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Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients

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KAPOSI'S sarcoma (KS) is frequently associated with human immunodeficiency virus-1 (HIV-1) infection[1]. Supernatants from HIV-1-infected T cells carrying the CD4 antigen promote the growth of cells derived from KS lesions of AIDS patients (AIDS-KS cells)[2], and the HIV-1 *tat* gene, introduced into the germ line of mice, induces skin lesions closely resembling KS[3]. Here we report that the *tat* gene product (Tat) is released from both HIV-1-acutely infected H9 cells and *tat*-transfected COS-1 cells. These Tat-containing supernatants specifically promote growth of AIDS-KS cells which are inhibited by anti-Tat antibodies; recombinant Tat has the same growth-promoting properties. Therefore a viral regulatory gene product can be released as a biologically active protein and directly act as a growth stimulator. These and previous data[3] indicate that extracellular Tat could be involved in the development or progression, or both, of KS in HIV-1-infected individuals.

We recently developed a system for the long-term culture of spindle-like cells derived from KS lesions of AIDS patients (AIDS-KS cells)[2] dependent on conditioned media (CM) derived from activated T cells carrying the CD4 antigen (ref. 2, and B. Ensoli *et al.*, manuscript in preparation) or HIV-1-infected cells[2]. Morphological and immunohistochemical studies indicated that AIDS-KS cells are of mesenchymal cell origin and share features with endothelial and smooth muscle cells (ref. 4; and H. A. Welch, S. Nakamura, S.Z.S., R.C.G. and J. Folkman, manuscript submitted), both potential progenitors of the suspected tumour cells (spindle cells) of KS[4,5-7]. Transplantation of AIDS-KS cells into nude mice produced mouse lesions closely resembling KS[4], probably by release of specific cytokines with effects relevant to the histogenesis of KS lesions, including neoangiogenesis (refs 4, 8, 9; and E. W. Thompson *et al.*, manuscript submitted). The presence of KS-growth promoting activity in CM from HIV-infected CD4$^+$ T cells, the absence of HIV-1 sequences in DNA from KS tissue or cultured cells[4-6] and the observation that transgenic mice carrying the *tat* gene develop KS-like lesions and express *tat* in the skin but not in the tumour cells[3], indicate that the role of HIV-1 in KS is indirect, and that Tat itself might be released by infected cells and promote activation and growth of target cells involved in the formation of KS.

To test this hypothesis, we examined the effect on growth of CM from HIV-1-infected T cells (H9)[10] on AIDS-KS cells (AIDS-KS3 cell clone)[2,4,8], and on normal control cells (human umbilical vein endothelial cells, H-UVE; and human newborn aortic smooth muscle cells, H-ASM). H9–HIV-1-derived CM, but not H9 CM, specifically stimulated AIDS-KS cell proliferation but had no effect on H-UVE or H-ASM growth after 6 days of culture (Fig. 1*a*), or in prolonged assays (2 weeks) (data not shown). The average stimulation of AIDS-KS cell growth (5–6 days assay) was 2.0-fold (1.7- to 3.3-fold) for HIV-1 CM as compared to 2.5-fold (1.9- to 3.6-fold) with the positive control (CM from human T-lymphotrophic virus-II (HTLV-II)-transformed CD4 T cells)[2]. The variability observed in different experiments was related to cell passage number and the level of basal cell growth.

The proliferation of AIDS-KS cells stimulated by H9–HIV-1 CM was specifically inhibited by anti-Tat antibodies, whereas there was no reduction of cell growth for control media or positive control (Fig. 1*b*). This indicates that Tat is responsible for the growth effect present in CM from HIV-1-infected H9 cells, implying that Tat is released into the extracellular fluid.

Using radioimmunoprecipitation analysis (RIPA) with affinity-purified anti-Tat antibodies, we detected a distinct band of the same apparent relative molecular mass (M_r) as intracellular Tat (M_r 15,000; 15 K)[11] in CM from H9–HIV-1-acutely infected cells (Fig. 1*c*). Extracellular Tat was detectable 8–16 days after cell-free infection of H9 cells, and its presence corre-

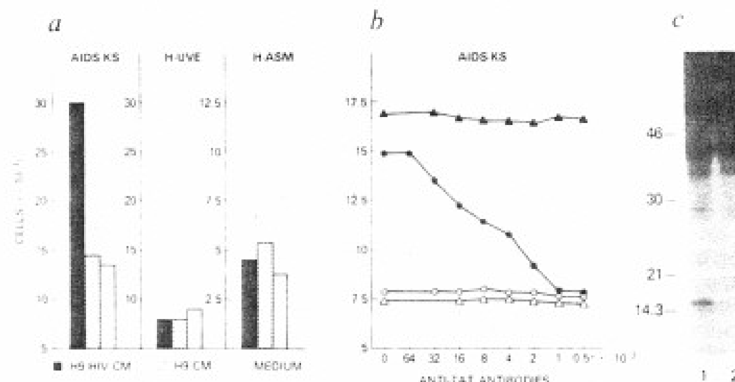

FIG. 1 Tat is involved in AIDS-KS cell response to HIV-infected T cells. *a*, Growth response of AIDS-KS and control cells to CM from HIV-1-infected or uninfected H9 cells. CM were diluted 1:4. *b*, Specific block of AIDS-KS cell growth response to H9–HIV-1-derived CM by anti-Tat antibodies. ●, H9–HIV-1-derived CM (1:16); ○, H9-derived CM (1:16); △, medium without CM; ▲, HTLV-II-derived CM (1:16). *c*, RIPA of Tat in CM from HIV-1-infected or uninfected H9 cells. Lanes 1 and 2, CM from HIV-1-infected or uninfected H9 cells, respectively. Arrow, Tat in CM from infected cells.

METHODS. CM from HIV-1-infected (IIIB isolate) or uninfected H9 cells (1×10^6 ml^{-1}) were collected from day 4–24 after cell-free infection, centrifuged at low (2,000*g*) and high speed (30,000 r.p.m.) for 1 h, stored at −20 °C and individually tested. Effects of CM 10 days after infection are shown. Cells were seeded in 25-cm^2 flasks (AIDS-KS, 5×10^4; H-UVE, 1×10^5) or in 12-well plates (H-ASM, 5×10^3 per well). Medium specimens were replaced on day 3. The cell number was determined after trypsinization of cells on day 5 or 6 of culture by both Trypan blue staining and Coulter-counter measurements. For AIDS-KS cells, CM from HTLV-II-transformed T cells (HTLV-II CM, dilution 1:4), and for H-UVE cells, ECGS (30 μg ml^{-1}) and heparin (45 μg ml^{-1}), were used as positive controls[2], with values of 35.2×10^4 and 38×10^4, respectively. Before each assay, AIDS-KS cells were maintained for 4–15 days in medium without HTLV-II CM and incubated in serum-free media for 4 h. Experiments were repeated twice with the same CM and several other times with different CM preparations. Similar results were also obtained on other AIDS-KS cell cultures (AIDS-KS1 and AIDS-KS2)[2,4]. *b*, AIDS-KS3 cells were cultured and plated as in *a*. CM or medium alone were preincubated (7 h to overnight) with or without serial dilutions of rabbit anti-Tat polyclonal antibodies (~7 μg μl^{-1}) obtained by immunization with recombinant purified Tat expressed in *E. coli*, and purified by affinity chromatography using purified Tat. Fresh media treated identically were replaced on day 3. The cell number was determined on day 5 or 6 of culture, as described. The experiments were reproduced with a Tat antiserum from the same source and with two different anti-Tat rabbit sera (obtained by immunization with a Tat synthetic peptide (residues 1–61 of N terminus). The effects of CM 12 days after infection are shown. Results were reproduced with other CM preparations (all between 10 to 15 days after infection). *c*, On day 12 post-infection, cells (1×10^6 ml^{-1}) were metabolically labelled with [^{35}S]methionine and [^{35}S]cysteine (150 μCi ml^{-1} each) for 24 h, supernatants were centrifuged (40,000 r.p.m., 1 h, 4 °C) and precleared for 5 h with normal rabbit serum and protein A/Sepharose (Boehringer). Supernatants (4 ml) were incubated overnight with anti-Tat affinity-purified antibodies (dilution, 1:100) and proteins separated by 15% SDS-PAGE[20] (3-day exposure). M_r markers (Rainbow, Amersham) are indicated ($M_r\times10^{-3}$).

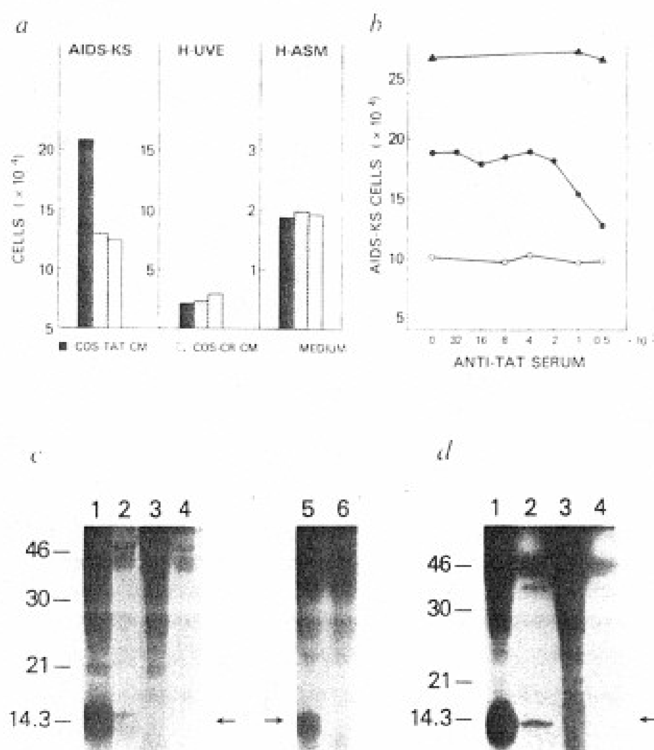

FIG. 2 Role of Tat in growth response of AIDS-KS cells to Tat-transfected COS cells. *a*, Growth response of AIDS-KS cells, H-UVE and H-ASM cells to CM from COS-1 cells transfected with a *tat*-expressing vector (COS-TAT). CM diluted 1:16. *b*, Specific block of AIDS-KS cell growth response to COS-TAT-derived CM by anti-Tat antiserum. ●, COS-TAT-derived CM (1:16); ○, media alone without CM; ▲, HTLV-II-derived CM (1:16). *c*, RIPA of Tat present in cell extract and supernatant from COS-TAT transfected and metabolically labelled cells, and competition assay of extracellular Tat. Lanes 1 and 2, cell extract and supernatant from COS-TAT cells; lanes 3 and 4, cell extract and supernatant from COS-CR cells, respectively, precipitated with anti-Tat antiserum; lanes 5 and 6, supernatants from COS-TAT cells precipitated with anti-Tat antiserum, before (lane 5) and after (lane 6) preincubation of the antiserum with recombinant purified Tat. The arrows indicate the Tat products. *d*, RIPA of Tat in COS-TAT-transfected cells. Lanes 1 and 3, cell extracts; lanes 2 and 4, supernatants from COS-TAT- and COS-CR-transfected cells, respectively, precipitated with affinity-purified anti-Tat antibodies.

METHODS. *a*, Cells were plated (AIDS-KS3, 5×10^4; H-UVE cells, 5×10^4 per $25\,cm^2$ flask; H-ASM cells, 2.5×10^3 per well) and cell number determined on day 5 (AIDS-KS), day 6 (H-ASM) or day 9 (H-UVE). In the experiment shown, the positive controls gave values of 28.5×10^4 for AIDS-KS cells (HTLV-II CM) and 20.8×10^4 for H-UVE cells (ECGS and heparin). COS-1 cells ($5-7 \times 10^6$ cells) were transfected by electroporation[21] with a plasmid expressing *tat* (pcv-tat) or a control plasmid DNA (30 μg each). Ten hours after transfection, cells were split (1:2), washed twice in PBS and incubated (46 h) in RPMI 1640 medium supplemented with 5% dialysed FCS and 1% Nutridoma-HU (Boehringer). CM were collected, centrifuged and stored as described in Fig. 1*a*. Growth assays were repeated once or twice for each transfection experiment (three). The experiments were also reproduced with AIDS-KS1 cells. *b*, Blocking experiment was performed on AIDS-KS3 cells as described in Fig. 1*b* and cells counted on day 6. The antiserum used was raised against a Tat synthetic peptide. The experiment was repeated twice and with a different Tat serum. *c*, *d*, COS-1 cells were transfected as described in *a*. Cells were metabolically labelled 56 h post-transfection for 6–8 h, as described in Fig. 1*c*. Cell extracts (0.5 ml) and CM (1.5 ml for lanes 1 to 4 and 4 ml for lanes 5 and 6) were precleared and incubated overnight with anti-Tat serum (1:100) used for the blocking experiments shown. For the competition assay, the anti-Tat serum (1:100) was preincubated (2 h) with 6 μg recombinant purified Tat (expressed in *E. coli*). Exposure was overnight. The experiments were reproduced with all the different anti-Tat sera available. M_r markers are indicated to the left ($M_r \times 10^{-3}$).

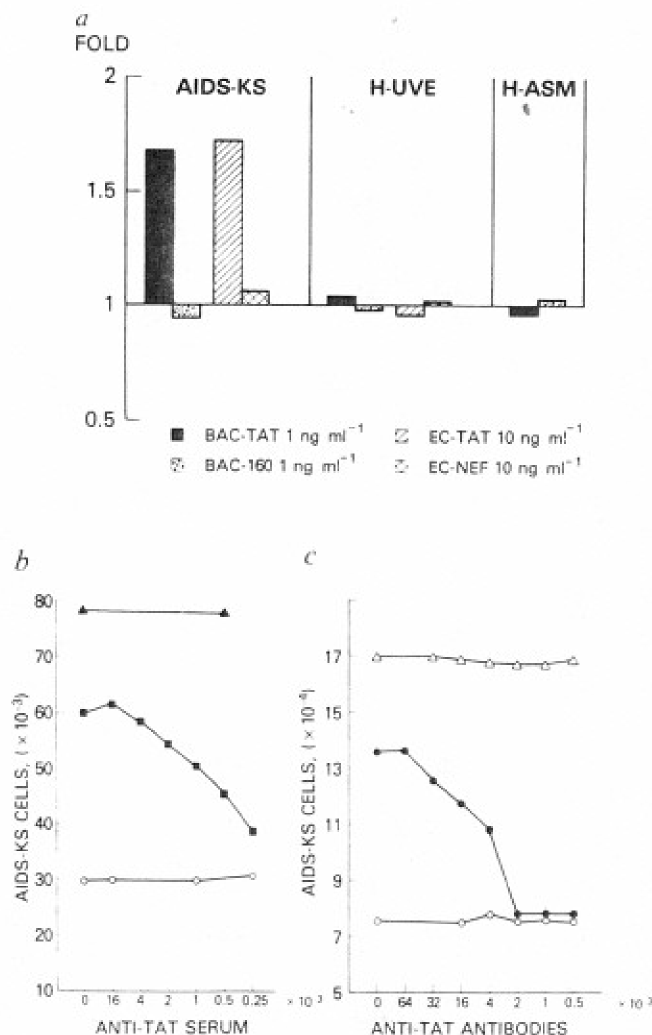

FIG. 3 Specific growth response of AIDS-KS3 cells to recombinant Tat. *a*, Growth response of AIDS-KS3 and control cells to recominant Tat proteins or to recombinant control proteins (gp160 and Nef). BAC-TAT and BAC-160, baculovirus-derived Tat and gp160 proteins at 1 ng ml^{-1}. EC-TAT and EC-NEF, *E. coli*-derived Tat and Nef proteins at 10 ng ml^{-1}. *b*, *c*, Block of AIDS-KS cell growth response to EC-TAT (*b*) or BAC-TAT proteins (*c*) by anti-Tat antibodies. ■, EC-TAT at the final concentration of 10 ng ml^{-1}; ●, BAC-TAT at the final concentration of 1 ng ml^{-1}; ○, media alone without CM; ▲, △, HTLV-II-derived CM (1:16).

METHODS. *a*, BAC-TAT and BAC-160 were expressed in the baculovirus system (Repligen) (~5% of total protein content); EC-TAT and EC-NEF were expressed in the *E. coli* system (Repligen) (~10% of total protein content). DTT (5 mM) was added to the stock protein preparations to prevent oxidation and equivalent dilutions of the same compound were also added to the positive and negative controls. Proteins and media were replaced on day 3. The cell number was determined on day 6 and expressed as fold increase compared with the values of basal cell growth (media alone, onefold). The positive controls gave a twofold increase for AIDS-KS3 cells (HTLV-II CM) and 2.05-fold for H-UVE cells (ECGS and heparin), respectively. Results shown are the average of experiments repeated five times at the same conditions for AIDS-KS3 cells (s.d. values $<5\%$) and twice for H-UVE cells and H-ASM cells. A similar growth response was also obtained with AIDS-KS1 cells, or after 21 days of assay for all cell types. Activity of both recombinant Tat proteins (10 μg) was tested on HeLa cells carrying the HIV-1 LTR-linked chloramphenicol acetyltransferase (CAT) gene or by transient transfections and quantified by CAT assays as previously shown[20]. The recombinant proteins were also tested by western blot analysis. The range of activity in inducing AIDS-KS cell proliferation was 0.5–10 ng per ml final total protein concentration for BAC-TAT with a peak between 1 and 5 ng ml^{-1}, and 1–20 ng ml^{-1} for EC-TAT with a peak between 5 and 10 ng ml^{-1}. Some variability in dose response was observed, generally dependent on aging, storage of proteins and cell culture conditions. Loss of activity is probably due to oxidation of Tat[19]. All the protein preparations were negative for endotoxin (<0.0001 pg μl^{-1}). *b*, *c*, AIDS-KS3 cells were plated at 1×10^4 per 25-cm^2 flask (*b*) or at 5×10^4 per 25-cm^2 flask (*c*). Anti-Tat serum raised against a Tat synthetic peptide was used for EC-TAT, whereas anti-Tat affinity-purified antibodies were preincubated with BAC-TAT. Cell number was determined on day 6. The experiments were repeated twice for each protein preparation. For BAC-TAT, other antibody preparations were also used.

lated with the time of optimal CM growth-promoting effect and with positive blocking effect by anti-Tat antibodies.

To study the proliferative effect induced by Tat and to avoid cell death commonly observed during HIV-1 infection of T cells, we examined the growth-promoting activity in CM of COS-1 cells transfected with *tat*-expressing plasmid (COS-TAT) (Fig. 2*a*). Specific stimulation of AIDS-KS, but not of H-UVE or H-ASM, cell growth by COS-TAT CM could be reproducibly observed, even in longer assays (2 weeks), and specifically blocked by several anti-Tat sera (Fig. 2*b*). The same antibodies also detected Tat in both cell extracts and CM of metabolically labelled COS-TAT cells (Fig. 2, *c* and *d*). Two protein species (M_r ~ 14 K and 16 K) were sometimes detected with several antibodies (Fig. 2*c*), and both bands were completely competed-out by purified recombinant Tat (Fig. 2*c*, lane 5). The doublet may correspond to the two forms of Tat expressed from spliced and unspliced messenger RNA[12].

We next tested the growth effects of recombinant Tat expressed in baculovirus (BAC-TAT) and in *Escherichia coli* (EC-TAT) on AIDS-KS cells and control cells. Both BAC-TAT and EC-TAT induced AIDS-KS cell proliferation, but not growth of control cells (for up to 3 weeks). The optimal concentrations were 1 ng ml^{-1} (BAC-TAT) and 10 ng ml^{-1} (EC-TAT) Fig. 3*a*). The same concentrations of baculovirus-expressed gp160 and *E. coli*-expressed Nef protein failed to induce AIDS-KS cell growth or growth of control cells. In these experiments there was a 1.7-fold increase of AIDS-KS cell number with Tat, and a 2-fold increase with the positive control. In several other experiments, greater stimulation was observed, and the ability of both Tat preparations to induce AIDS-KS cell proliferation was specifically neutralized by preincubation with several anti-Tat sera (Fig. 3*b* and *c*). Because AIDS-KS cells are primary cell cultures of mesenchymal origin and cells of comparable origin (endothelial and smooth muscle cells) normally do not proliferate *in vivo* and need particular conditions to grow *in vitro* (cell culture system and growth factors), the reproducible (about twofold) stimulation of AIDS-KS cells by Tat seems to be significant.

These *in vitro* data show that Tat may provide the primary growth stimulus to AIDS-KS cells, and may have a role *in vivo* in the pathogenesis of HIV-1-associated KS. This does not rule out contribution by other factors at different stages of infection and disease development. Because normal endothelial and smooth muscle cells are not responsive to Tat, the normal KS cell progenitor may require a previous or simultaneous 'priming' event, or both, before becoming Tat-responsive. Alternatively, a different subtype of endothelial or smooth muscle cells, not available at present for *in vitro* study, may be the progenitor of KS spindle cells.

The mechanisms by which Tat is released and promotes growth are unknown. Because cell death is minimal both after infection of H9 cells and in Tat-transfected COS cells, release of Tat is unlikely to be due to cell death. Tat may be a member of the class of proteins that, although lacking a classical signal peptide, are released by cells through an unknown mechanism(s) (interleukin-1α and -1β (refs 13, 14), platelet-derived endothelial cell growth factor[15] and basic and acid fibroblast growth factors[16,17]). Similar to the effects of Tat on AIDS-KS cells, all these cytokines induce their effects on target cells at very low doses (pg–ng).

Exogenous Tat can be taken up by cultured cells, localizes in the nucleus[18] and can depress antigen-mediated T-cell activation response[19], further indicating a role of exogenous Tat in cellular functions. But the amount of Tat necessary for biological effects (μg) is several times that necessary for the growth-promoting effect[18,19], indicating that *in vivo* growth stimulation of some mesenchymal target-cells by Tat may be more biologically relevant.

Our present results show that a viral regulatory gene product can be released as a biologically active protein and directly act as a growth stimulator for cells derived from KS lesions of AIDS patients. Although our *in vitro* data do not definitively establish a general mechanism for the *in vivo* genesis of KS, these and previous results[3] indicate that, by inducing AIDS-KS cell proliferation, Tat may contribute to the induction or progression, or both, of KS in HIV-1-infected individuals, thus representing one of the links between HIV-1 infection and KS. □

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