associated with HIV infection of the brain (63). Neurological apoptosis is not specific for HAD, but is a feature of many different types of dementia caused by infectious agents and other neurodegenerative diseases, including Alzheimer’s disease (41). Using the murine anti-FLJ21908 Ab, punctate green staining consistent with the presence of the FLJ21908 protein was detected in patients with HAD, but not in normal, Alzheimer’s disease or non-HIV-1 encephalitis patients (Fig. 10). We also stained lymph nodes from the same patients for the presence of the FLJ21908 protein that was used to study brain tissue. Similar to the results obtained with the brain tissue, the FLJ21908 protein was present in lymph nodes from the HIV-1-infected patients (Fig. 11).

Discussion

We have extended our previous studies that first described apoptosis in T and B cells induced by chronically HIV-1-infected human macrophage hybridomas. We have identified a proapoptotic factor, the FLJ21908 protein (Fig. 1, B and C), produced by the chronically HIV-1-infected human macrophage hybridoma cell
line, 43 HIV. We have demonstrated that the FLJ21908 protein is the same as our previously described factor because anti-proapoptotic Abs generated against partially purified 43 HIV supernatant can block the apoptotic activity of the FLJ21908 protein (Fig. 2). Apoptotic activity was demonstrated in the supernatant of Bosc and 43 cells after transfection with the FL14767485 gene that encodes for the FLJ21908 protein, while the full-length FLJ21908 protein has a molecular mass of 66 kDa that can be demonstrated in the lysate of 43 HIV, Bosc, and 43 cells transfected with the FL14767485 gene. In the supernatant, a 6000d protein consistent with the size of our previously described proapoptotic factor was present (Fig. 5A). We can isolate a purified 6000d peptide from E. coli expressing the FL14676485 gene. RNA for the FL14676485 gene can be demonstrated in the 43 HIV cells, but not in 43 cells (Fig. 5B). A subpopulation of primary monocytes infected with HIV-1 BaL produces the FLJ21908 protein (Fig. 5C). FLJ21908-mediated apoptosis is induced through the activation of caspase-3 through caspase-9 (Fig. 6, A, B, and C). The FLJ21908 protein has the same biological activity as our initially described factor. It induces apoptosis in human CD4+ and CD8+ T and B cells (Fig. 7) and also in murine T cells (Fig. 8). The FLJ21908 protein also induces apoptosis in the SH-SY5Y neuronal cell line, as determined by Western blotting with anti-caspase-3 and anti-PARP Abs (Fig. 9). Furthermore, the FLJ21908 protein can be detected in brain and lymphoid tissue from patients with HAD (Figs. 10 and 11).

The Homo sapiens hypothetical protein FLJ21908 has a molecular mass of 66 kDa, but has eight open reading frames encoding for proteins with molecular mass of 9900, 6490, 4400, 4290, 4070, and 3890. By Western blotting with anti-caspase-3 Abs, and by reverse transcriptase-polymerase chain reaction, FLJ21908 RNA can be demonstrated in the brain and lymphoid tissue of patients with HAD (Figs. 10 and 11).

**Table I. Characteristics of the HAD patients**

<table>
<thead>
<tr>
<th>Age</th>
<th>Gender</th>
<th>Duration of Dementia (years)</th>
<th>Therapy</th>
<th>CD4 Counts Cells/mm³</th>
<th>Viral Load Copies/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>M</td>
<td>2</td>
<td>HAART</td>
<td>135</td>
<td>3,000</td>
</tr>
<tr>
<td>37</td>
<td>F</td>
<td>2.7</td>
<td>HAART</td>
<td>75</td>
<td>7,000</td>
</tr>
<tr>
<td>57</td>
<td>M</td>
<td>1.5</td>
<td>HAART</td>
<td>110</td>
<td>15,000</td>
</tr>
<tr>
<td>34</td>
<td>M</td>
<td>2</td>
<td>No therapy</td>
<td>50</td>
<td>14,567</td>
</tr>
</tbody>
</table>

*Demographics, duration of dementia, therapy, CD4 counts, and viral loads of patients studied in Figs. 10 and 11. HAART is highly active antiretroviral therapy.*
4100, 3960, and 3740 Da (National Center for Biotechnology Information Open Reading Frame Finder). In addition, motif analysis of the FLJ21908 protein revealed two tetratricopeptide (TPR) repeats, which is a repeat structure of 34 aa first described in yeast and later found to occur in a large number of proteins (64) (Fig. 1a). A common feature of TRP repeats is protein-protein interactions (65). It has also been proposed that TPR proteins preferentially interact with WD-40 repeat proteins, but in many instances TPR aggregate to form multiprotein complexes (64). A common feature of TPR repeats is protein-protein interactions (65). It has also been proposed that TPR proteins preferentially interact with WD-40 repeat proteins, but in many instances TPR aggregate to form multiprotein complexes (64). TPR repeats have been implicated in apoptosis (66). In our initial characterization of the proapoptotic activity from the FL14676485 gene, we determined by molecular mass fractionation that proapoptotic activity was present from the fractions that corresponded to a molecular mass less than 10,000 Da (49). Furthermore, fractionation of supernatants from HIV-1 infected monocytes that induced apoptosis in target PBMC revealed that activity was also present in those fractions with a molecular mass less than 10 Da similar to the 43HIV cell line (49). The presence of full-length 66-kDa FLJ21908 protein in apoptosis is unclear; however, the 66-kDa protein is not secreted in 43HIV cells and is uninfected 43 and Bosc cells after transfection with the FL14676485 gene (Fig. 5A). We are presently attempting to obtain sufficient quantities of the 6000D fragment of the FLJ21908 protein for in vitro sequence analysis.

The regulation of the FLJ21908 protein as it relates to apoptosis involves not only the induction of the FL14676485 gene, but also its cleavage into its proapoptotic form. Bosc cells transfected with the FL14676485 gene produced the 6000-Da peptide, demonstrating that normal host enzymes are capable of cleaving the parent FLJ21908 protein (Fig. 5). Apoptotic activity from the cell line occurs only after prolonged infection (greater than 35 days) (49). As noted above, in the transfection experiments uninfected 43 cells and uninfected 43 cells produced the 6000-Da peptide, proving that the 66-kDa protein is cleaved by host proteases. It is possible that other factors may be involved in the generation of the 6000-Da peptide from the parent FLJ21908 protein. One possibility is HIV-1 protease that is well known to cleave host cell proteins and transfected HIV-1 protease kill host cells (67). HIV-1 protease inhibitor-based therapy to treat AIDS inhibits apoptosis (68). HIV-1 protease inhibitors have had little impact on HAD because they have limited penetration across the blood-brain barrier.

FIGURE 10. Detection of the proapoptotic factor in patients with HAD. Immunofluorescence staining was performed using tissue sections from normal brain, HAD, Alzheimer's disease, and non-HIV-1 encephalitis. The sections were stained with murine FITC-labeled anti-FLJ21908 Abs and analyzed by confocal microscopy. Isotype-matched FITC-labeled IgG1 served as the negative control. Two observers routinely observed 10 separate fields.

FIGURE 11. Detection of the FLJ21908 protein from lymph nodes. Immunofluorescence was also performed on sections of lymph nodes from the same patients in Fig. 10. The sections were stained with FITC-labeled murine anti-proapoptotic factor Ab and analyzed by confocal microscopy. Isotype-matched FITC-labeled IgG1 served as the negative control. Two observers routinely observed 10 separate fields.
barrier (69, 70). The FLJ21908 protein may be induced by other pathogens and not be specific to HIV-1. However, HIV-2 and influenza infection of 43 cells did not result in the production of the FLJ21908.

The FLJ21908 protein induces apoptosis through the activation of caspase-9 (Fig. 6). Members of the Bcl-2 family control signaling of apoptosis through caspase-9. Studies are in progress to better characterize the apoptotic signaling of the FLJ21908 protein. The FLJ21908 protein induces apoptosis in CD4+ and CD8+ T and B cells (Fig. 7) and can induce apoptosis in murine splenocytes (Fig. 8). The FLJ21908 protein may have a broader biological role. Although we identified the FLJ21908 protein as being produced by HIV-1-infected macrophages, it is possible that other cell types have the capacity to produce this protein, and in fact it may be a normal constituent of cell growth and regulation. This may not only play a role in the T cell loss during HIV-1 infection, but may be important in the regulation of inflammatory responses.

Although it has been proposed that HIV-1 disease progression is associated with a shift in cytokine profile from type 1 to type 2 profiles in vitro (71), the dominant in vivo shift is that of induced TNF-α, IFN-γ, IL-10, and IL-6 with reduced IL-2, as determined by studying cytokine mRNA expression in lymph nodes (72). These cytokines have been implicated in the induction of apoptosis and may further contribute to HIV-1 disease progression by augmentation of apoptosis induced by the FLJ21908 protein. The key question in our studies is whether the FLJ21908 protein contributes to the cell loss in HIV-1-infected patients. One area of particular interest is the neurological complications of HIV-1 infections. As noted above, in HIV-1 infection of the CNS, the virus is selectively localized within the perivascular and meningeal areas, where inflammatory lesions are associated with select populations of macrophages that are activated by cytokines and chemokines. Blood-borne macrophages and microglia (73). In particular, a subpopulation of macrophages, CD16high CD14 low has been identified as being important in the neurological manifestations of AIDS (74). This subpopulation of inflammatory macrophages tends to establish chronic inflammation in the brain and cause neurotoxicity. Interestingly, the FLJ21908 protein on HIV-1-infected macrophages adhere to the normal endothelium of the blood-brain barrier, transmigrate, and then trigger apoptosis of neural tissues.

The discrepancy between the number and localization of HIV-1-infected cells and the severity of the neurological abnormalities in AIDS has suggested that soluble factors produced by the infected brain macrophages may be responsible for the pathology seen (41). Different cytokines, including IL-1 and TNF-α, chemokines (75), along with excitotoxic substances that cause excessive activation of NMDARs that damage neurons contribute to neurotoxicity. We are extending these preliminary experiments to better characterize the mechanism by which the FLJ21908 protein induces apoptosis in both lymphoid and neuronal cells, and to study more patients.

In conclusion, we have previously described a soluble 6000d peptide produced by an HIV-1-infected human macrophage hybridoma, clone 43HV, that induces apoptosis in bystander, Ag-, and mitogen-stimulated T cells, as well as B cells. We have identified this factor as the novel cDNA clone FL14676485 that encodes for the human hypothetical factor FLJ21908. Apoptosis induced by the FLJ21908 protein may contribute to the apoptosis and dementia observed in AIDS patients.

References


Letter of Retraction

We wish to retract the manuscript titled “Induction of Apoptosis by HIV Infected Monocytic Cells” by Kirk Sperber, Prarthana Beuria, Netai Singha, Irwin Gelman, Patricia Cortes, Houchu Chen, and Thomas Kraus, The Journal of Immunology, 2003, 170: 1566–1578. The manuscript contains errors in the presentation of data in some of the figures.

In Fig. 2, where we demonstrated the ability of the murine and rabbit Abs to block apoptosis, the FACS histograms 1, 5, and 6 as well as histograms 3 and 4 were mistakenly duplicated. We have repeated these experiments and demonstrate that both the mouse and rabbit Abs block the apoptotic activity of a bacterial supernatant containing FLJ21908. We have localized the site of the pro-apoptotic activity on the protein by making a fusion protein from aa 330 to aa 665 of the FLJ21908 gene and have identified a 6000-Da breakdown product consistent with the molecular mass of the originally described factor. We have also used Flag-tagged fusion proteins to isolate the apoptotic activity to the terminal 150 aa.

In Fig. 6C, demonstrating that FLJ21908 induces apoptosis through caspase 9, the breakdown products of the positive control and the 50% supernatant appear to be the same. We have repeated these experiments using the FLJ21908 fusion protein and Flag-tagged protein containing the last 150 aa and have demonstrated that caspase 9 is activated in the H-9 T cell line and in the SH-SY5Y neuronal cell line.

The gels in Fig. 9 demonstrating caspase 3 and PARP activation after treatment with the FLJ21908 protein appear the same. We have repeated these experiments using different concentrations of the FLJ21908 Flag-tagged protein and demonstrate caspase 3 and PARP activation.

The flow cytometry control data from Fig. 4B demonstrating that FLJ21908 is necessary and sufficient for the induction of apoptosis look the same but are in fact not identical.

Despite these errors, the message of the manuscript remains unchanged. We report the identification of an apoptotic peptide produced by an HIV-infected human macrophage cell line. However, given the errors made in the figures we wish to retract the manuscript.

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