Expression of Transforming Growth Factor β and Transforming Growth Factor β Receptors on AIDS-associated Kaposi’s Sarcoma

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ABSTRACT

Several humoral growth factors may contribute to the development and growth of AIDS-associated Kaposi’s sarcoma (KS). They are either provided by chronically activated cells of the immune system or in an autocrine/paracrine manner by the neoplastic cells themselves. Transforming growth factor β (TGF-β) may directly enhance the growth of KS cells and tumor matrix formation. To mediate a signal both TGF-β receptors type I and type II (TβR-I and TβR-II) have to be expressed. We investigated the expression of TGF-β, TGF-β receptors types I and II, and endoglin, a nonsignaling-type TβR-III, by means of immunohistochemistry on skin biopsies from patients with AIDS-related KS. We found that the TGF-β ligand was expressed by KS cells in 9 of 11 samples. TβR-II was strongly expressed in 10 of 12 samples, but none of the investigated tumor samples stained for TβR-I. Endoglin was weakly expressed on all KS lesions and stained the endothelium of tumor-associated vessels in 92% of the samples. These findings show that most KS lesions have the ability to produce TGF-β and that KS cells maintain a high expression of TβR-II in the absence of TβR-I, which may allow KS to escape growth inhibitory effects of endocrine or paracrine TGF-β.

INTRODUCTION

KS3 is the most frequently diagnosed malignancy in patients with AIDS and occurs in up to 15% of patients (1). KS cells are thought to be derived from the endothelium and exhibit characteristics of vascular differentiation. Whether KS represents a true malignancy or a diffuse vascular proliferation is under debate (2, 3). Furthermore, levels of certain growth factors such as basic fibroblast growth factor (4) and TGF-β (5) have been shown to be elevated in patients with KS. The cells composing KS lesions may exhibit a specific receptor pattern for growth factors that could be involved in the progression to KS, and these receptors could serve as targets for specific growth antagonists or immunotherapies.

TGF-β usually mediates growth suppression (6), but may paradoxically play an important role in promoting the development of KS since it has been reported to stimulate the growth of KS cells in vitro (4), and serum levels of TGF-β are elevated in HIV-infected individuals (5). The reason for this effect is not known. The level of circulating TGF-β in normal serum is within a range that exceeds the minimal concentration for a biological effect, suggesting that TGF-β may act both locally and systemically (7). There are two essential components of functional TGF-β receptors: type I and II receptors (TβR-I and TβR-II), both of which are needed to mediate a growth-suppressing signal (8, 9). TβR-II is a constitutively active serine/threonine kinase which phosphorylates TβR-I in the presence of the ligand to signal growth suppression (10). Expression of the two components of the TβR has not been carefully studied in KS, and aberrant expression might explain the lack of growth-suppressive effects of TGF-β on these cells.

In this study, we investigated whether previously reported growth-enhancing properties of TGF-β would correlate with the expression of the components of the TβR on KS lesions, or whether KS cells escape from the growth inhibitory effects of TGF-β by loss of TβR (11). We showed that TGF-β itself is expressed in most KS lesions in vivo and that while KS cells demonstrate a high level of expression of TβR-II (and TβR-III/endoglin), they do not express TβR-I. This partial expression represents a potential mechanism for the aberrant responses to TGF-β that were observed.

MATERIALS AND METHODS

Tissue Specimens. Skin biopsies from 12 HIV-seropositive Caucasian male patients with clinically suspected KS were used to establish a pathological diagnosis. Half of the specimen was snap frozen for immunohistochemical studies, and frozen sections were air-dried and then fixed in acetone. The specimens were stored at −30°C until used for immunohistochemistry. The other half of the tissue was placed in 3% formalin solution for routine processing. Skin biopsies were performed on lesions from the chest, abdomen, arm, leg, and back. Lesions ranged from 5 × 6 mm to 30 × 10 mm in diameter.

Antibodies. A murine IgG1 antibody recognizing human TGF-β1 and TGF-β2 at a working concentration of 125 μg/ml (Genzyme, Cambridge, MA) was used. This antibody effectively inhibits the biological activity of TGF-β. Affinity-purified
Expression of TGF-β and TGF-β Receptors on AIDS-associated KS

Table 1  Results of staining for factor VIII, TGF-β, TβR-I, TβR-II, and endoglin*  

<table>
<thead>
<tr>
<th>Patient</th>
<th>Histological stage</th>
<th>nc</th>
<th>pc</th>
<th>Factor VIII (tumor)</th>
<th>TGF-β (KS cells)</th>
<th>TGF-β (tumor vessels)</th>
<th>TβR-I (KS cells)</th>
<th>TβR-II (tumor vessels)</th>
<th>Endoglin (tumor vessels)</th>
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<tr>
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<td>+</td>
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<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

*No staining was defined as --, staining of 49% or less of the lesion was scored as +, staining of 50–74% of the lesion was scored as ++, and staining of 75% or more was scored as +++.

nc, negative controls stained with antibody from a mouse antihuman natural killer cell IgM-secreting hybridoma (HNK-1); pc, positive controls with antibody F8/86 mouse IgG1 antihuman von Willebrand factor.

rabbit polyclonal antibodies R-20 and L-21 were used to stain for TβR-I and TβR-II, respectively, and were used according to the manufacturer’s guidelines (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). R-20 recognizes an internal epitope corresponding to amino acids 482–501 of the TβR-I, and L-21 recognizes an epitope mapping to an internal domain of TβR-II corresponding to amino acids 246–266. A murine IgM mAb, TEC 11, used to stain for TβR-III (endoglin) was produced as described previously and was kindly provided by Dr. P. Thorpe (12). The positive control antibody used here was F8/86 mouse IgG1 antihuman von Willebrand factor, diluted 1:75 (DAKO Ltd., High Wycombe, United Kingdom). As a negative control to determine background staining, we used a mouse antihuman natural killer cell IgM-secreting hybridoma (HNK-1; ATCC, Rockville, MD). The primary antibodies were incubated for 40 min at room temperature.

Alkaline Phosphatase and Immunoperoxidase Staining.

Frozen section slides were rehydrated in PBS. Staining with anti-TGF-β was done at a working concentration of 100 μg/ml. Washes were performed with Tris buffer. Secondary biotinylated antimonouse antibody was used at 1:100 (DAKO, Carpinteria, CA). Streptavidin-alkaline phosphatase complex was used at a dilution of 1:150, and slides were subsequently incubated with substrate red (Vector Laboratories, Burlingame, CA).

To stain for receptors I and II, sections were rehydrated in PBS and incubated with primary antibody against TβR-I (dilution 1:100) and TβR-II (dilution 1:75). Sections were then washed with PBS and 0.1% Tween 20 and incubated with a biotinylated antirabbit immunoglobulin (dilution 1:300) for 60 min. Biotinylated goat antimonouse IgM (Vector Laboratories) was used to stain for endoglin (pure supernatant, provided by Dr. P. Thorpe). Sections incubated with F8/86 were incubated (dilution 1:100) with biotinylated F(ab)2 sheep antimonouse IgG(H + L) (Sigma, St. Louis, MO). The slides were washed with PBS and 0.1% Tween 20 and incubated with streptavidin-biotin-horseradish peroxidase complex, diluted 1:100 for 60 min (DAKO Ltd.). After repeated washes, bound peroxidase was developed with 1:100 diluted 3-amino-9-ethylcarbazole in N,N-dimethylformamide (Sigma) for a maximum of 20 min to produce a brown reaction product. The two different staining systems were recommended by the vendor for these antibodies. Sections were counterstained with hematoxylin and embedded in crystal mount.

All specimens were evaluated with a ×20 objective. Staining was scored as -- if no staining was appreciated, + if 49% or less of the vascular proliferation was highlighted, ++ if from 50 to 74% of the lesion was highlighted, and +++ if there was 75% or greater staining. Staining for von Willebrand factor was used as a positive control, and the sample quality was judged by the staining of the endothelial cells of preexisting blood vessels in a granular, cytoplasmatic pattern. As a positive control for antibody R-20 (anti-TβR-I) and anti-TGF-β12, frozen sections of a human epidermoid carcinoma cell line A431 (ATCC) were used, for antibody L-21 (anti-TβR-II) frozen sections of a multipotential hematopoetic cell line K562 (ATCC) were used, and as controls for TEC11 frozen sections from human cancer biopsies served.

RESULTS

Patient Demographics and Histology. Four patients had clinically patch-stage KS (Patients 3, 8, 9, and 11), and the other eight patients had plaque-stage KS. Patient 2 was receiving α-IFN (12 million IU/day for the preceding 4 months). All patients were HIV seropositive, with homosexuality as their primary risk factor for HIV disease. All had been confirmed as HIV positive by Western blot. The diagnosis of KS was confirmed in all cases using histological criteria that have been delineated elsewhere (13). The histological subtypes are listed in Table 1.

Expression of TGF-β. Nine of 11 investigated KS biopsies expressed TGF-β ligand as assessed by immunohistochemistry (Table 1). Strong staining was observed with A431 cells, which served as positive controls (data not shown). Staining for TGF-β (Fig. 1, a and d) mainly showed granular cytoplasmic staining. Further significant staining could be observed on the cell membranes that faced the endoluminal side of a KS-associated vessel or cavernous structures within the KS lesions. This finding suggests that not only do the majority of KS lesions produce TGF-β, but also that the endoluminal mem-

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branes in KS lesions may bind TGF-β from the circulation. The epidermis overlying KS lesions was negative for TGF-β as was normal skin (data not shown). This is in concordance with previous reports that show expression of TGF-β isoforms 1 and 2 only in regenerating skin (14).

**Expression of TBR-I and TBR-II.** Staining for TBR-I could not be detected on any KS lesion (Table 1 and Fig. 2, c and h). In each case positive controls of A431 cells stained strongly, and normal positive staining in the epidermis served as an additional internal positive control. Strong staining for TBR-I could be detected in the epidermis above the KS lesions in 67% of patients, whereas the epidermis did not stain for TBR-II in any case (Fig. 2, c and d). TBR-II positivity was found in 10 (83%) of 12 KS lesions and was very strong in 67% of cases (Table 1 and Fig. 2, d and i). Positive cells exhibited a membrane-related cytoplasmic staining pattern that was not limited to vessel lumina and independent of histological subtype (Fig. 2i). No correlation of staining pattern with CD4 counts, duration of KS lesions, location of lesions, or inflammatory cells in the specimen was observed. Interestingly, both patients not display-

**Fig. 1** Immunohistochemical staining for TGF-β in KS. Frozen sections were stained with primary antibody recognizing TGF-β, a and c, negative staining for TGF-β on biopsy from Patient 3. b and d, positive staining revealing granular cytoplasmic staining on the biopsy from Patient 8. a and b, ×100; c, ×400; d, ×200.
Fig. 2  Expression of TβR-I, TβR-II, and endoglin. KS stained without primary antibody: a, ×100; f, ×400. Staining with F8/86 for von Willebrand factor: b, ×100; g, ×400. Staining with antibody R-20 for TβR-I: c, ×100; h, ×400. Staining with antibody L-21 for TβR-II: d, ×100; i, ×400. Staining with TEC11 for endoglin: e, ×100; j, ×400.
ing any T\(\beta\)R-I and T\(\beta\)R-II receptors stained positively for TGF-\(\beta\) ligand (Patients 4 and 6).

**Expression of T\(\beta\)R-III/Endoglin**. Stains for endoglin were positive in all KS lesions (Fig. 2, e and j). Endoglin expression was often observed in the cells lining the lumina of pathological vessels, whereas those KS cells in aggregations apart from direct contact with vessels mostly failed to stain (Fig. 2j). Normal vessels within KS lesions stained for endoglin in most cases (92%, Table 1), and endoglin was not expressed in any structures in normal skin. The observation that normal vessels coming in contact with KS or induced by KS show a positivity for endoglin is consistent with previous reports demonstrating expression in tumor neovasculature within solid tumors (12).

**DISCUSSION**

Several cytokines and growth factors have been implicated in the pathogenesis of KS. TGF-\(\beta\) is highly expressed by KS cells in vitro (15) and is produced by peripheral blood monoclonal cells infected with HIV-1 (5). TGF-\(\beta\) is a growth factor with effects which differ according to the target cell type (for reviews, see Refs. 15 and 16). Normal cells, especially epithelial cells, seem to be growth inhibited by TGF-\(\beta\), whereas the growth of transformed cells is frequently not inhibited by TGF-\(\beta\) and may even be enhanced (16). Malignancies such as adult T-cell leukemia (17) or thyroid epithelial neoplasms (18) have been shown to secrete a high level of TGF-\(\beta\), raising the possibility of autocrine growth stimulation. Furthermore, metastases may express higher levels than the primary tumor or surrounding benign tissue (6, 19). Growth of KS cells can be enhanced by various factors such as interleukin 6 (20, 21), interleukin 1 (22), basic fibroblast growth factor (22), vascular endothelial growth factor (23), platelet-derived growth factor (24), and oncostatin M (25, 26). TGF-\(\beta\) also has been shown to promote growth of KS in vitro (16). The growth-enhancing effect of TGF-\(\beta\) could be directly mediated by the T\(\beta\)Rs themselves or may result from a TGF-\(\beta\)-mediated increase in the expression of other growth factors such as platelet-derived growth factor and vascular endothelial growth factor (27, 28) initiating an autocrine stimulatory loop.

Detection of TGF-\(\beta\) expression in vivo by frozen section immunohistochemistry allows assessment without the artifacts associated with in vitro growth. We show here that the ability of KS cells to secrete TGF-\(\beta\) can vary from patient to patient, although the majority of KS stain for TGF-\(\beta\). Effector cells of the immune system are significantly inhibited by TGF-\(\beta\) (29), which may allow KS cells to evade immune recognition. Furthermore, TGF-\(\beta\) is known to alter the tumor matrix in a manner that is advantageous for tumor growth (30). Others have also shown that growth factors released by KS cells enhance endothelial growth, but it remains to be shown which cytokine or which combination of growth factors is responsible for this matrix modulation in vivo (31).

Endoglin is an essential component of the nonsignaling T\(\beta\)R-III complex of human endothelial cells and preferentially binds TGF-\(\beta\)1 and 3 (32). It is expressed primarily by proliferating endothelial cells in chronic inflammatory disorders and tumor neovasculature, but not by resting endothelial cells (12). Recent studies suggest that immunotargeting of T\(\beta\)R-III with toxin-linked mAbs may be used to selectively destroy tumor-associated vessels (12, 33). In this study we used an antiedoglin antibody (TEC11) currently being tested for clinical trials to assess its potential usefulness in KS. We found that endoglin is expressed on most KS cells, especially those lining the lumina of pathological vessels which are easily accessible to systemically introduced immunoconjugates. In addition to the expression of endoglin on KS cells themselves, we found expression in the normal neovasculature within KS lesions, which may also serve as a target for immunotoxin therapy. Immunoconjugates of TEC 11 have been shown to bind to the endothelium of vessels and cause coagulation of these tumor-associated vessels, leading to local ischemia and tumor necrosis. This critically limits the blood flow to the tumor and inhibits its growth (33).

Interestingly, vessels coming in contact with KS cells demonstrate a high level of endoglin expression similar to that observed in the neovasculature induced by solid tumors (12). The findings in this study support the further evaluation of TEC11 for the targeted immunotherapy of KS.

In this study T\(\beta\)R-I was absent in all specimens. This absence could potentially allow KS cells to evade inhibitory signals from TGF-\(\beta\), as both receptor components T\(\beta\)R-I and T\(\beta\)R-II are needed for the transmission of an antiproliferative signal (9). If the growth of KS cells is stimulated by TGF-\(\beta\) in vitro (16), it is thus unclear which receptor pathway mediates this effect. There would have to be another pathway with a different receptor substituting for the lack of T\(\beta\)R-I, or alternatively, it has been suggested that TGF-\(\beta\) could bind to T\(\beta\)R-II alone and mediate a transcriptional response in the absence of the classical type I receptor type (34–36). Furthermore, homodimer formation of T\(\beta\)R-II has been shown, and whether such a complex is involved in growth regulation remains to be confirmed (37). Other neoplastic cell lines, such as T-cell malignancies, gastric cancer, and osteosarcoma have been shown to escape from the growth inhibitory effects of TGF-\(\beta\) (11, 38, 39). The mechanism of this evasion of growth inhibitory signals seems to be a loss of the function or expression of T\(\beta\)R-II in all cases. We find that while T\(\beta\)R-II remains highly expressed in most cases of KS in this series, the specific loss of T\(\beta\)R-I represents a potentially novel mechanism that allows KS to escape from the growth inhibitory effects of TGF-\(\beta\).

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Expression of transforming growth factor beta and transforming growth factor beta receptors on AIDS-associated Kaposi's sarcoma.

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