Propranolol Decreases Proliferation of Endothelial Cells Transformed by Kaposi’s Sarcoma-Associated Herpesvirus and Induces Lytic Viral Gene Expression

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Kaposi’s sarcoma (KS) is common in Africa, but economic constraints hinder successful treatment in most patients. Propranolol, a generic β-adrenergic antagonist, decreased proliferation of KS-associated herpesvirus (KSHV)-infected cells. Downregulation of cyclin A2 and cyclin-dependent kinase 1 (CDK1) recapitulated this phenotype. Additionally, propranolol induced lytic gene expression in association with downregulation of CDK6. Thus, propranolol has diverse effects on KSHV-infected cells, and this generic drug has potential as a therapeutic agent for KS.

FIG 1 Propranolol reduced the number of KSHV-infected but not mock-infected EC. Solid lines, KSHV-infected cells; dashed lines, mock-infected cells. (A) By colorimetric measurement of XTT metabolism following 48 h of exposure to propranolol, the drug reduced the number of KSHV- but not mock-infected cells in a dose-dependent manner (P < 0.0046; mixed linear regression, quadruplicate data). Data were normalized to the level of XTT metabolism for untreated, mock-infected cells. (B) KSHV- and mock-infected cells metabolized XTT equally (P = 0.155; mixed linear regression, quadruplicate data). Data were normalized to the level of XTT metabolism for 1 × 10^5 mock-infected cells.
genes and infection with KSHV were performed as described previously (11). E6 and E7 allow for extended culture of EC without inducing a transformed phenotype; following infection with KSHV, however, E6/E7-expressing cells develop a fully transformed phenotype, including postconfluent and anchorage-independent growth (3,4, 11–13). The presence of E6 and E7 also allows for prolonged culture (in these experiments, 48 h) of EC in low-serum medium without recombinant growth factors, conditions that would otherwise induce apoptosis of other EC types due to growth factor withdrawal (14,15). Therefore, while this in vitro model does not fully recapitulate the KS tumor microenvironment and the behavior of explanted KS cells in culture, it is uniquely suited for the evaluation of putative progrowth autocrine signaling pathways and assessment of the mechanisms by which KSHV is able to transform cells.

To measure the effect of propranolol on postconfluent growth, mock- and KSHV-infected cells were plated at confluence (2.5 × 10^4 cells/well) on 96-well Primaria plates (Corning, Tewksbury, MA) in 100 μL of endothelial basal medium 2 plus 1% fetal bovine serum (EBM-2-1%). After 24 h, cells were re-fed EBM-2-1% with or without propranolol (Fisher, Pittsburg, PA) and incubated for 48 h. Cell number was determined by colorimetric measurement of XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] bioreduction (Cayman Chemical Company, Ann Arbor, MI) (5). All data presented in this paper are representative of at least three independent experiments. Numbers of KSHV-infected cells but not mock-infected cells were significantly decreased in a dose-dependent fashion by propranolol (Fig. 1A). Equal metabolism of XTT by KSHV- and mock-infected cells was confirmed by measuring XTT metabolism 6 h after plating (Fig. 1B).

Decreased numbers of cells could have resulted from decreased proliferation or increased cell death. Accordingly, lactate dehydrogenase (LDH; Cayman) was measured in supernatants as a marker of cell death. No significant increase in LDH release was observed with propranolol treatment (Fig. 2A). A slight decrease in cell viability was noted using LIVE/DEAD cell viability stain (Molecular Probes, Pittsburg, PA), assessed using a FACSCanto flow cytometer (BD, San Jose, CA) (Fig. 2B), but cell death was not associated with high levels of cleaved caspase 3 or cleaved poly(ADP-ribose) polymerase (PARP) (Fig. 2C). Together, these data demonstrate that propranolol decreased the number of KSHV-infected cells in postconfluent cultures primarily by decreasing proliferation.
CDK1 and cyclin A2 contribute to postconfluent proliferation of KSHV-infected cells. In normal cells, cyclin A2 binds to CDK2 and CDK1 to mediate events during the G_1-S and G_2-M transitions, respectively (16, 17). Upregulation of cyclin A2 drives proliferation of EC released from contact inhibition and is a characteristic of numerous tumors (16, 17). In KS, cyclin A2 expression is upregulated by KSHV infection in vitro (3, 18) and cyclin A2 levels correlate with advanced tumor stage (19). Therefore, we assessed the expression levels of cyclin A2 and other related cell cycle mediators.

Immunoblotting for multiple CDKs, CDK inhibitors, and cyclins demonstrated that KSHV-infected EC upregulated expression of CDK2 and cyclins A2 and E2 and that propranolol treatment decreased expression of CDK4, CDK6, and cyclins A2 and E2 (Fig. 3A). Downregulation of CDK4 and CDK6 by propranolol was associated with a reduction in phosphorylation at three sites on the retinoblastoma protein (Rb) (Fig. 3B). The CDK1 and CDK2 inhibitor roscovitine (20) also decreased the numbers of KSHV-infected EC but not mock-infected EC in a dose-dependent manner (Fig. 3C). To determine which CDKs were required for KSHV-infected cell proliferation, cells were transfected with Silencer Select small interfering RNAs (siRNAs; Ambion, Pittsburg, PA) and the specificity of knockdowns was assessed by immunoblotting (Fig. 3D). KSHV-infected EC were untransfected and untreated, untransfected and treated with propranolol, or transfected with control or target siRNA, and cell number was assessed by measuring XTT metabolism (Fig. 3E). As expected, 50 μM propranolol reduced the number of cells compared with that of untreated controls. Multiple siRNAs reduced cell numbers, but knockdown of CDK1 and cyclin A2 had the largest effects. Cell cycle profiles of KSHV-infected cells were analyzed following DNA staining with 7-aminoactinomycin D (7-AAD; BD) by flow cytometry, and subpopulations were quantified using Dean-Jett-Fox modeling in FlowJo software (FlowJo, Ashland, OR). Propranolol treatment resulted in an increased number of cells in S phase and a decreased number of cells in G_2-M compared with numbers for untreated cells (Fig. 3F). Knockdown of cyclin A2 and CDK1 both resulted in increased percentages of cells in G_2-M, and knockdown of cyclin A2 also showed an increase in the percentage of cells in S phase to a level similar to that of cells treated with propranolol. Taken together, these data suggest that proliferation of cells transformed by KSHV requires endogenous CDK1/cyclin A2 complexes and that downregulation of cyclin A2 by propranolol in part mediates the observed reduction in proliferation following drug treatment. Importantly, the slight but statistically significant reductions in proliferation observed following knockdown of CDK2, CDK4, and cyclin B1 suggest that these cell cycle regulators also contribute to KS cell proliferation. It is notable that the cell cycle subpopulations observed in cells treated with propranolol differ markedly from those in cells in which CDK1 or cyclin A2 was knocked down, supporting the interpretation that propranolol has inhibitory effects at multiple stages of the cell cycle.

Propranolol induces KSHV lytic gene expression. CDK6 has been shown to participate in the maintenance of latency by binding to the viral cyclin homolog and phosphorylating nucleophosmin (21). Since propranolol decreased expression of CDK6, we used semiquantitative reverse transcription (RT)-PCR to assess lytic gene expression in KSHV-infected cells treated with propranolol (18S rRNA forward [fwd] primer, GTA ACC CTT TGA ACC CCA TT; 18S rRNA reverse [rev] primer, CCA TCC AAT CAG TAG TAGGG; RTA fwd primer, CAA GGT GTG CTT TGA GTT AGA GA; RTA rev primer, TCC CAA AGA GGT ACC AGG TG; viral interleukin-6 [vIL-6] fwd primer, TGCTG TTC AAG ATG TGG TTC; vIL-6 rev primer, ATG CCG GTG CCA GAA CAG AG; K8.1 fwd primer, CAG CAC AGA ACT GAC CGA CCA; K8.1 rev primer, TGG CAC AGC GTT ACT AGC AC; ORF65 fwd primer, GGA TGA GAG GTT GTG GTA AAT; ORF65 rev primer, CTC GGG AAG CAG TAT AAC CAC; viral interferon regulatory factor [vIRF] fwd primer, GGA AGA ACA ATG CTT GGA ATG; and vIRF rev primer, CTA GTG GCT TGT CTT GAT TA). Propranolol induced expression of lytic viral genes from each kinetic class in a time-dependent (Fig. 4A) and dose-dependent (Fig. 4B) manner. Knockdown of CDK6 with siRNA, confirmed by immunoblotting (Fig. 4C), induced expression of lytic viral genes from each kinetic class (Fig. 4D). Together, these data suggest that induction of viral lytic gene expression by propranolol is mediated in part by downregulation of CDK6.

The focus of this investigation was on CDKs and cyclins because these cellular proteins drive the specific events of cell cycle progression following stimulation by myriad mitogenic signaling pathways. Further experimentation is required to elucidate which upstream proangiogenic pathways induced by β-adrenergic signaling, such as vascular endothelial cell growth factor (VEGF) and signal transducer and activator of transcrip-
tion 3 (STAT3), mediate the observed proliferative advantage of KSHV-infected cells and contribute to the maintenance of viral latency (22–24).

The dose range of propranolol used here, though higher than concentrations in serum achievable using standard clinical dosing, is consistent with the concentrations of the drug used in in vitro studies of IH cells (23–25). Importantly, however, there is a disconnect between effective in vitro and in vivo dosing of propranolol, as the effective and safe oral dose of propranolol for IH is 1 to 3 mg/kg of body weight/day (9, 10), where 2 mg/kg/day results in average and peak blood levels of 0.234 ± 0.096 μM and 0.277 ± 0.113 μM, respectively, in term and preterm infants (26). Notably, however, cells in the experiments reported here were treated with a single dose of propranolol during 48 h of incubation, whereas clinical use would employ multiple daily doses.

Propranolol and similar agents have been explored for the treatment of malignancies other than IH, and various phenotypes, including decreased proliferation and migration and increased sensitivity to cytotoxic chemotherapy agents, have been described previously (22, 27–29). Notably, a topical preparation of timolol, another β-adrenergic antagonist, was effective in treating two cases of classic KS (30). Given that propranolol has a favorable safety profile and excellent oral

**FIG 4** Propranolol induced expression of lytic viral genes. (A) Semiquantitative RT-PCR revealed that expression of KSHV lytic genes of all kinetic classes was induced by 50 μM propranolol (solid lines) in a time-dependent manner to levels similar to those induced by 20 nM PMA (dashed lines). (B) After 48 h of exposure to propranolol, maximum induction of lytic gene expression was achieved at a concentration of 50 μM. (C) Knockdown of CDK6 using siRNA was confirmed by immunoblotting. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (D) Knockdown of CDK6 induced expression of KSHV lytic genes of all kinetic classes.
bioavailability, this drug may be a useful adjunct for chemotherapy against more aggressive forms of KS. Furthermore, propranolol is included on the World Health Organization’s list of essential medicines and is therefore expected to be available for use in limited-resource settings (http://www.who.int/medicines/publications/essentialmedicines/en/).

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S.C.M. designed the research and wrote the manuscript. S.C.M., R.S.H., and R.D.M. conducted experiments.

We declare that we have no conflicts of interest.

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