HIV-1 Transactivator Protein Tat Induces Proliferation and TGFβ Expression in Human Articular Chondrocytes

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Abstract. The human immunodeficiency virus-1 (HIV-1) protein Tat binds to cell surface antigens and can regulate cellular responses. Tat has similar immunosuppressive effects as transforming growth factor-β (TGFβ) and both inhibit lymphocyte proliferation. TGFβ is expressed by primary human articular chondrocytes and is their most potent growth factor. The present study analyzed the interactions of TGFβ and HIV Tat in the regulation of human articular chondrocytes. Synthetic or recombinant full-length Tat (1-86) induced chondrocyte proliferation and this was of similar magnitude as the response to TGFβ. Tat peptides that did not contain the RGD motif had similar chondrocyte stimulatory activity as full-length Tat. Among a series of Tat peptides, peptide 38-62 which contains the basic domain was the only one active, suggesting that this region is responsible for the effects on chondrocyte proliferation. Full-length Tat and peptide 38-62 synergized with TGFβ and induced proliferative responses that were greater than those obtained with any combination of the known chondrocyte growth factors. Further characterization of the interactions between Tat and TGFβ showed that Tat increased synthesis and TGFβ activity and TGFβ1 mRNA levels. The stimulatory effects of Tat and peptide 38-62 on chondrocyte proliferation were reduced by neutralizing antibodies to TGFβ and by TGFβ antisense oligonucleotides. These results identify a virally encoded protein and a synthetic peptide derived from it as novel and potent chondrocyte growth stimuli which act at least in part through the induction of TGFβ.

HIV-1 Tat is a virally encoded regulatory protein which stimulates HIV gene expression (8) through binding to a specific binding motif, tar, in the HIV promoter located in the long terminal repeat (LTR) (4). Tat is encoded by two exons and the mature protein contains 86 amino acids. Domains that are functionally important in HIV replication have been mapped to the first 72 amino acids encoded by exon 1 and include an acidic, a basic, and a cysteine-rich domain (9). The acidic NH2-terminal domain has been proposed to function as activation domain; the region between amino acids 22 and 37 contains 7 cysteine residues that are thought to mediate dimer formation and metal binding; and a basic domain between amino acids 49 and 57 is required for nuclear and nucleolar localization and binding to tar (5). The COOH-terminal 14 amino acids that are encoded by exon 2 do not appear to be essential in HIV replication but contain the RGD sequence which is a motif present in extracellular matrix proteins and involved with binding to cell surface adhesion receptors (2, 28). Tat requires interaction with cellular proteins to express its maximal effect on HIV replication and several intracellular proteins that participate in interactions with Tat have been identified (6, 14-18, 20, 23, 31, 32). Tat can activate the HIV promoter when it is synthesized in the same cell that contains the HIV LTR. In addition, a paracrine mechanism has been suggested that involves synthesis and secretion of Tat, binding to the cell surface, internalization and stimulation of the HIV LTR (12). A paracrine function of Tat may also be responsible for the development of Kaposi's sarcoma-like lesions in Tat transgenic mice which occurred in the absence of intracellular Tat-expressing cells (41). Furthermore, proliferation of Kaposi's sarcoma cells in vitro was increased by Tat (7). In contrast, growth-inhibitory effects have been reported in lymphocytes where Tat reduced antigen-induced T cell responses (38).

Mechanisms responsible for these Tat effects are not completely characterized. Binding of Tat to cell surface antigens may be nonspecific, and adsorptive endocytosis has been suggested as a mechanism responsible for cellular uptake of Tat (10). However, Tat has also been shown to bind to specific cell surface receptors such as the αvβ5 integrin (39) and a
Materials and Methods

HIV Tat and the cytokine TGFβ are qualitatively similar immunosuppressive factors and both inhibit lymphocyte proliferation (19, 38). One possible explanation for the antiproliferative effects in T cells was that it induced the production of TGFβ which is one of the most potent endogenous growth inhibitors for these cells (29). TGFβ as a bifunctional regulator of cell function (26), is a potent stimulator of proliferation in human articular chondrocytes (11, 21). The present study shows that Tat and a peptide containing the basic domain are potent chondrocyte growth factors which act in part through the induction of TGFβ.

Chondrocyte Isolation and Culture

Primary human articular chondrocytes were isolated as described (37). Cartilage was obtained at autopsy and from the University of California, San Diego tissue bank from donors without known history of joint disease. For all experiments reported here, cartilage from the femoral condyles and tibial plateaus of the knee joints was used. Care was taken to obtain morphologically normal and full thickness cartilage and to avoid inclusion of subchondral bone. The cartilage surface was gently scraped with a scalpel to remove cells from joint fluid potentially adhering to cartilage. The slices were incubated in DMEM (Whittaker MAB Bioproducts, Walkersville, MD) containing 10% FBS for 15 min at 37°C (30 min), washed with DMEM (Whittaker MAB Bioproducts, Walkersville, MD), and transferred to 3 h on a gyratory shaker until the tissue fragments were dissolved. The cells were washed three times and cultured for 3 h on a gyratory shaker until the tissue fragments were dissolved. The cells were washed three times and cultured for 3

For studies on TGFβ production, primary chondrocytes were cultured in T175 flasks for 24 h after isolation in DMEM 5% FBS. The cells were nonadherent at that time point; they were collected, washed two times in serum-free media, and plated in 96-well plates for studies on proliferation or TGFβ production.

Chondrocyte Proliferation Studies

Chondrocytes were plated in 96-well flat bottom tissue culture plates in DMEM containing 5% FBS at 5,000 cells per well. Tat and growth factors were added and the cells were incubated for 96 h. Proliferation was determined by [3H]thymidine uptake during the final 12 h of culture. Cells were frozen at -70°C for 2 h. After thawing, the cells were harvested on an automated cell harvester (Cambridge Tech, Watertown, MA).

Northern Blot Hybridization

Northern blot hybridizations were performed after each HPLC step and during storage as Tat 1-86 was found to be readily oxidized and inactivated. The Tat 1-86 was further purified by semipreparative reverse phase HPLC (using a Vydac C18 column, 250 x 10 mm, with 5 µM, 300 A pore size packing; Separations Group, Hesperia, CA) as previously described (3). The gradient was 0-60% water-acetonitrile, 0.1% trifluoroacetic acid over 240 min. The Tat peptides corresponding to the amino acid positions 1-21, 1-42, 38-62, 55-72, and 70-86 in the polypeptide sequence were synthesized using 4-methyl benzhydryl amine resin (Applied Biosystems Inc.) on a peptide synthesizer (431A; Applied Biosystems). The recombinant plasmids were linearized and transcribed with the appropriate RNA polymerase (SP6 to T7) defining a 216-bp fragment of the cDNA were synthesized, with the restriction sites EcoRI and HindIII added at their 5' ends, respectively. After amplification by PCR the fragment was inserted into pGEM-4z. The 503-bp PstI-KpnI fragment of TGFβ1 cDNA (kindly provided by Dr. I. Braude, Cetus Corp., Emeryville, CA) was subcloned into the transcription vector pGEM-4z (Promega Corp., Madison, WI). β-actin. Two primers (5'-CGTCGTCGACAACGG-3' and 5'-GACCGTGAGAAGACCGG-3') defining a 216-bp fragment of the cDNA were synthesized, with the restriction sites EcoRI and HindIII added at their 5' ends, respectively. After amplification by PCR the fragment was inserted into pGEM-4z.

Probe Preparation

The recombinant plasmids were linearized and transcribed with the appropriate RNA polymerase (SP6 to T7) to obtain antisense probe. The probes were labeled with [32P]UTP (Amersham Corp., Arlington Heights, IL) and separated from unincorporated nucleotides by gel filtration (Centri-Sep columns; Princeton Separations Inc., Adelphi, NJ).
Growth Factors and Other Reagents

TGFβ1, PDGF-AA, bFGF, IGF-I, and IL-6 were all recombinant human preparations and purchased from R&D Systems. Neutralizing rabbit antibody to human TGFβ was also obtained from R&D Systems. Phosphorothioate anti-sense oligonucleotides with the sequence 5'-GAA GTC AAT GTA CAG-Y corresponding to a region that is conserved among the three isoforms of TGFβ and the matching sense oligonucleotide were synthesized on a Pharmacia Gene Assembler (Pharmacia Diagnostics Inc., Fairfield, NJ) and purified on HPLC.

Results

HIV Tat Stimulation of Chondrocyte Proliferation

HIV-1 Tat was tested in microproliferation assays with primary human articular chondrocytes. Synthetic or recombinant preparations of full-length HIV-1 Tat, dose dependently stimulated chondrocyte DNA synthesis (Fig. 1 a). Maximal Tat effects were of similar magnitude as the effects of TGFβ, the most potent growth factor for primary human articular chondrocytes. To determine the specificity of these effects, Tat was oxidized and this abrogated its chondrocyte stimulatory activity (Fig. 1 b). The NH₂-terminal 14 amino acids of HIV-1 Tat encoded by exon II contain an RGD motif which defines interactions with cell surface integrins (2). A Tat peptide which contained only the amino acids encoded by exon I retained similar chondrocyte growth factor activity (Fig. 1 b), indicating that the RGD motif is not involved with this Tat effect. Comparison of the effects of HIV-1 Tat and TGFβ with other growth factors is shown in Fig. 2. The maximal effects of Tat were of comparable magnitude as those of TGFβ and greater than the effects seen with bFGF, PDGF, or IGF-I. The analysis of the interactions between Tat and the other chondrocyte growth factors showed that Tat and TGFβ synergistically stimulated chondrocyte proliferation while the effects of Tat and the other factors were additive (Fig. 2). Tat not only stimulated [³H]thymidine uptake but also increased cell numbers to a similar extent as TGFβ (Table I).

Analysis of HIV Tat Domains

To analyze which domains of Tat are required for the effects on chondrocyte proliferation, several peptides (20–22 mers) corresponding to different regions of the molecule were synthesized. The Tat peptide 38-62 stimulated chondrocyte proliferation (Fig. 3 a) while the other peptides had no significant effect. In the analysis of its interactions with...
Table I. TGFβ, Tat and Chondrocyte Replication

|     | Media | TGFβ | Tat | TGFβ + Tat |
|-----|-------|------|-----|-----------|
| Experiment 1 | 68,585 | 133,243 | 98,732 | 193,720 |
| Experiment 2 | 86,596 | 183,021 | 176,532 | 275,301 |

Primary chondrocytes were plated at 50,000 cells per well in 6-well plates and cultured in media alone (DMEM supplemented with 5% FBS) or stimulated with TGFβ1 (10 ng/ml), recombinant Tat 1-86 (10 μg/ml) or both. Cells were collected after 5 d, stained with trypan blue and counted. Results represent mean values of triplicate determinations.

TGFβ, Tat 38-62 also showed the synergy that was seen with full-length Tat (Fig. 3 b) and induced levels of chondrocyte proliferation that exceeded those induced by any other combination of growth factors tested (not shown). Among the Tat peptides tested, Tat 38-62 is highly basic (net charge +7) and a recent report suggested that the effects of a similar Tat peptide were due to this feature (22). Therefore, we tested protamine, a highly basic polypeptide, but it had no effect on chondrocyte proliferation (not shown), suggesting that the effect of peptide 38-62 is not only a function of its basic charge.

Tat Stimulates TGFβ Expression in Chondrocytes

Chondrocytes express the TGFβ1, TGFβ2, and TGFβ3 genes and produce the corresponding proteins (35, 36). Most of the regulatory factors that stimulate chondrocyte proliferation also stimulate TGFβ production (11). To analyze whether the Tat effects occur through TGFβ-dependent mechanisms we tested the effect of Tat on TGFβ production by chondrocytes. Tat stimulated the release of increased levels of TGFβ activity (Fig. 4). Furthermore, Tat induced TGFβ1 mRNA levels and showed a strong synergy with TGFβ1 which autoinduces its mRNA expression (Fig. 5). Analysis of TGFβ1 mRNA levels by a semiquantitative PCR assay was performed on several additional chondrocyte cultures and showed induction of TGFβ1 by Tat as well as the marked synergy with TGFβ1 (not shown).

Figure 3. Effects of HIV Tat peptides on chondrocyte proliferation. TGFβ (ng/ml) and Tat peptides (μg/ml) were added at initiation of the proliferation studies. A shows effects of peptides alone; B shows the combination with TGFβ. Results represent mean cpm ± SEM of three experiments performed in triplicate.

Figure 4. Induction of TGFβ activity by HIV Tat. For the analysis of TGFβ production, chondrocytes were plated in serum-free media and stimulated with Tat (concentrations in μg/ml) or IL-6 (10 ng/ml), a known inducer of TGFβ. Conditioned media were collected after 24 h and tested in the CCL64 assay for active and latent TGFβ. Results were obtained from three chondrocyte cultures. Each supernatant was tested in the CCL64 assay in triplicate.

Figure 5. Regulation of TGFβ1 mRNA levels. Chondrocytes were stimulated with TGFβ1 (10 ng/ml) or Tat peptide 38-62 (10 μg/ml) for 8 h. Total RNA was isolated and analyzed for TGFβ1 mRNA by Northern blotting. To document the amount of RNA loaded, filters were probed for β-actin mRNA. Lane 1, media control; lane 2, TGFβ; lane 3, Tat; lane 4 TGFβ plus Tat.
Table II. Cell Specificity of Tat Effects

| Stimulus | Media | Tat | TGFβ | bFGF |
|----------|-------|-----|------|------|
| Cell type |       |     |      |      |
| Primary chondrocytes | 439 | 5,833 | 6,982 | 1,432 |
| Subcult. chondrocytes | 678 | 809 | 1,372 | 4,907 |
| Fibroblasts | 902 | 1,273 | 891 | 5,328 |

Primary chondrocytes, subcultured chondrocytes (passage 5), and human skin fibroblasts (passage 9) were cultured in media alone (DMEM supplemented with 5% FBS) or stimulated with recombinant Tat 1-86 (10 μg/ml), TGFβ1, or bFGF (both at 10 ng/ml). Proliferation was determined after 5 d by [3H]thymidine incorporation and is shown as mean cpm.

Figure 6. Involvement of TGFβ in Tat stimulation of chondrocyte proliferation. (A) Chondrocytes were stimulated with Tat 38-62 (10 μg/ml) and antibodies to TGFβ or control rabbit IgG (antibody concentrations are shown as μg/ml) were added at the same time. (B) Cells were preincubated with oligonucleotides (concentrations shown in μM) for 8 h and then stimulated with Tat 38-62 (10 μg/ml). Proliferation in all cultures was analyzed 96 h after the addition of Tat. TGFβ antibodies and oligonucleotides were added only once at initiation of culture. Results represent mean cpm ± SEM of two experiments with different chondrocyte preparations each performed in triplicate.

Tat Induced TGFβ Expression and Chondrocyte Proliferation

To test the role of TGFβ in the Tat effects on chondrocytes, the cells were cultured in the presence of Tat and an antisera which neutralizes the biological activity of the TGFβ isoforms 1-3. This antisera dose dependently and specifically reduced the stimulatory effects of Tat (Fig. 6A). These effects were observed when antibodies were added only at initiation of culture. These inhibitory effects were reversible. Chondrocytes cultured in the presence of Tat and TGFβ antibody were collected after 4 d and replated. Their response to subsequent stimulation with Tat or TGFβ was similar to that of cells that had been precultured in media. As an alternative approach to interfering with endogenous TGFβ, antisense oligonucleotides corresponding to conserved regions in the three isoforms of human TGFβ were synthesized. The antisense oligonucleotide dose dependently reduced the Tat stimulation of chondrocyte proliferation while the corresponding sense oligonucleotide had no detectable effect (Fig. 6B). These results suggest that the endogenous production of TGFβ is at least in part responsible for Tat-induced chondrocyte proliferation.

Cell-lineage Specificity of Tat Effects on Proliferation

Chondrocytes undergo functional and phenotypic changes during in vitro subculture, a process that is referred to as dedifferentiation to a fibroblast-like phenotype (1). TGFβ responsiveness with respect to growth stimulation decreases during in vitro subculture and TGFβ is not the most potent stimulator of fibroblast proliferation in monolayer culture (11). It was thus tested whether the ability of Tat to stimulate chondrocyte proliferation is a function of cell type and differentiation. Table II shows results from a comparative analysis of primary and subcultured chondrocytes and human skin fibroblasts. Tat and TGFβ stimulated proliferation of primary chondrocytes but not of subcultured cells or fibroblasts. The effects of bFGF are shown as a positive control for the stimulation of fibroblast proliferation. Certain fibroblast cell lines such as 3T3 show increased proliferation in response to TGFβ. However, in experiments where TGFβ caused a mean 4.7-fold increase in proliferation, Tat did not show significant effects alone or in combination with TGFβ (not shown).

Discussion

This study demonstrates that the HIV-1 Tat protein stimulates proliferation of normal human articular chondrocytes. This is associated with the expression of TGFβ and the growth promoting effect of the Tat protein are at least in part dependent on the induction of TGFβ. A 24-amino acid peptide containing the basic domain of the Tat protein induces similar levels of chondrocyte proliferation as TGFβ.

The first suggestion that Tat may be involved with cellular responses other than HIV replication was based on observations with transgenic mice carrying the Tat gene which developed dermal lesions that were similar to Kaposi's sarcoma.
Although the Tat transgene was expressed in the skin, it was not expressed in the tumor cells, suggesting that the Tat-expressing cells produced factors that stimulated proliferation of the tumor cells. In a long-term follow-up study the Tat transgenic mice also had a higher incidence of liver cancer which may be initiated by growth signals from extrahepatic cells expressing the Tat gene (40). Collectively, these findings suggest that Tat may directly or indirectly induce the expression of growth signals. This could either be a function of the Tat protein itself (this notion is supported by the ability of Tat to stimulate proliferation of Kaposi’s sarcoma cells [7]) or to inhibit antigen-induced lymphocyte proliferation (38). Alternatively, Tat may induce expression of cellular genes involved with the regulation of proliferation.

The Tat protein and TGFβ have similar immunosuppressive and antiproliferative effects on lymphocytes (19, 38). This provided the basis for the present hypothesis that an interaction between Tat and the bifunctional growth regulator TGFβ (26) should result in growth stimulation in cell systems where TGFβ stimulates cell proliferation. Human articular chondrocytes were chosen as a model since TGFβ is one of their most potent growth factors (11, 21).

The results show that Tat induces proliferation of human articular chondrocytes. At optimal doses Tat induced similar levels of chondrocyte proliferation as TGFβ.

Analysis of different mesenchymal cell types showed that the Tat effects on proliferation appear to be cell-type specific. It is only seen in primary, differentiated but not in subcultured, dedifferentiated cells, nor in human skin fibroblasts or fibroblast cell lines such as 3T3 which respond to TGFβ with increased proliferation. These observations are consistent with the growth factor response profile that characterizes these three different cell types (11, 21).

Induction of chondrocyte proliferation is a function of TGFβ but also of a series of other growth factors. To characterize the specificity of the Tat effects we tested a chondrocyte response that is specific for TGFβ. Articular chondrocytes produce inorganic pyrophosphate and TGFβ is the only known stimulator of this response (27). Treatment of human chondrocytes with the Tat peptide 37-62 increased inorganic pyrophosphate levels in these cultures to levels that were similar to those induced by TGFβ (Rosen, F., and M. Lotz, unpublished observations).

Tat and TGFβ showed strong synergy in the stimulation of chondrocytes. The level of chondrocyte proliferation induced by Tat and TGFβ was greater than that induced by any other combination of growth factors tested. This synergy was seen in measurements of [3H]thymidine incorporation as well as in cell counts. Within 24-48 h TGFβ/Tat-treated cultures were microscopically distinguishable by a greater cell density. TGFβ/αTat autoinduces the expression of its gene and Tat also synergized with TGFβ in increasing mRNA levels.

Since chondrocytes produce TGFβ (35, 36), we tested whether the Tat effects were mediated through the induction of TGFβ. Under conditions where Tat stimulated proliferation of these cells it also increased expression of endogenous TGFβ mRNA and chondrocytes released similar levels of TGFβ in response to IL-6, a known inducer of TGFβ expression (35).

The proliferative effect of Tat was at least in part dependent on the induction of TGFβ as endogenous growth factor TGFβ1 as it was reduced by neutralizing antibodies to TGFβ and by TGFβ antisense oligonucleotides. However, Tat is likely to induce other signals in chondrocytes in addition to TGFβ since synergistic effects could also be observed under conditions where optimal amounts of exogenous TGFβ were added.

With the use of different synthetic peptides we identified the region in the Tat protein that was responsible for the stimulation of chondrocytes. A protein that contained only the first 72 amino acids encoded by exon I gave similar stimulation of chondrocytes. This peptide 1-72 lacks the 14 amino acids encoded by exon II which contains an RGD sequence that is capable of mediating cell adhesion (2). This motif and the NH2-terminal 14 amino acids were not required for the Tat effects on chondrocyte proliferation. A series of peptides corresponding to the COOH-terminal part of the Tat protein showed that peptide 38-62 was the only one active in the stimulation of chondrocytes. This peptide contains the basic domain of Tat which has been shown to be involved with binding of Tat to the cell surface (22). Although the receptor responsible for the Tat effects on chondrocytes has not been identified it is possible that the αvβ5 integrin may be involved. This integrin has recently been shown to function as a receptor for Tat, and the region in Tat that is responsible for binding is contained within the peptide that we have shown to have chondrocyte stimulatory activity. In further support of this notion, we have recently shown human articular chondrocytes express this integrin (43). It is thus possible that Tat acts at the cell surface and induces intracellular signals that activate the TGFβ gene and/or promote cell cycle progression. Tat may also enter chondrocytes and bind directly or in cooperation with host cell factors to the promoter of the TGFβ1 gene.

In summary, HIV Tat stimulates chondrocyte proliferation. These effects are associated with and in part dependent on the induction of TGFβ as endogenous growth factor. The basic domain of Tat which has been shown to mediate binding to cell surface antigens is also responsible for the effects of Tat on chondrocytes. Stimulation of TGFβ production and the resulting increase in chondrocyte proliferation are a cell type-specific function of the Tat/TGFβ interaction.

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