

Cannabinoid Modulation of Kaposi's Sarcoma–Associated Herpesvirus Infection and Transformation

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Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV; also named human herpesvirus 8) is necessary but not sufficient for the development of Kaposi's sarcoma. A variety of factors may contribute to the pathogenesis of Kaposi's sarcoma in addition to KSHV. Marijuana is a widely used recreational agent, and Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the major active component of marijuana, is prescribed for medicinal use. To evaluate how cannabinoids may affect the pathogenesis of Kaposi's sarcoma, we studied primary human dermal microvascular endothelial cells (HMVEC) exposed to KSHV. There was an increased efficiency of KSHV infection in the presence of low doses of Δ^9 -THC. We also found that Δ^9 -THC increased the viral load in KSHV-infected HMVEC through activation of the KSHV lytic switch gene, the *open reading frame 50*. Furthermore, we observed that Δ^9 -THC stimulated expression of the KSHV-encoded viral G protein-coupled receptor and Kaposi's sarcoma cell proliferation. Our results indicate that Δ^9 -THC can enhance KSHV infection and replication and foster KSHV-mediated endothelial transformation. Thus, use of cannabinoids may place individuals at greater risk for the development and progression of Kaposi's sarcoma. [Cancer Res 2007;67(15):7230–7]

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), the etiologic agent of Kaposi's sarcoma, is not sufficient for the development of Kaposi's sarcoma. This is highlighted by the observation that most KSHV infections are asymptomatic, and >95% of persons who become infected never develop Kaposi's sarcoma or other KSHV-related cancers (1, 2). It seems that certain cofactors participate in the development of Kaposi's sarcoma mainly through suppression of the immune system (3, 4). Whereas the immune system of healthy adults usually holds the virus in check, AIDS patients (5), transplant recipients (6), and other immunocompromised patients are at risk for Kaposi's sarcoma (7) if they are KSHV infected.

Marijuana, the prototypical cannabinoid, has been used as a recreational, ceremonial, and therapeutic substance throughout history. Although cannabinoids display a series of promising therapeutic potentials (8, 9), marijuana and other cannabinoids may impair both cell-mediated and humoral immune system function, leading to decreased resistance to infection by viruses

and bacteria (10–12). Hence, marijuana smoking has been postulated to act as a possible cofactor in the development of Kaposi's sarcoma, as it predisposes human immunodeficiency virus–positive individuals to opportunistic infections and Kaposi's sarcoma (13–15). A previous study showed that Δ^9 -tetrahydrocannabinol (Δ^9 -THC) inhibited the lytic replication of KSHV in B cells by modulating the expression of *open reading frame 50* (*ORF50*; ref. 16). However, these results cannot be extrapolated to the case of Kaposi's sarcoma because its lesions are mainly composed of spindle cells, which seem to be of lymphatic endothelial origin (17). Furthermore, the transcriptional profiling of KSHV genes is different in endothelial cells and B cells (18). Thus, the effects of cannabinoids on KSHV are not yet characterized. Δ^9 -THC is highly lipophilic and can change membrane permeability (19), suggesting that this cannabinoid could modulate KSHV entry into the endothelium. Moreover, Δ^9 -THC initiates a signaling cascade that could modulate cellular susceptibility to KSHV and alter the viral life cycle, as well as promote viral strategies of immune evasion. For these reasons, the potential risks versus benefits of cannabinoids need to be explored in KSHV-associated diseases.

Here, we report that low doses of Δ^9 -THC, similar to those achieved *in vivo*, facilitated KSHV infection in endothelial cells through enhancement of cell-cell interactions and endocytosis. We further show that Δ^9 -THC up-regulated the expression of both the lytic switch gene *ORF50* and the carcinogenic KSHV G protein-coupled receptor (GPCR), thereby increasing viral titers in culture and inducing endothelial cell transformation. These results suggest that low doses of Δ^9 -THC may foster the initiation of Kaposi's sarcoma and contribute to its progression and spread.

Materials and Methods

Cells. Primary human dermal microvascular endothelial cells (HMVEC; adult) were purchased from Cambrex, Inc. and maintained in EBM-2 medium with EGM-2MV SingleQuots. Recombinant green fluorescent protein (GFP)-KSHV-carrying BCBL-1 cells (GFP-BCBL-1) were a gift from Dr. Jeffrey Vieira (Department of Laboratory Medicine, University of Washington, Seattle, WA). BJAB is a line of KSHV- and EBV-negative human B cells. GFP-BCBL-1 and BJAB cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 10 mmol/L HEPES (pH 7.5), 100 units/mL penicillin, 100 μ g/mL streptomycin, and 2 mmol/L L-glutamine.

Drugs. Δ^9 -THC was purchased as a stock solution of 1 mg/mL in methanol from Sigma-Aldrich Co. The CB1 selective antagonist AM251 was purchased from Cayman Chemical Co. The CB2 selective antagonist AM630 was purchased from Tocris Bioscience. O-1918 [(–)-4-(3-3, 4-*trans-p*-menthadien-(1, 8)-yl)-orcinol] was synthesized as described (20) and dissolved in ethanol.

Reverse transcription-PCR. Total cell RNA was extracted by using the RNeasy Mini kit from Qiagen, Inc. Primers for CB1 [5'-GCCTGGCGG-TGGCAGACCTCC-3' (upstream) and 5'-GCAGCACGGCGATCACAATGG-3' (downstream)] or for CB2 [5'-CATGGAGGAATGCTGGGTGAC-3' (upstream) and 5'-GAGGAAGCGGATGAACAGGAG-3' (downstream)] were synthesized by Integrated DNA Technologies, Inc. One-step reverse transcription-PCR

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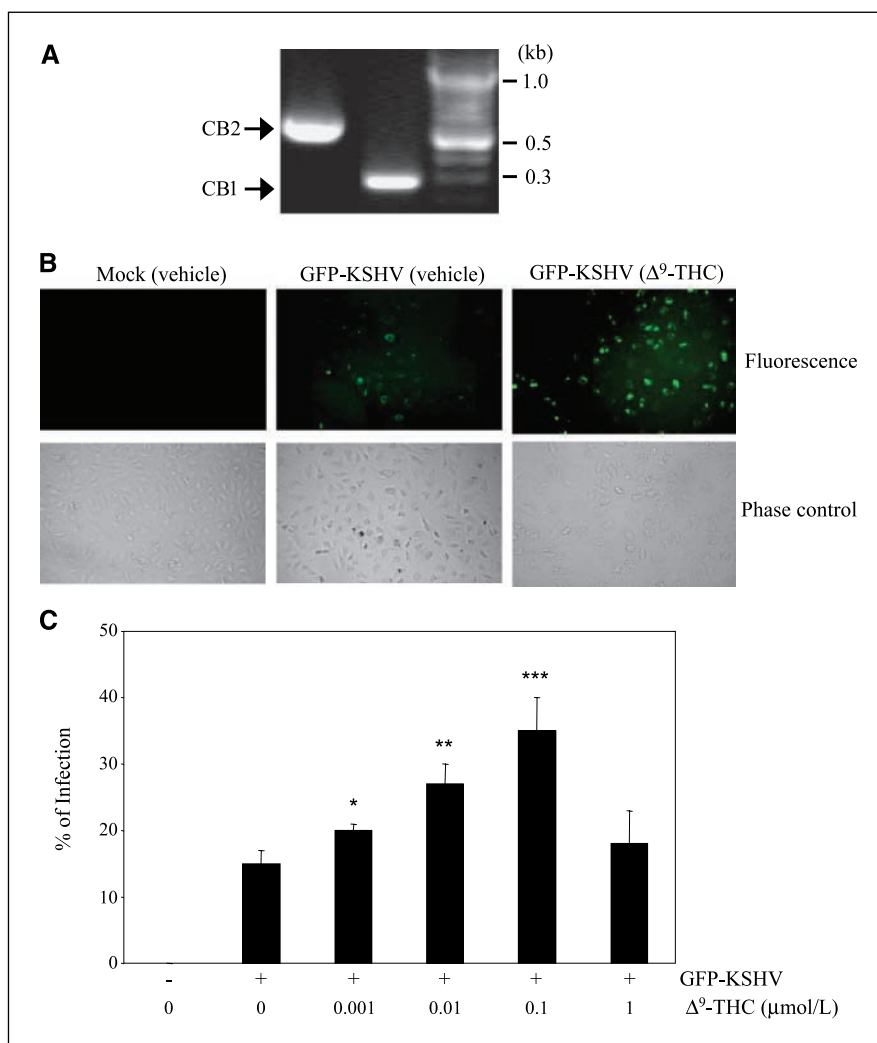


Figure 1. Low doses of Δ^9 -THC increase KSHV infection in HMVEC. **A**, HMVEC express the cannabinoid receptors, CB1 and CB2. **B**, green fluorescent cells (indicative of GFP-KSHV infection) were counted under a fluorescent microscope. Magnification, $\times 100$. Representative photographs. **C**, percentage of GFP-KSHV infection was quantitated. Columns, mean; bars, SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for the treatment with Δ^9 -THC versus vehicle control.

(RT-PCR) was done by using the Titanium One-Step RT-PCR kit (BD Biosciences) following the manufacturer's instructions.

Viral infection. GFP-KSHV was purified and used for the viral infection assay as described previously (21, 22). About 60% confluent cells were either mock infected or infected with GFP-KSHV in the presence or absence of different concentrations of Δ^9 -THC in 24-well plates at 37°C for 3 h, then washed, and incubated at 37°C for 3 days with growth medium. The percentage of infection was calculated and shown.

Real-time DNA PCR and real-time RT-PCR. KSHV-infected HMVEC and GFP-BCBL-1 cells were cultured in the presence or absence of Δ^9 -THC or vehicle control for 7 days. Viral DNA from the supernatant and cells was then isolated by using the viral nucleic acid purification kit (Roche Applied Science). KSHV *latency-associated nuclear antigen 1 (LANA-1)* gene copies were measured by using real-time DNA PCR technology. The primers and Taqman probe were synthesized as described previously (23). In the meantime, total cell RNA was extracted from KSHV-infected HMVEC. Real-time RT-PCR technology was used to determine the expression of KSHV *ORF50*. The primers were as follows: 5'-CGCAATGCGT-TACGTTGTG-3' (forward primer) and 5'-GCCCGACTGTTGAATCG-3' (reverse primer). The Taqman probe was 6FAM-ACCTGTGCCCCC-TCTTCGACACC-TAMRA. The primers and Taqman probe were synthesized by BioScience International, Inc. A real-time PCR assay was done in duplicate for each standard and sample by using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The amplification of 18S RNA served as an endogenous control for *ORF50* expression. The relative

quantification value was calculated by using the $\Delta\Delta C_t$ method from Applied Biosystems.

Western blotting. Cells were lysed in radioimmunoprecipitation buffer containing protease inhibitors (Roche Applied Science) and phosphatase inhibitors. Equal amounts of total proteins were resolved by SDS-PAGE and subjected to Western blot analysis using the enhanced chemiluminescence system (Amersham Pharmacia Biotech). Goat polyclonal anti-phospho-paxillin (Tyr³¹), rabbit anti-platelet/endothelial cell adhesion molecule-1 (PECAM-1; or CD31), rabbit anti-intercellular adhesion molecule-1 (ICAM-1; or CD54), mouse anti- β -catenin, mouse anti-phospho-extracellular signal-regulated kinase 1/2, and rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal anti-phospho-p130Cas (Tyr⁴¹⁰) was purchased from Cell Signaling Technology. Rabbit anti-KSHV GPCR polyclonal antibody was purchased from Cell Sciences, Inc.

Confocal scanning. Cells were cultured in eight-well chamber slides (to ~40–60% confluence), then serum starved, and treated as indicated. After stimulation, cells were immediately fixed with 4% (v/v) paraformaldehyde for 1 h at room temperature and permeabilized for 2 min on ice. Cells were first incubated with primary antibodies or normal IgG controls for at least 1 h at 4°C and then softly washed three times with $1\times$ PBS. Next, FITC-conjugated secondary antibodies were added for 30 min at 4°C . After discarding the staining solution with secondary antibody, propidium iodide staining solution (10 $\mu\text{g/mL}$) was added for 5 min at room temperature.

Cells were carefully washed again three times with $1\times$ PBS. Finally, the chambers were removed and coverslips were mounted on slides and examined under a Leica TCS-NT laser scanning confocal microscope (Leica Microsystems). Antibodies used were goat anti-phospho-paxillin antibody (Tyr³¹), rabbit anti-KSHV GPCR polyclonal antibody, and fluorescein antigoat and antirabbit IgG (H+L) secondary antibodies (Vector Laboratories). Propidium iodide solution was from Sigma-Aldrich.

Adhesion assay. HMVEC and GFP-BCBL-1 cells were pretreated with different concentrations of Δ^9 -THC or vehicle control as indicated. GFP-BCBL-1 cells were then cocultured with HMVEC for 30 min on a shaker at mild speed (50 rpm) in 96-well plates. GFP-BCBL-1 cells were next removed and carefully washed three times with $1\times$ PBS. The adhesion of GFP-BCBL-1 cells to HMVEC was determined by using a Victor 1420 MultiLabel counter from Perkin-Elmer Co. The adhesion index was calculated and shown.

Transformation assay. HMVEC were exposed to KSHV or mock controls and cultured in the presence of Δ^9 -THC or vehicle control along with different antagonists in soft agar medium for 21 days, following the protocol provided by Chemicon International, Inc. Colonies were then stained and counted under a microscope.

Transfection and proliferation assay. HMVEC were transiently transfected with plasmids containing wild-type KSHV GPCR (pcDNA3vGPCR) or vector alone (pcDNA3) by using Superfect transfection reagent from Qiagen. At day 2 after transfection, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay was done in the presence or absence of Δ^9 -THC by using the Cell Proliferation kit (I) from Roche Applied Science, per the manufacturer's instructions.

Statistical analysis. Each treatment was done at least in triplicate and repeated three times. Statistical significance was determined by using the ANOVA test ($P < 0.05$).

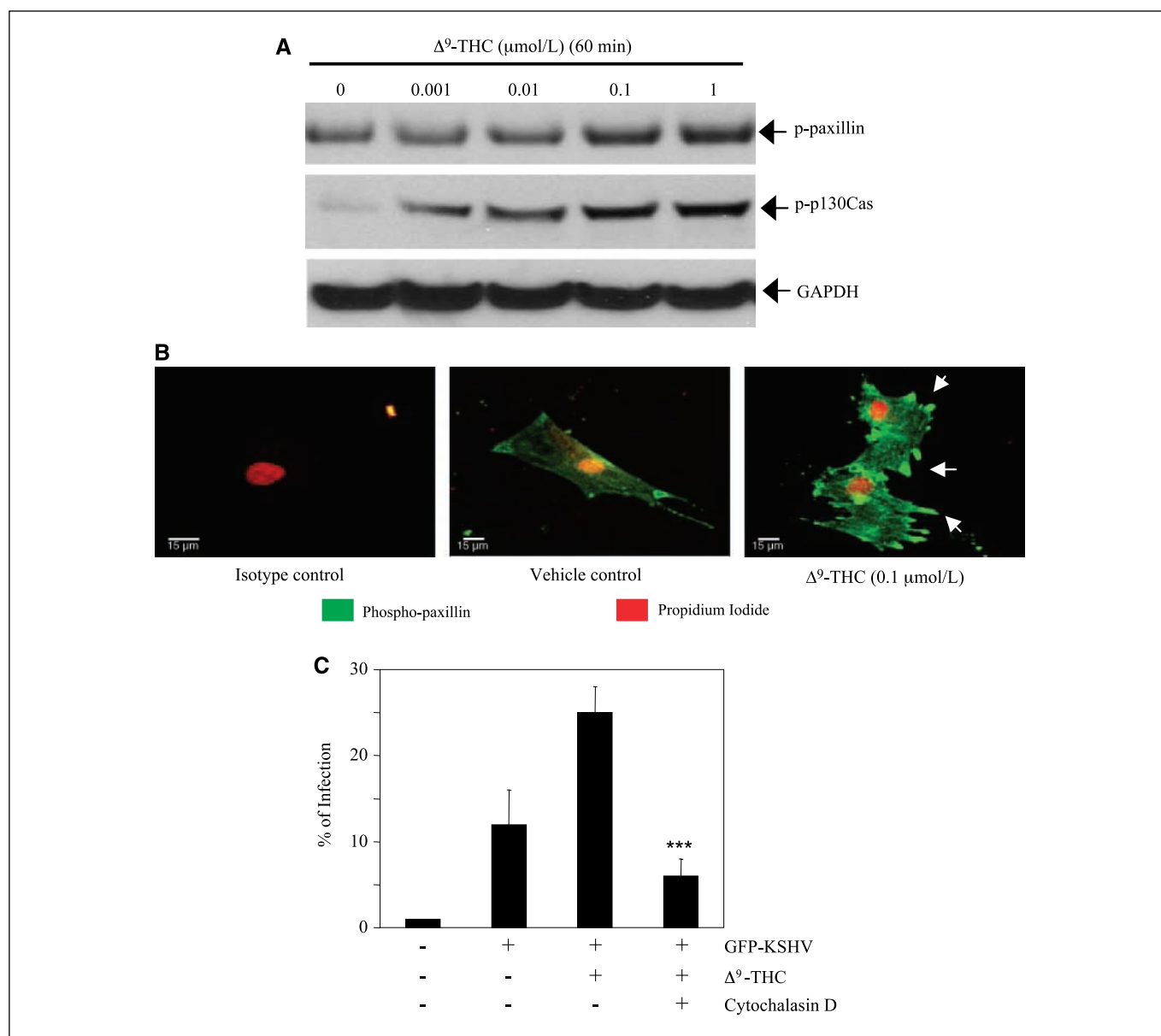


Figure 2. Δ^9 -THC induces the tyrosine phosphorylation of cytoskeletal proteins p130Cas and paxillin. **A**, HMVEC were starved and stimulated with Δ^9 -THC, as indicated. Total cell lysate was then collected for the Western blotting. Phosphorylation levels of paxillin (*p-paxillin*) and p130Cas (*p-p130Cas*) at 60 min. GAPDH served as a loading control. **B**, activation of paxillin on stimulation with Δ^9 -THC is represented by the bright green color based on confocal scanning. Propidium iodide was used to stain the nucleus. Arrows, sites of antibody-labeled paxillin. Bar, 15 μm . **C**, pretreatment with cytochalasin D (30 min) diminishes the Δ^9 -THC-induced increase in KSHV infection.***, $P < 0.001$ for the treatment with cytochalasin D versus DMSO. Columns, mean; bars, SD.

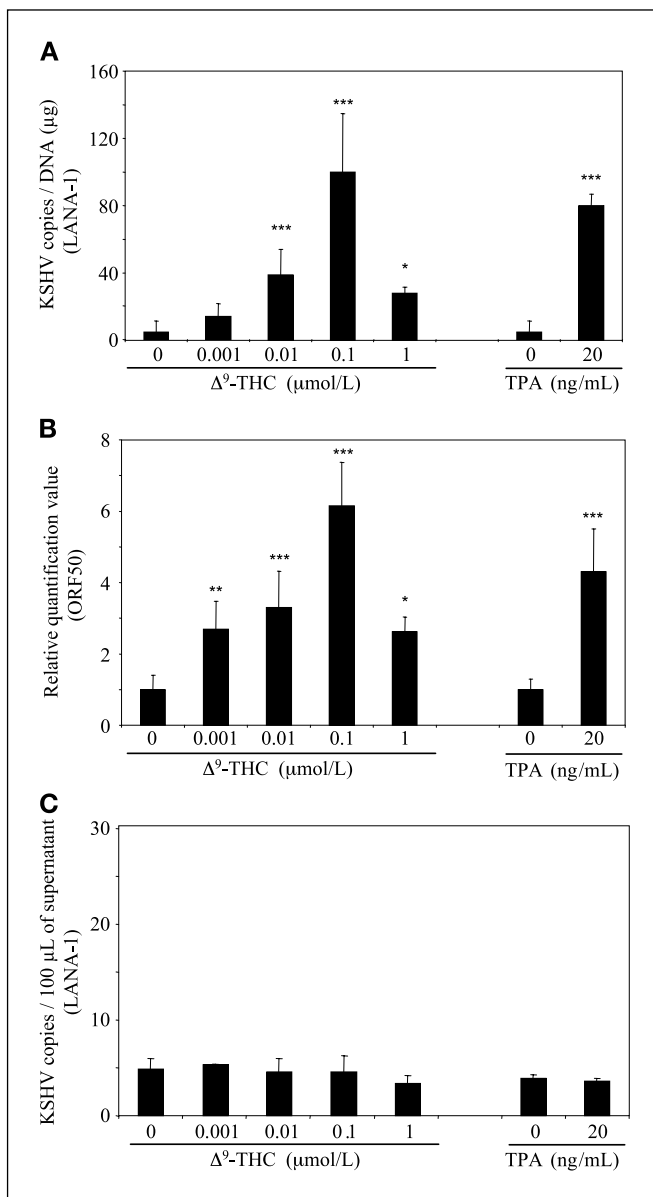


Figure 3. Low doses of Δ^9 -THC stimulate KSHV replication. Cellular DNA and total RNA samples were prepared from KSHV-infected HMVEC after the indicated treatments. **A**, KSHV *LANA-1* copies as determined by real-time DNA PCR analysis. **B**, the expression of KSHV *ORF50* based on real-time RT-PCR analysis. Relative quantification value was calculated based on the $\Delta\Delta C_t$ method. **C**, *LANA-1* copies in the supernatant of KSHV-infected HMVEC based on real-time DNA PCR analysis. Columns, mean; bars, SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for the treatment with Δ^9 -THC or TPA versus vehicle control.

Results

Low doses of Δ^9 -THC increase KSHV infection. We first observed robust expression of the cannabinoid receptors, CB1 and CB2, in HMVEC (Fig. 1A). Next, to investigate whether Δ^9 -THC affects KSHV infection, HMVEC were treated with different doses of Δ^9 -THC (0, 0.001, 0.01, 0.1, and 1 $\mu\text{mol/L}$) or vehicle control for 1 h before exposure to GFP-KSHV. Low doses of Δ^9 -THC (<1 $\mu\text{mol/L}$) increased KSHV infection in a concentration-dependent manner compared with the vehicle treatment (Fig. 1B and C). High doses of Δ^9 -THC (>1 $\mu\text{mol/L}$) caused considerable cell death in combination with KSHV exposure (data not shown). In addition, we found that

KSHV infection of endothelial cells slightly up-regulated the expression of CB1 and CB2 (data not shown). However, we did not observe any significant changes in the expression of integrin $\alpha_3\beta_1$, vascular endothelial growth factor (VEGF) receptor (VEGFR)-3, or DC-SIGN on treatment with Δ^9 -THC (data not shown).

We next sought to determine whether Δ^9 -THC may modulate KSHV entry into endothelial cells. KSHV mainly enters target cells through endocytosis, which is controlled by reorganization and rearrangement of the cytoskeleton (21, 24). To this end, we studied the effects of Δ^9 -THC on the cytoskeletal system. By using Western blot analysis, we found that two important cytoskeletal proteins, p130Cas and paxillin, were significantly phosphorylated after exposure to Δ^9 -THC (Fig. 2A). To further confirm the above observations, we did confocal scanning to detect activated paxillin under the same conditions. As shown in Fig. 2B, activated paxillin exhibited staining with fluorescein. The basal level of activated paxillin was observed to be uniform in the endothelial cytoplasm in quiescent cells. After Δ^9 -THC stimulation, significantly more paxillin was activated and aggregated beneath the cell surface. Cell membranes seemed invaginated and some pseudopodia-like dendrites were formed. These morphologic changes in cell shape can increase the interaction of cells with viruses, enhance endocytosis, and could also account for the observed increase in KSHV infection. In this regard, a specific endocytosis inhibitor, cytochalasin D, was used to block the activation of the cytoskeletal system induced by Δ^9 -THC. Pretreatment with cytochalasin D (1 $\mu\text{mol/L}$; Sigma-Aldrich) significantly decreased KSHV infection in the presence of Δ^9 -THC (Fig. 2C), suggesting that Δ^9 -THC modulates the process of endocytosis and leads to higher KSHV infection efficiency in HMVEC.

Low doses of Δ^9 -THC induce KSHV replication in endothelial cells. The status of the KSHV life cycle is closely linked to the course of KSHV-associated diseases. The major latency-associated nuclear antigen of KSHV (*LANA-1*, ORF73) is expressed by infected cells and has been commonly used as a marker of KSHV load in real-time PCR assays (23). Chemical agents like 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induce KSHV and have been used as a positive control to study cannabinoid effects on viral latency. We found a significant increase in the number of *LANA-1* copies in KSHV-infected HMVEC on treatment with Δ^9 -THC but not in the culture supernatants (Fig. 3A and C). Viral loads were observed to increase over time within a week after exposure to Δ^9 -THC (data not shown). Basal titers of KSHV in the HMVEC were much lower than those in the GFP-BCBL-1 cells. However, the effect of Δ^9 -THC on viral titers was more significant in the KSHV-HMVEC than in the GFP-BCBL-1 cells (data not shown). These results suggest that the KSHV life cycle is cell dependent and is differentially modulated in response to certain stimuli like cannabinoids.

We then assessed the expression of *ORF50*, the major switch gene for KSHV from latency to the lytic cycle, after treatment of endothelial cells with Δ^9 -THC. We observed that *ORF50* expression was up-regulated by Δ^9 -THC in KSHV-infected HMVEC (Fig. 3B). These results indicate that Δ^9 -THC-induced *ORF50* expression can promote KSHV lytic replication in HMVEC.

Low doses of Δ^9 -THC foster KSHV-infected HMVEC to form colonies in vitro. Δ^9 -THC has been reported to enhance tumor cell proliferation and to accelerate cancer progression (25–27). We found a biphasic activity of Δ^9 -THC on KSHV-mediated transformation. Low doses of Δ^9 -THC stimulated colony formation, whereas high doses of Δ^9 -THC inhibited colony formation (Fig. 4A). To decipher how Δ^9 -THC may change the growth status of tumor

cells, we investigated the expression levels of KSHV GPCR, one of the key elements in KSHV-mediated cell transformation. We observed a pattern of KSHV GPCR distribution on the cell surface similar to that of normal GPCRs. Isolated KSHV GPCR pits or vesicles were observed on the cell membrane in KSHV-infected HMVEC in the absence of cannabinoid treatment. However, significantly more and brighter KSHV GPCR vesicles aggregated on the cell surface of spindle-shaped cells after exposure to Δ^9 -THC (Fig. 4B). The increased expression of KSHV GPCR was further

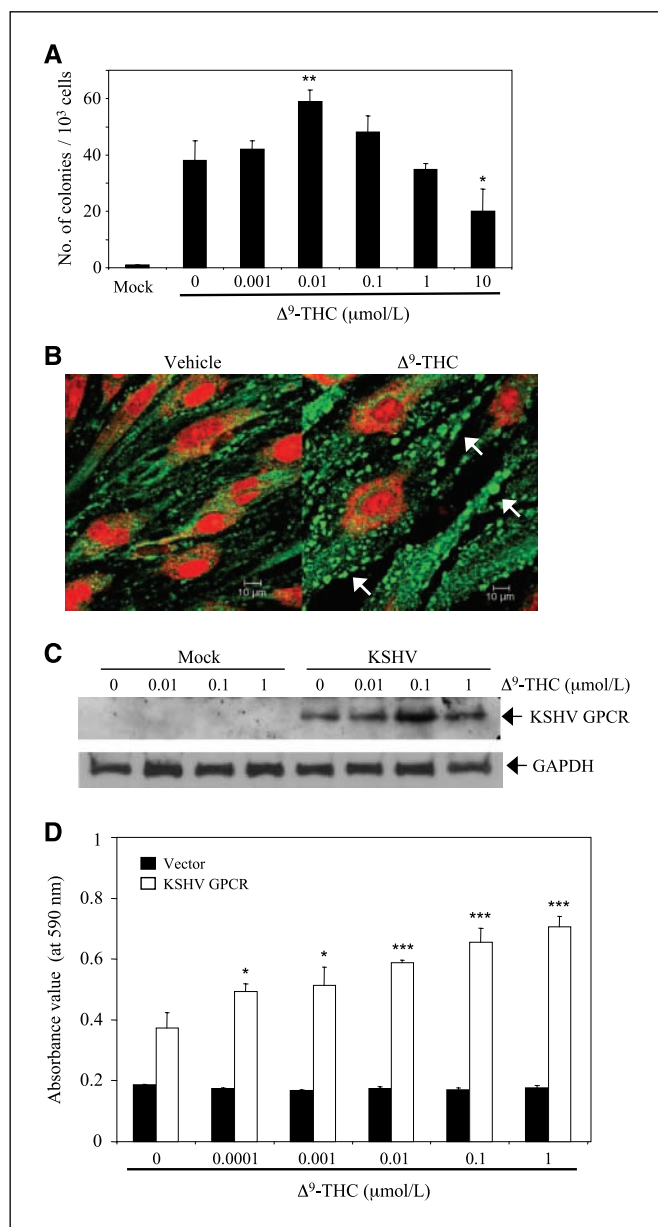


Figure 4. Low doses of Δ^9 -THC induce the growth of KSHV-infected HMVEC. **A**, the number of colonies of KSHV-infected HMVEC grown in soft agar in the presence or absence of Δ^9 -THC was counted under a microscope. *Columns*, mean; *bars*, SD. *, $P < 0.05$; **, $P < 0.01$ compared with the vehicle-treated HMVEC containing KSHV. **B**, Δ^9 -THC enhances the activation of KSHV GPCR in KSHV-infected spindle cells. *Arrows*, active KSHV GPCR vesicles. *Bar*, 10 μm . **C**, the expression level of KSHV GPCR in the presence or absence of Δ^9 -THC is shown by Western blotting. GAPDH served as a loading control. **D**, Δ^9 -THC induces cell proliferation in KSHV GPCR-transfected HMVEC. *Columns*, mean; *bars*, SD. *, $P < 0.05$; ***, $P < 0.001$ for the treatment with Δ^9 -THC versus vehicle control.

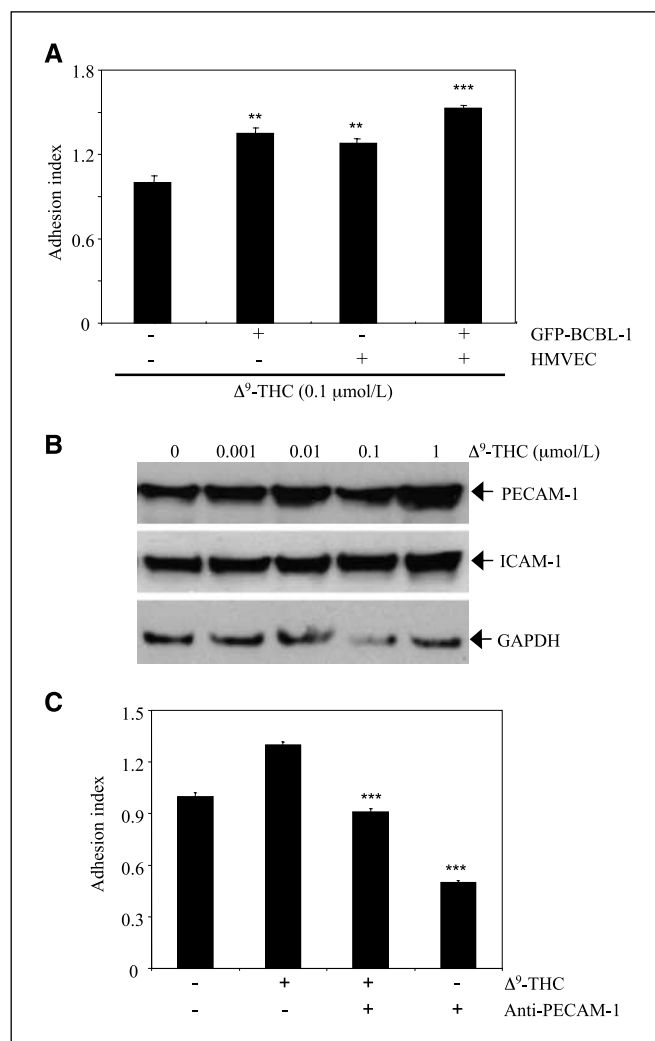


Figure 5. Δ^9 -THC enhances KSHV-infected GFP-BCBL-1 cell adhesion to endothelial cells. **A**, HMVEC and GFP-BCBL-1 cells were treated for 30 min with Δ^9 -THC, TPA, or their vehicle controls, as indicated. GFP-BCBL-1 cells were then cocultured with HMVEC for another 30 min. After vigorous washing, attached GFP-BCBL-1 cells were quantitated by fluorescent counter. **, $P < 0.01$; ***, $P < 0.001$ compared with the untreated group. **B**, the expression levels of PECAM-1 and ICAM-1 as analyzed by Western blotting. GAPDH was used to show uniform protein loading. **C**, blockade of PECAM-1 inhibits Δ^9 -THC-induced adhesion. Cells were pretreated with neutralized anti-PECAM-1 antibody (15 $\mu\text{g}/\text{mL}$) or IgG control for 30 min and then used for the adhesion assay, as described in Materials and Methods. *Columns*, mean; *bars*, SD. ***, $P < 0.001$ compared with the control group.

confirmed by Western blotting (Fig. 4C). These results suggest the involvement of KSHV GPCR in the transforming effects of Δ^9 -THC.

To show whether KSHV GPCR is integrally involved in Δ^9 -THC-induced cell proliferation, we transiently transfected HMVEC with plasmids encoding either KSHV GPCR or vector alone and then quantitated and compared the effect of Δ^9 -THC on cell proliferation. We found a significant increase in cell proliferation following Δ^9 -THC treatment of the KSHV GPCR-expressing cells but no such effect with the control cells (Fig. 4D). In addition, we observed no significant changes in growth-regulated oncogene- α or interleukin-8 (IL-8) production in the cultured KSHV-infected HMVEC (data not shown). These two growth factors have been reported to stimulate Kaposi's sarcoma growth through binding to their cognate receptors and KSHV GPCR. These data indicate that

neither autocrine nor paracrine loops contribute to the effects of Δ^9 -THC, but rather that up-regulation of the constitutively active KSHV GPCR mediates the effects of Δ^9 -THC on Kaposi's sarcoma cell proliferation.

Low doses of Δ^9 -THC induce the adhesion of KSHV-infected B cells to endothelial cells. Latent-infected lymphocytes constitute the major pathologic reservoir of KSHV *in vivo*. When KSHV latency switches into lytic replication in B cells and lytically infected lymphocytes circulate or home to adjacent lymph nodes, interactions between lymphocytes and endothelial cells frequently occur, which may enhance viral secondary transmission. To address whether Δ^9 -THC could modulate viral transmission, we used an *in vitro* static adhesion assay to investigate the effects of Δ^9 -THC on the interaction between lymphocytes and endothelial cells. Lytically infected GFP-BCBL-1 cells were prepared by using TPA (20 ng/mL) and then coincubated with HMVEC. Both GFP-BCBL-1 cells and HMVEC were treated in the absence or presence of Δ^9 -THC, as indicated. We found that Δ^9 -THC significantly enhanced the adhesion of GFP-BCBL-1 cells to HMVEC (Fig. 5A), leading to increased KSHV infection in HMVEC compared with the vehicle control (data not shown). Further experiments showed that the Δ^9 -THC-induced adhesion was due to up-regulated PECAM-1 expression (Fig. 5B), as neutralized anti-PECAM-1 antibody blocked the Δ^9 -THC-induced effects (Fig. 5C). These data suggest that Δ^9 -THC stimulates expression of the endothelial-specific focal adhesion molecule PECAM-1, resulting in enhanced cell-cell interactions.

Different cannabinoid receptors are involved in Δ^9 -THC-mediated signal transduction. To investigate which cannabinoid receptors might play an important role in the Δ^9 -THC-induced effects, we blocked cannabinoid receptors with specific antagonists of the receptors. We observed that Δ^9 -THC induced significant expression of PECAM-1 and β -catenin but not ICAM-1 in KSHV-infected HMVEC. Specific antagonists, including AM251, AM630, and O-1918, only partially blocked these effects (Fig. 6). Our results suggest that different cannabinoid receptors are involved in Δ^9 -THC-mediated signal transduction.

Discussion

KSHV infection is prevalent and lifelong, and identification of cofactors that may contribute to the pathogenesis of Kaposi's

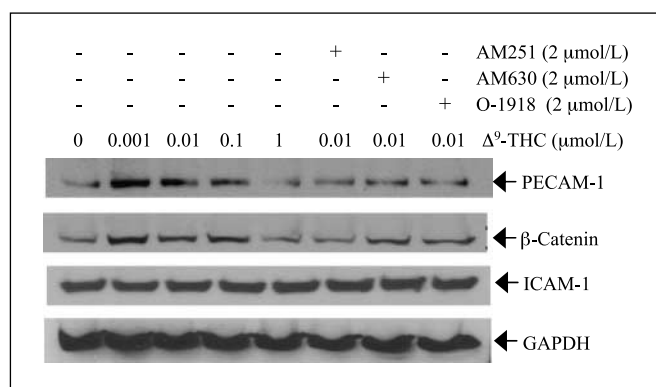


Figure 6. Δ^9 -THC induces the expression of PECAM-1 and β -catenin in KSHV-infected HMVEC. Cells were pretreated with specific antagonists as indicated and then incubated with Δ^9 -THC or vehicle overnight. The expression of various proteins was then examined sequentially as shown. GAPDH was used to check protein loading.

sarcoma could assist in its prevention (28). Based on epidemiologic studies, drug addiction increases the risk of acquiring infectious diseases (15). In addition, marijuana use is common in U.S. and European populations, and medicinal treatment with cannabinoids is increasing. Thus, in this report, we address the role of cannabinoids in the pathogenesis of Kaposi's sarcoma. Our studies strongly suggest that Δ^9 -THC could worsen KSHV infection and foster development of the neoplasm.

We found that Δ^9 -THC modulated host-virus interactions via its effects on endocytosis. We also observed that low doses of Δ^9 -THC (<1 μ mol/L) activated the cytoskeletal system in HMVEC, with significant phosphorylation of p130Cas and paxillin, two critical components of the focal adhesion complex. These proteins are required for the control of actin organization and the formation of microvilli-like cellular protrusions and for the internalization of bacteria as well as the migratory responses of cells (29–31). In particular, p130Cas protein has been reported to participate in the uptake of adenovirus, human respiratory syncytial virus, and *Yersinia* through either endocytosis or phagocytosis (32–34). Furthermore, both p130Cas and paxillin proteins interact with focal adhesion kinase (FAK) and are tyrosine phosphorylated during integrin-mediated adhesion. Integrins and FAK are involved in KSHV entry into target cells (21, 35). Our findings indicate that cannabinoids like Δ^9 -THC may facilitate KSHV infection through activation of certain cytoskeletal proteins.

We further showed that Δ^9 -THC modified KSHV status through regulation of the key viral gene *ORF50*. Maintenance of latent status and reactivation of lytic replication are essential for Kaposi's sarcoma occurrence. The latent infection contributes to immune evasion, whereas lytic infection leads to the production of infectious viral progeny. KSHV latency usually dominates the KSHV life cycle, and KSHV starts its lytic cycle through unclear mechanisms. Because *ORF50* expression is sufficient for KSHV lytic viral reactivation and *ORF50* targeting efficiently inhibits KSHV replication, it has been recognized as a major switch gene for KSHV reactivation (36, 37). In prior studies, TPA and hypoxia were found to stimulate the lytic replication of KSHV via *ORF50*, and methotrexate was shown to inhibit TPA-mediated lytic replication also through *ORF50* (38–40). Therefore, Δ^9 -THC-induced *ORF50* gene expression and subsequent KSHV lytic replication may significantly change the course of KSHV-associated diseases.

Moreover, we showed that Δ^9 -THC strongly induced the expression of KSHV GPCR on the cell surface and significantly fostered endothelial transformation *in vitro*. Kaposi's sarcoma is a typical vascular tumor expressing high levels of VEGF and VEGFR. Δ^9 -THC has been reported to inhibit the angiogenesis and tumorigenesis of gliomas via the VEGF/VEGFR pathway (41). However, this does not seem to be the case in Kaposi's sarcoma because the dose-dependent inhibitory effects of Δ^9 -THC on VEGF/VEGFR were only observed in HMVEC and not in KSHV-infected HMVEC and Kaposi's sarcoma cells (data not shown). In contrast, we found that Δ^9 -THC induced the expression of KSHV GPCR, a homologue of the IL-8 receptor. It has been reported that constitutively active KSHV GPCR triggers the autonomous proliferation of endothelial cells, causes Kaposi's sarcoma-like lesions in mice, and initiates the development and progression of Kaposi's sarcoma (42–45). The differing effects of Δ^9 -THC on KSHV observed in our study and other reports may be due to the varied transcriptional activation of KSHV genes in different cell

types (endothelial cells versus B cells) after infection. In addition, different concentration ranges of Δ^9 -THC (low versus high dosage) as well as varied technological approaches were used in our study as compared with the previous report. Hence, it seems that distinct pathogenetic pathways may exist for KSHV-associated neoplasms.

Cannabinoids are a family of multifunctional compounds and their targets are not fully understood. It is known that Δ^9 -THC binds to the CB1 and CB2 receptors with equal affinity. However, Δ^9 -THC may also bind to other unknown receptors because vanilloid receptor 1 and GPCR55 also serve as cannabinoid receptors (46, 47). Here, we found that both CB1 and CB2 receptors as well as an endothelial cannabinoid receptor distinct from CB1 and CB2 (20) may be involved in the Δ^9 -THC-mediated effects on KSHV because selective antagonists for each of these receptors only partially blocked the Δ^9 -THC-induced effects. Further investigation is needed to clarify the multifunctional effects of cannabinoids.

In conclusion, our study indicates that Δ^9 -THC in concentrations easily achieved *in vivo* may enhance KSHV infection, accelerate KSHV replication, and/or foster KSHV-mediated transformation. These observations suggest that Δ^9 -THC may accelerate the development, progression, and spread of Kaposi's sarcoma. It is

important to emphasize that marijuana is not a single drug but a complex botanical substance. Although Δ^9 -THC is the major psychoactive component of marijuana, many of its other components are not well elucidated. The clinical pharmacology of the constituents of marijuana is likely complicated, particularly when the plant is smoked or eaten in its natural form. Indeed, both viral enhancers and inhibitors may coexist within marijuana. Hence, for the medical use of purified cannabinoids like Δ^9 -THC, our study suggests that their administration may place patients at greater risk for KSHV infection and Kaposi's sarcoma and that treatment should be evaluated on a case by case basis under close medical supervision. Further epidemiologic studies and clinical research are needed to clarify the importance and safety of administering cannabinoids like Δ^9 -THC.

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