

crust is sufficient to lead to an 'inverse boudinage' style of folding (Fig. 6).

The total amount of strain is difficult to estimate. If, based on the fit to the coherence data, the upper mantle deforms as a 50-km elastic plate, the amount of shortening obtained by 'unfolding' the 500-km wavelength undulations is at most a few per cent. In the upper-crustal layer, however, the elastic strength is likely to have been exceeded everywhere, such that it deforms as a viscous or plastic layer. The fact that the coherency at 100–200 km (Fig. 5b) involves both positive and negative correlation between gravity and topography means that folding as well as layer thickening has occurred. Thus the strain in the upper crust could be substantial, as is also the case for the lower-crustal decoupling zone.

Testing models for Tibet

This interpretation of the Bouguer gravity and topography data for Tibet in terms of folding of layers in the upper crust and

uppermost mantle permits a reassessment of the merits of the three simple models presented in Fig. 1. Our layered strength profile with a decoupling zone in the lower crust and a rigid upper mantle is more in keeping with the predictions of the Asian subduction (Fig. 1a, c) models than with that of the rigid indenter model (Fig. 1b), in that the upper mantle appears too strong to shorten by the amount needed to double the thickness of the crust and thus must subduct. Either the Indian plate or the Asian lithosphere might be expected to display an effective elastic thickness of ~40–50 km. Thus our rigidity estimate does not distinguish the different polarities of subduction for the models shown in Fig. 1a and c but does preclude a weak upper mantle as in Fig. 1b. It is intriguing to note, however, that the arcuate gravity low centred at 34° N, 90° E (Fig. 2) is very similar to the negative gravity anomalies over oceanic trenches. If the oceanic analogue holds, the fact that the low is convex to the north would imply subduction of the Asian mantle as proposed by Willett and Beaumont⁷. □

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Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma

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Basic fibroblast growth factor (bFGF) and human immunodeficiency virus type 1 (HIV-1) Tat protein synergize in inducing angiogenic Kaposi's sarcoma-like lesions in mice. Synergy is due to Tat, which enhances endothelial cell growth and type-IV collagenase expression in response to bFGF mimicking extracellular matrix proteins. The bFGF, extracellular Tat and Tat receptors are present in HIV-1-associated KS, which may explain the higher frequency and aggressiveness of this form compared to classical Kaposi's sarcoma where only bFGF is present.

KAPOSI'S sarcoma (KS) is an angioproliferative disease which is rare and mild in elderly men of Mediterranean origin (classical KS) but frequent and aggressive in association with HIV-1 infection (acquired immunodeficiency syndrome (AIDS)-KS)^{1,3}. Both forms, however, are characterized by similar histology^{1,3}, including the presence of proliferating spindle-shaped cells considered to be the 'tumour' cells of KS, angiogenesis, inflamma-

tory cell infiltration and oedema. The spindle cells represent a heterogeneous population dominated by endothelial cells mixed with cells of dendritic and monocytic origin⁴.

Several lines of evidence suggest that KS is a cytokine-mediated disease, at least in early stages⁵, and that angiogenic factors and, in particular, basic fibroblast growth factor (bFGF)^{6,7}, play a role in lesion development. For example, spindle cells of endo-

thelial origin derived from KS lesions of AIDS patients (AIDS-KS cells)^{8,10} induce angiogenesis in the chick chorioallantoic membrane assay and, after injection in nude mice, they induce vascular lesions of mouse cell origin closely resembling KS⁹. One of the prominent cytokines produced by AIDS-KS cells is bFGF, which promotes growth of these cells in an autocrine fashion¹⁰ and, after release, stimulates endothelial cell migration, invasion and proliferation^{10,11}, events required for angiogenesis⁶. In fact, antisense oligodeoxynucleotides directed against bFGF messenger RNA block KS cell growth and lesion formation in nude mice¹¹. Because bFGF mRNA is expressed *in vivo* in spindle cells both of AIDS-associated and of classical KS¹², these data suggest that bFGF plays a key role in the pathogenesis of both forms. However, AIDS-KS is more frequent and more aggressive than classical KS, suggesting that other factors act with bFGF in individuals infected with HIV-1. One such factor may be the HIV-1 Tat protein which is released by infected T cells^{13,14}. Some *tat*-transgenic mice develop KS-like lesions⁴³ and extracellular Tat promotes growth, migration, invasion and adhesion of endothelial cells, precursors of KS spindle cells and AIDS-KS cells themselves^{13,17}. However, with normal endothelial cells, the effects of Tat also require earlier cell activation with inflammatory cytokines such as γ -interferon, interleukin-1 and tumour necrosis factor (refs 15–17 and V.F. *et al.*, manuscript submitted). These cytokines induce endothelial cells to acquire features of KS cells, including the spindle morphology and the expression of the integrin receptors $\alpha_5\beta_1$ and $\alpha_v\beta_3$ which, in turn, mediate the effects of extracellular Tat on these cells^{15,16}. The same cytokines are increased in individuals infected with HIV-1^{15,18,19}, and homosexual men, who are at highest risk for developing KS, show evidence of immune activation and may have higher cytokine levels even before HIV-1 infection^{15,20}. Inflammatory cytokines also activate production and release of bFGF from endothelial cells^{21,22}, suggesting a mechanism for the increased bFGF expression in KS.

Here we provide evidence that bFGF and Tat synergize in inducing KS development and that extracellular Tat may represent the factor increasing the frequency and aggressiveness of KS in individuals infected with HIV-1.

bFGF, Tat and integrins in AIDS-KS lesions

To verify whether bFGF and Tat are present in KS, frozen sections from AIDS-associated KS lesions, classical KS lesions and control tissues were stained with affinity-purified antibodies directed against these proteins. Factor VIII-related antigen (FVIII-RA) was monitored to identify vessels and endothelial spindle cells. HIV-1 p24 core antigen was evaluated to identify virus infected cells. bFGF was detected in all AIDS-KS (10/10) and classical KS lesions (2/2) examined in both vessels and spindle cells (Table 1a), whereas bFGF was present only around the vessels in control tissues probably stored in the basement membrane²³ (Fig. 1a). Anti-FVIII-RA antibodies stained the vessels in all tissues (KS and controls) and many of the spindle cells in both AIDS-KS and classical KS (Fig. 1b). The endothelial origin of these spindle cells was also confirmed by using antibodies directed against cadherin-5, CD34, EN-4 and ELAM-1 (data not shown). These results indicated that bFGF is expressed by spindle cells of both AIDS-associated and classical KS and suggested that most of the bFGF-producing spindle cells are endothelial.

The same tissues were then stained with anti-Tat and anti-p24 antibodies. Tat was detected in 7/10 and p24 in 4/7 AIDS-KS lesions examined, but not in classical KS or control tissues (Table 1a). Stronger Tat staining was associated with more advanced lesions and was localized more often in spindle cells whereas p24 was mostly positive in mononuclear cells (Fig. 1c, d). This confirmed previous data from *in situ* hybridization on the presence of cells infected with HIV-1 in AIDS-KS²⁴ and raised the question of the nature of the Tat-positive spindle cells. In particular, these cells may be uninfected cells that have taken

up Tat released by infected cells, as found *in vitro* with KS spindle cells and normal endothelial cells¹⁴ or in mice injected with Tat²⁵. To verify this, an AIDS-KS lesion was doubly stained with anti-Tat and anti-FVIII-RA, -CD14, -CD4, -p24 or -HIV-1 reverse transcriptase (RT) antibodies combined. Over 50% of the total Tat-positive cells (22%) were uninfected endothelial spindle cells (Table 1b). Spindle-shaped and mononuclear HIV-1-infected macrophages and infected lymphocytes represented 10%, 5% and 33% of the total Tat-positive cells, respectively. Other HIV-1-infected mononuclear cells (9%) (CD14+, p24+ and RT+) did not express detectable Tat (data not shown). Thus, Tat released by infected cells^{13,14} is taken up by KS spindle cells *in vivo*, as found *in vitro*¹⁴.

Uptake of Tat by KS cells or cytokine-activated endothelial cells appears to be mediated by the same integrin receptors (A. Holmes *et al.*, manuscript in preparation) that are highly expressed by KS spindle cells and are inducible by inflammatory cytokines on endothelial cells¹⁶. These integrins, $\alpha_5\beta_1$ and $\alpha_v\beta_3$, function as the receptors for Tat and mediate its effects on these cell types by binding to the RGD (arginine-glycine-aspartic acid) sequence of the protein¹⁶, as they normally do with extracellular matrix (ECM) proteins, such as fibronectin (FN) and vitronectin (VN)²⁶. The same integrins are expressed by endothelial cells *in vivo* during angiogenesis induced by bFGF or inflammatory cytokines²⁷. Therefore, KS lesions and control tissues were stained with antibodies recognizing the RGD-binding region of the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ receptors¹⁶ alone or combined with anti-Tat antibodies. Both integrins were found to be highly expressed by spindle cells and vessels of AIDS-KS (Table 1c). Classical KS showed lower positivity with prominent $\alpha_v\beta_3$ staining, whereas in control tissues only some vessels were positive. In addition, Tat-positive spindle cells of AIDS-KS also stained for β_1 or β_3 integrins confirming the presence of both Tat and its receptors on the same cells.

bFGF induces angiogenic KS-like lesions

To determine whether bFGF can promote the histological changes found in KS, increasing amounts of the protein were injected subcutaneously into nude mice and compared to the effect of AIDS-KS cells (Table 2 and Fig. 2a). Human umbilical vein endothelial (H-UVE) cells, bovine serum albumin (BSA), and buffer or media were used as negative controls (Table 2). bFGF, but not BSA or buffer, induced the formation of macroscopic vascular lesions at the site of injection in a dose-dependent manner (Fig. 2a and Table 2). Specifically, 0.1 μ g bFGF induced only microscopic alterations, 1 μ g bFGF induced macroscopic lesions in 44% of the animals and 10 μ g or greater amounts induced macroscopic lesions in 100% of the mice. Lesions were very similar to those induced by AIDS-KS cells, both developing within 3–4 days and reaching their maximum size by day 6–7. Histologically, both lesions presented alterations characteristic of early stage KS such as angiogenesis, spindle cell proliferation, oedema and inflammatory cell infiltration (Table 2 and Fig. 2A). The frequency and the intensity of these features depended on the amount of bFGF inoculated (Table 2), except for acute (neutrophilic) and chronic (mononuclear) inflammatory cell infiltration and oedema which were always less prevalent with bFGF compared to AIDS-KS cells (Table 2). This is consistent with data indicating that AIDS-KS cells also produce cytokines mediating inflammatory effects¹⁰.

bFGF and Tat synergize to induce KS-like lesions

Because bFGF is expressed at high levels both in AIDS-associated and classical KS^{10,12} (Table 1 and Fig. 1), it is likely that this cytokine contributes to the histogenesis of both forms. However, AIDS-KS is more frequent and aggressive than classical KS. As Tat and bFGF are both present in AIDS-KS (Table 1 and Fig. 1), the role of Tat in KS lesion formation was investigated by injecting nude mice with Tat protein alone or combined with bFGF (Table 3). In the absence of bFGF, Tat stimulated histo-

logical alterations similar to those found with 0.1 µg bFGF (Fig. 2Ba, b). These alterations were evident with 1–10 µg Tat and did not increase with higher doses (up to 100 µg; data not shown). This is consistent with the requirement of other factors (that is, inflammatory cytokines) inducing the receptors for Tat^{14–17} and with recent findings indicating that the effects of

Tat on KS and endothelial cells may be mediated by mechanism(s) other than the direct transactivation of gene expression, which is dose-dependent and requires much higher concentrations of the protein^{13–17,28}. However, when Tat and bFGF were injected simultaneously, both the incidence of macroscopic lesions and the histopathology markedly increased as compared

TABLE 1 Staining of bFGF, FVIII-RA, HIV-1 Tat and p24 (a), characterization of Tat-positive cells (b) and $\alpha_5\beta_1$, $\alpha_v\beta_3$ integrin (Tat) receptor expression (c) in AIDS-KS, classical KS and control tissues

(a) Staining results

Specimen	Average % (range) positive cells			
	bFGF*	FVIII*	TAT	p24
AIDS-KS				
Lymph node	28 (20–34)	34 (21–45)	22 (13–31)	24 (11–33)
Skin				
1	19 (17–20)	39 (35–44)	27 (12–38)	23 (21–25)
2	22 (16–30)	47 (30–78)	18 (12–32)	13 (10–15)
3	33 (21–42)	49 (27–67)	12 (6–18)	16 (11–21)
4	27 (23–31)	41 (36–47)	—	—
5	4 (2–6)	10 (6–13)	—	—
6	15 (10–28)	68 (59–76)	7 (3–10)	ND
7	29 (21–43)	51 (41–58)	12 (10–13)	ND
8	7 (5–9)	39 (34–44)	8 (5–11)	ND
9	10 (6–14)	18 (14–22)	—	—
Classical KS				
Skin				
1	28 (26–30)	41 (33–50)	—	—
2	9 (7–10)	30 (24–34)	—	—
Controls				
Tonsil				
1	—	—	—	—
2	—	—	—	—
Skin				
1	—	—	—	—
2	—	—	—	—
3	—	—	—	—

(b) AIDS-KS lesion (lymph node)

Tat-Positive Cells % (Range)	Phenotype FVIII-RA	CD14	CD4	p24	RT	Morphology	HIV-1 infection
11% (7–16)	+	—	—	—	—	Spindle	—
2% (0.5–3)	—	+	+	+	+	Spindle	+
7% (5–9)	—	—	+	+	+	Mononuclear	+
1% (0–2)	—	+	+	+	+	Mononuclear	+

(c) Average % (range) of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ positive spindle cells and double positive for Tat†

Specimen†	α_5	β_1	α_v	β_3	β_1 /Tat	β_3 /Tat	Tat
AIDS-KS	24 (13–35)	15 (10–34)	41 (21–52)	50 (34–69)	5 (3–7)	12 (10–13)	14 (9–19)
Classical KS	3 (1–7)	3 (1–13)	24 (15–32)	25 (13–48)	—	—	—
Tonsil	—	—	—	—	—	—	—
Control skin	—	—	—	—	—	—	—

Frozen sections from skin and tonsil of individuals not infected with HIV-1, from involved skin and lymph node of AIDS-KS patients and from involved and uninvolved skin of classical KS patients were fixed in cold acetone and single- or double-stained by the alkaline-phosphatase-anti-alkaline phosphatase (APAAP) and/or the peroxidase-anti-peroxidase (PAP) methods^{14,16} by using affinity-purified mouse monoclonal or rabbit polyclonal antibodies alone or combined (APAAP revealed the monoclonal and PAP the polyclonal antibodies). For single-staining with APAAP, slides were incubated (20 min, room temperature) with rabbit anti-mouse antibodies (1:25) (Dako, Glostrup, Denmark). After washing, the APAAP complex (1:25) was applied for 20 min. The second and third steps were repeated twice and the conjugate was preadsorbed with standard immunoglobulins (Boehringer-Mannheim Standards). For PAP and double-stainings (APAAP/PAP) the universal Dako double-stain kit system 40 was used by manufacturer protocol. Monoclonal antibodies were: anti-bFGF (1:200) (Promega); anti-FVIII-RA (1:100) (Dako); anti-Tat¹⁴ (1:250); anti-Tat (1:1,000) and anti-RT (1:50) (American Biotechnology); anti-p24 (1:5), anti-CD14 (1:20) and anti-CD4 (1:10) (Dako); anti- α_5 (CDW49e), anti- β_1 (CD29), anti- α_v (CD51) and anti- β_3 (CD61) (1:50) (AMAC, Cambridge, MA). Polyclonal antibodies were: anti-FVIII (1:500) (Dako), anti-CD4 (1:250) and anti-p24 (1:250) (American Biotechnology) and anti-Tat¹⁴ (1:500). All antibodies were characterized for specificity before use. Stainings were done with each antibody directed against the same antigen with identical results. All combinations of monoclonal and polyclonal antibodies were used for double stainings. Reported are the average results and the range (minimal and maximal values) of the percent of positive cells in 3–10 high-power microscopic fields from 3 or more experiments. Results were also confirmed by immunofluorescence.

* Vessels stained positive for FVIII and bFGF in all tissues.

† Specimens were 2 AIDS-KS (lymph node and skin), 4 classical KS (skin), 1 tonsil, 2 normal skin.

‡ All positive cells had spindle morphology. Most vessels were positive for both integrins with prevalence for $\alpha_v\beta_3$ in all KS tissues analysed with highest positivity in AIDS-KS; some vessel positivity was seen in control tissues.

ND, Not done; (—), negative; (+), positive.

with each protein alone (Table 3 and Fig. 2Bc). For example, when Tat (10 µg) and bFGF (0.1 or 1 µg) were combined, macroscopic lesions developed in 35% and 71% of the mice, respectively, reaching effects similar to 1 µg and 10 µg bFGF alone (Table 3). Tat also increased the intensity of the histological alterations (particularly spindle cell growth, angiogenesis

and oedema) for each dose of bFGF inoculated, reaching values generally observed with about 10-fold higher bFGF concentrations (Table 3 and Fig. 2B). In contrast, heat-inactivated Tat had no synergistic effects with bFGF (0.1 or 1 µg) (Table 3). Similarly, synergy was blocked by anti-bFGF or anti-Tat antibodies (Table 3). Because after each injection in mice Tat was

TABLE 2 Injection of bFGF in nude mice induces KS-like lesions in a dose-dependent fashion

bFGF	Lesion*	Angiogenesis†	Spindle cells†	Acute inflammation†	Chronic inflammation†	Oedema†
0.1 µg	0% (47)	32% (1)	75% (1)	50% (1)	60% (1)	75% (1)
1 µg	44% (43)	86% (2.7)	95% (3)	62% (1)	62% (2)	76% (2)
10 µg	100% (11)	100% (5)	100% (5)	70% (1)	100% (2)	100% (2)
30 µg	100% (19)	100% (6)	100% (6)	100% (1)	100% (2)	100% (2)
90 µg	100% (10)	100% (7)	100% (7)	100% (1)	100% (2)	100% (2)
BSA 90 µg	0% (2)	0%	0%	0%	0%	0%
Buffer	0% (36)	0%	0%	0%	0%	0%
AIDS-KS cells 3 × 10 ⁶	100% (10)	100% (4)	100% (4)	100% (6)	100% (4)	100% (3)
H-UVE cells 3 × 10 ⁶	0% (6)	0%	0%	0%	0%	0%

Recombinant purified human bFGF (Boehringer-Mannheim), BSA (Sigma), AIDS-KS cells or H-UVE cells (3×10^6)⁸⁻¹⁰ were injected subcutaneously into the lower back (right side) of BALB/c nu/nu athymic mice⁹, whereas their negative controls (protein buffer [PBS, 0.1% BSA] or media) were injected into the left side of the same mice. To increase efficiency, proteins, cells or buffer (200 µl) were mixed with 200 µl of matrigel (Collaborative Research, Bedford, MA)⁴¹ before inoculation. Mice were killed at 6–7 days after injection. With bFGF or AIDS-KS cells, lesions developed within 3–4 days reaching maximal size (4 × 5 to 9 × 12 mm) by day 6–7, whereas none of the 86 animals inoculated with the negative controls (buffer or media in matrigel) developed lesions. Tissue samples were fixed in formalin and analysed microscopically after a haematoxylin-eosin (H and E) staining. The histological changes observed at the site of injection, blood vessel formation, spindle cell proliferation, acute (neutrophilic) and chronic (mononuclear) inflammatory cell infiltration and oedema, were evaluated by comparison with the negative controls and graded according to intensity with values ranging from 1–7 with the minimal alteration observed given a value of one (intensity value). Each intensity value reported is the average result from all mice presenting that alteration per experimental condition. Vascular lesions and histological changes were also observed in the absence of matrigel.

* Percentage of mice developing vascular macroscopic lesions. The number of mice inoculated is in parentheses.

† Percentage of mice inoculated developing histological alterations. Average 'intensity' value for each histopathological feature observed per experimental condition is in parentheses.

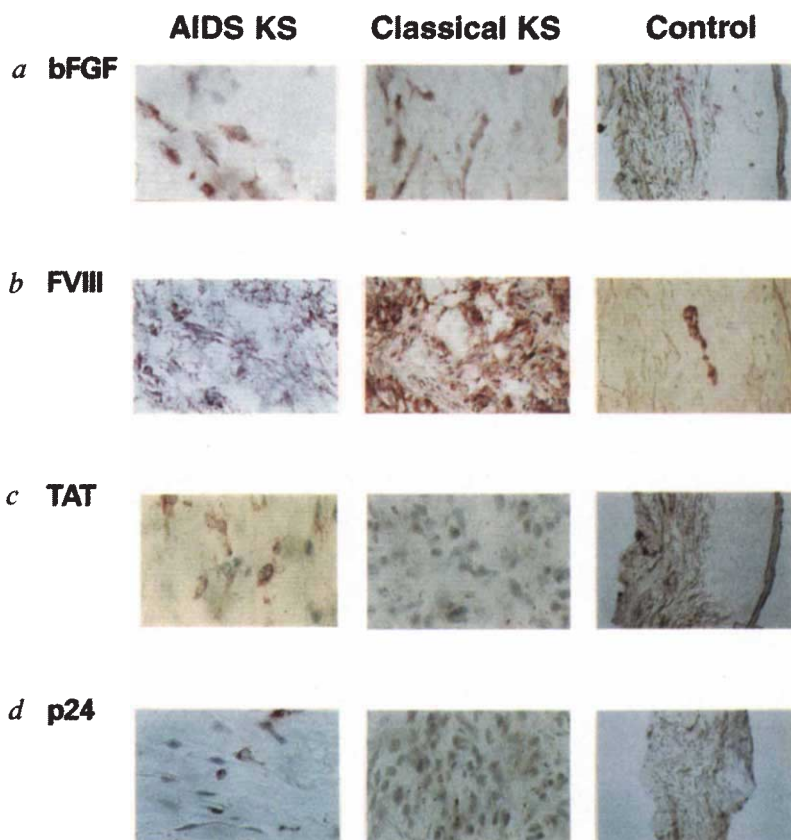
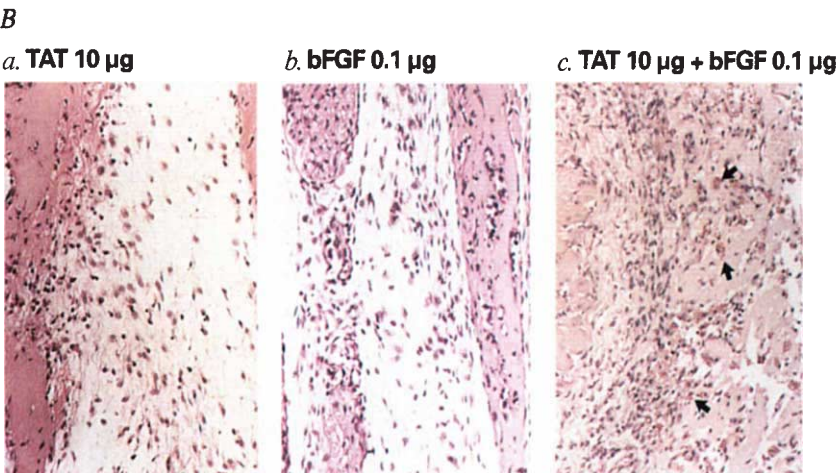
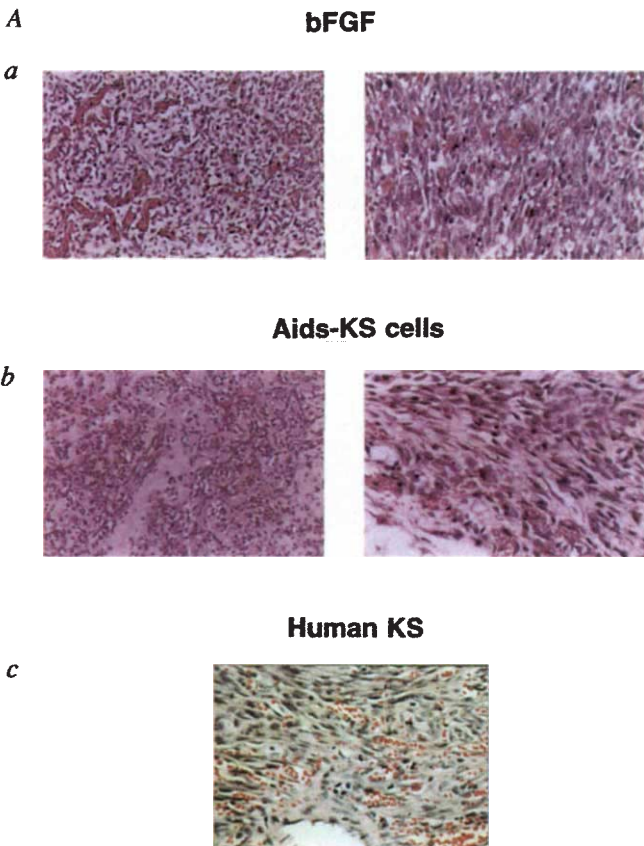


FIG. 1 bFGF, FVIII-RA, HIV-1 Tat and HIV-1 p24 staining in AIDS-KS, classical KS and control tissues. a, bFGF, b, FVIII-RA, c, TAT and d, p24 staining of a representative AIDS-KS skin lesion (left panels), classical KS skin lesion (middle panels) and control skin (right panels), respectively. Shown are examples of the staining with the antibodies reported in Table 1a. Magnifications are ×22 or ×54 for KS lesions and ×5.4 or ×22 for control skin. For methodology see the legend to Table 1.

fully active, these data indicate that biologically active protein is needed for the synergistic KS-promoting effect with bFGF and that the effects of both Tat and bFGF are specific.

To investigate the mechanism of this synergistic effect, bFGF, Tat or buffer were injected on day 0 and the other protein of the combination was inoculated in the same sites 2 days later. When bFGF was injected before Tat, synergy was more evident than the simultaneous inoculation, reaching 45% and 100% of lesion formation with 0.1 or 1 μg bFGF and Tat, respectively. In contrast, when Tat was injected on day 0 and bFGF on day 2, no synergy occurred (supplementary information available non request). This indicates that bFGF is essential for lesion formation and that Tat is the factor enhancing bFGF activity. This observation may partially explain the high frequency of KS in homosexual men who may have increased levels of inflammatory cytokines activating bFGF expression^{21,22} even before HIV-1 infection^{15,18-20}.



Tat mimics the effect of ECM molecules

There may be several mechanisms by which Tat enhances bFGF activity. Endothelial cells require two signals in order to grow during angiogenesis: one from angiogenic factors, such as bFGF, which trigger growth and integrin expression, and one from ECM molecules, such as FN, which induce cell adhesion and modulate cell responsiveness to bFGF^{27,29}. FN regulates endothelial cell growth (both basal and bFGF-induced) by modulating cell shape and by activating intracellular signalling pathways mediated through its RGD sequence and the $\alpha_5\beta_1$ receptor²⁹. Our previous data indicated that, like ECM molecules, Tat mediates vascular and AIDS-KS cell adhesion by the interaction of its RGD sequence with the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$, the receptors for FN and VN, respectively¹⁶, and that this effect is increased by the basic region of the protein which interacts with heparin complexes of the cell surface (ref. 16 and our unpublished data).

To determine whether Tat could mimic the effect of ECM molecules on endothelial cell growth, H-UVE cells were seeded onto non-adhesive plates coated with Tat (0.01, 0.1 or 1 $\mu\text{g ml}^{-1}$), 0.1% BSA (negative control) or 30 $\mu\text{g ml}^{-1}$ FN (positive control). The day after, the percentage of adherent cells was counted, and then [³H]thymidine incorporation was evaluated 48 h after the addition of bFGF (0.01, 0.1 or 1 ng ml^{-1}) or buffer. Tat could support cell adhesion and spreading in a dose-dependent fashion and at 1 $\mu\text{g ml}^{-1}$ reached the effect of equimolar concentrations of FN (Fig. 3Aa). Tat also increased both basal and bFGF-induced cell growth at the same levels observed with FN (Fig. 3Ab, c). Thus, Tat can support endothelial cell adhesion, spread and growth, and can increase the cell growth response to bFGF mimicking the effect of FN²⁹. As Tat receptors are expressed by endothelial and spindle cells of KS (Table 1c) and Tat can compete against endothelial and KS spindle cell adhesion to FN and VN¹⁶, these results suggest that Tat can exert these activities *in vivo*.

FN and TAT have similar effects *in vivo*

To determine whether FN could induce effects similar to Tat *in vivo*, the molecule (30 μg) was injected into mice alone or with bFGF (0.1 or 1 μg). As found with Tat, FN alone did not induce lesions in mice; however, in the presence of bFGF, lesions developed in 40% and 80% of the mice, respectively (supplementary information available on request). When bFGF was injected on day 0 and FN on day 2, lesion development was further enhanced to 60% and 100% of the mice, respectively. These results indicated that Tat can mimic the effect of ECM proteins such as FN both *in vitro* and *in vivo*.

FIG. 2 A, Injection of bFGF into nude mice induces vascular lesions closely resembling the lesions induced by AIDS-KS spindle cells or primary human KS. Different fields ($\times 200$ and $\times 400$ magnifications) of the histology of the mouse lesions induced by bFGF (a) or AIDS-KS cells (b), compared to a human primary KS lesion (c) ($\times 400$ magnification). Slides were stained by H and E. See the legend to Table 2 for methodology. Angiogenesis and spindle cells are evident in all panels, whereas oedema and inflammatory cell infiltration are more pronounced after injection of AIDS-KS cells (b) and in human KS (c). B, Tat and bFGF have synergistic effects in inducing KS-like lesions. H&E staining of representative tissues samples from mice injected with a, 10 μg Tat; b, 0.1 μg bFGF; c, 10 μg Tat and 0.1 μg bFGF. Magnifications, $\times 86$. See the legends to Tables 2 and 3 for experimental procedures. Arrows point to angiogenesis. In the presence of Tat and bFGF combined, both the frequency and the intensity of the histological alterations (particularly angiogenesis, spindle cell growth and oedema) are increased as compared to each protein alone.

Activation of type-IV collagenase expression

Picomolar concentrations ($10\text{--}20\text{ ng ml}^{-1}$) of Tat induce endothelial cells to migrate and to invade the basement membrane and this is associated with degradation of collagen IV¹⁷. The degradation of the basement membrane can be mediated by several metalloproteinases^{30,31}. Among them we investigated type-IV collagenase mRNA encoding the 72K species because this form is involved in angiogenesis and tumour invasion³¹, and is induced by bFGF³². Tat enhanced collagenase mRNA expression in endothelial cells which peaked at 10 ng ml^{-1} Tat (2-fold increase; Fig. 3Ba). Phorbol-myristyl-acetate (PMA) (positive control)³³ resulted in a 2.8-fold activation (Fig. 3B). The effect of Tat (10 ng ml^{-1}) was increased synergistically by the presence of 0.1 or 1 ng bFGF (Fig. 3Bb), and reached 2.6- and 7.2-fold increase compared to bFGF alone which induced 1.2- and 3-fold activation, respectively (Fig. 3Bb). Thus, bFGF and Tat synergize in inducing collagenase IV gene expression in endothelial cells.

The most likely mechanism for Tat-activation of type-IV collagenase is the binding to the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins¹⁶ which activate this enzyme³⁴⁻³⁶. In contrast, direct transactivation of gene expression by Tat requires higher concentrations of extracellular protein^{14,28} than those needed for stimulating collagenase expression and endothelial cell invasion. Because collagenase IV is involved in the degradation of the ECM and because the effect of Tat is synergistic with bFGF, the data suggest another pathway by which bFGF and Tat may have synergistic KS promoting effects *in vivo*.

The data presented here indicate that both Tat and bFGF are present in AIDS-KS lesions and that they synergize in inducing angiogenesis and histological alterations typical of KS¹⁻⁵. As Tat is released from infected cells^{13,14} and its receptors (integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$)¹⁶ are expressed by spindle cells and endothelial

cells of KS lesions, Tat may be the factor responsible for the higher frequency and aggressiveness of KS in individuals infected with HIV-1 as compared to the classical form of KS in which only bFGF is present.

Tat synergizes with bFGF by at least two pathways: increased endothelial cell growth and invasion in response to bFGF, mimicking the effect of ECM proteins such as FN and VN.

A possible explanation for the lack of *in vivo* effect of Tat or FN injected alone in mice is that their receptors are already occupied by endogenous ECM proteins. However, simultaneous injection of bFGF triggers angiogenesis and integrin expression²⁷, increasing the availability of the receptors for these proteins which now are able to bind and to increase bFGF activity in a synergistic fashion. In fact, injection of bFGF before Tat or FN further increases lesion development. As bFGF and Tat are both present in AIDS-KS lesions and integrins are over-expressed in both vessels and spindle cells, it is likely that synergy by Tat occurs in individuals infected with HIV-1.

It is still unclear why KS is more frequent in infected homosexual males compared to other infected people. This could be due to greater immune activation and consequent inflammatory cytokine production in these individuals¹⁸⁻²⁰. Inflammatory cytokines induce endothelial cells to acquire the features of the KS cell phenotype, including morphology¹⁵, bFGF production and release^{21,22}, induction of Tat receptors and responsiveness to its angiogenic effects¹⁵⁻¹⁷. Furthermore, cytokine-activated endothelial and KS spindle cells express adhesion molecules which mediate contact with T cells, macrophages, and dermal dendrocytes^{4,37,38}, all of which are targets of HIV-1 infection^{24,39,40}. Because infected cells release Tat and in AIDS-KS lesions they are in contact with spindle cells, extracellular Tat may cooperate with bFGF in increasing KS lesion formation and progression. □

TABLE 3 bFGF and the HIV-1 Tat protein have synergistic angiogenic and KS-like promoting effects

	Lesion*	Angiogenesis†	Spindle cells†	Acute inflammation†	Chronic inflammation†	Oedema†
Tat 10 μg + buffer	0% (17)	41% (1)	94% (1)	57% (1)	100% (1)	94% (1)
bFGF 0.1 μg + buffer	0% (47)	32% (1)	75% (1)	50% (1)	60% (1)	75% (1)
Tat 10 μg + bFGF 0.1 μg	35% (31)	58% (3)	84% (2)	80% (1)	93% (1)	81% (2.5)
bFGF 0.1 μg + heat-inactivated Tat 10 μg	0% (5)	60% (1)	60% (2)	60% (1)	60% (1)	60% (1)
bFGF 0.1 μg + Tat 10 μg + anti-Tat antibodies	0% (10)	60% (1)	80% (1)	80% (1)	80% (1)	80% (1)
bFGF 0.1 μg + anti-bFGF antibodies + Tat 10 μg	0% (5)	20% (1)	40% (1)	40% (1)	40% (1)	60% (1)
bFGF 1 μg + buffer	44% (43)	86% (2.7)	95% (3)	62% (1)	62% (2)	76% (2)
Tat 10 μg + bFGF 1 μg	71% (31)	94% (3.5)	100% (4)	100% (1)	100% (2)	100% (3)
bFGF 1 μg + heat-inactivated Tat 10 μg	40% (5)	80% (2)	80% (3)	80% (1)	80% (2)	80% (2)
bFGF 1 μg + Tat 10 μg + anti-Tat antibodies	40% (5)	40% (2)	80% (3)	60% (1)	60% (2)	80% (2)
bFGF 10 μg + buffer	100% (11)	100% (5)	100% (5)	70% (1)	100% (2)	100% (2)
Tat 10 μg + bFGF 10 μg	100% (2)	100% (6)	100% (6)	100% (1)	100% (3)	100% (5)
Buffer + buffer	0% (70)	0%	0%	0%	0%	0%

Tat (10 μg), heat-inactivated Tat (10 μg), increasing amounts of bFGF (0.1, 1, 10 μg), Tat/anti-Tat antibodies, bFGF/anti-bFGF antibodies or protein buffer (with or without antibodies) were injected alone or in combination into mice as described in the legend to Table 2. METHODS: recombinant purified Tat protein, expressed in *E. coli*, was resuspended in the same buffer used for bFGF, handled and tested for activity as previously described¹⁴. As Tat oxidizes easily and loses biological activity¹³⁻¹⁷, the protein was tested¹⁴ before and after each injection to insure that fully active Tat was inoculated. Tat protein was inactivated by incubation at 56°C for 5 h. Biological activity was 5-7% of the initial activity. To block Tat activity, affinity-purified anti-Tat polyclonal antibodies^{13,14} (300 μl at $1.7\text{ }\mu\text{g }\mu\text{l}^{-1}$) were incubated with 10 μg of Tat (at 4°C , in the dark) for 6-12 h with gentle shaking. Antibodies incubated in protein buffer and Tat alone were used as negative and positive controls, respectively. After incubation, bFGF and matrigel were added to the tubes and the mixtures injected in mice. *In vitro* testing¹⁴ indicated that more than 96% of Tat activity was blocked by the antibodies. To block bFGF, 1 mg neutralizing affinity-purified anti-bFGF polyclonal antibodies (R&D Systems, Minneapolis, MN) were incubated with 0.1 μg bFGF in 300 μl total volume, or with buffer. Then Tat and matrigel were added and mixtures inoculated in mice. A second injection of the antibody (1 mg) was done in the same sites at day 2. Antibodies had no effects when injected alone (data not shown).

* Percentage of mice developing macroscopic vascular lesions. In parentheses, number of mice inoculated.

† Percentage of mice inoculated developing histological alterations. In parentheses, average 'intensity' value for each histopathological feature observed per experimental condition.

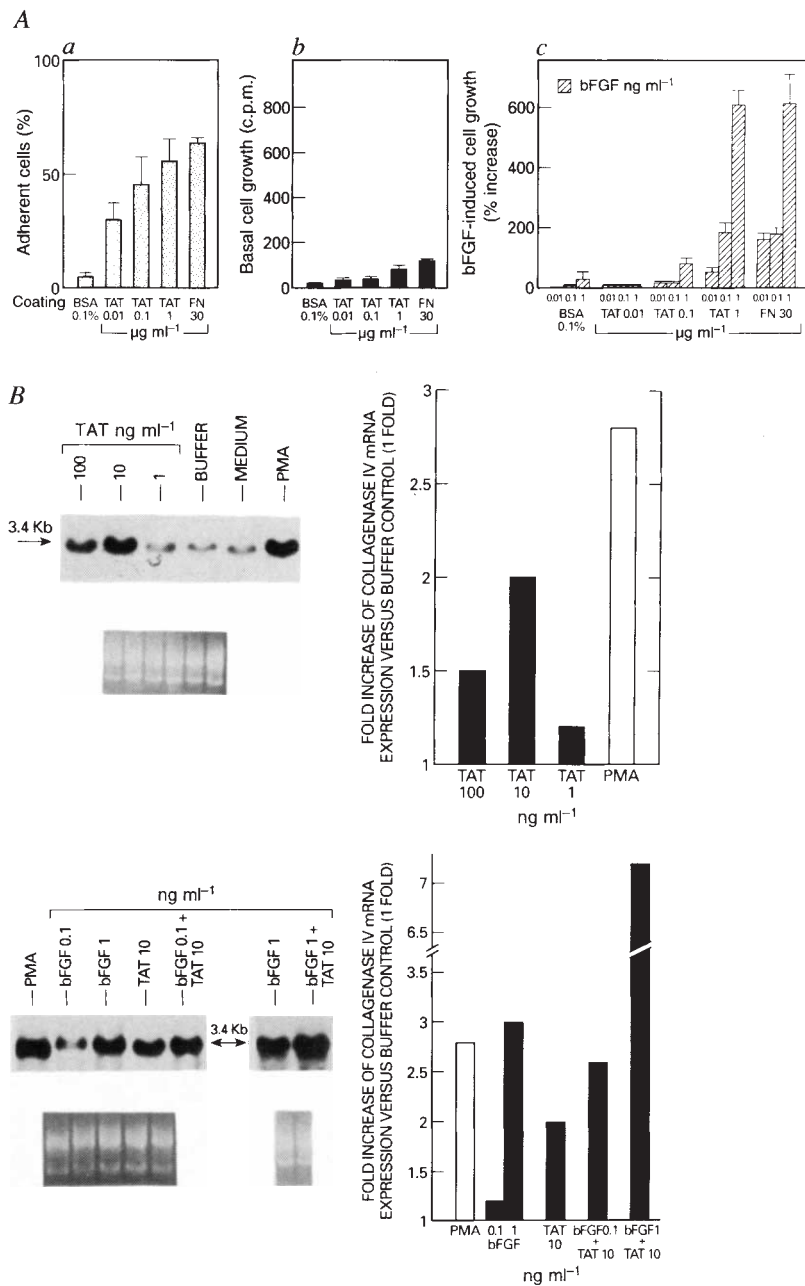


FIG. 3 A, Tat supports endothelial cell adhesion and increases growth to bFGF mimicking the effect of FN. a, Percentage adhesion; b, basal growth (c.p.m.); and c, bFGF-induced growth (percent increase) of H-UVE cells seeded onto BSA-, Tat- or FN-coated plates (average of 3 experiments). b, HIV-1 Tat protein activates endothelial type-IV collagenase expression. b, Synergistic collagenase IV activation by bFGF and Tat. Shown are northern blots (left panels) and fold-increase of collagenase IV mRNA (over control buffer) by densitometry (right panels) with H-UVE cells treated with Tat (100, 10 or 1 ng ml^{-1}), buffer, medium or PMA (20 ng ml^{-1}) (a); bFGF (0.1 and 1 ng ml^{-1}) and Tat (10 ng ml^{-1}), alone or combined, and PMA (b). Ethidium-stained gels are shown to confirm equivalent RNA amounts.

METHODS. H-UVE cells (p4-p9) (Cell System Inc., Kirkland, WA) were cultured as described previously^{8,10,13-17}. Non-tissue culture-treated 96-well plates (Linbro, Flow Laboratories, McLean, VA) were coated with 100 μl PBS-BSA 0.1% with or without Tat (0.01, 0.1 or 1 $\mu\text{g ml}^{-1}$) or FN (30 $\mu\text{g ml}^{-1}$) in the dark (4 °C). After 16–20 h, plates were washed, 200 μl PBS-BSA 1% added for 1 h (4 °C) and 30 min (room temperature), and washed again. Cells (8×10^2) were plated in medium alone, adherent cells counted at 24 h, and cell growth evaluated 48 h after the addition of [³H]thymidine (1 μCi per well) and BSA (0.1%) or bFGF (0.01, 0.1 or 1 ng ml^{-1}). Tat, bFGF, buffer or medium were added to H-UVE cells for 12–14 h and PMA (20 ng ml^{-1}) for 5–7 h. RNA was extracted and analysed by northern blot (10 μg for each lane)¹⁰ by using a ³²P end-labelled oligodeoxynucleotide corresponding to the sequence +59 to +99 (5'-AACTCTTTGTCCGTTTGGG-GGCGACATCGCCGGGAACT-3') of collagenase IV cDNA encoding for the 72K form⁴², which detects a 3.4 kilobase transcript. Experiments were repeated 3–6 times.

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