Roscovitine Inhibits STAT5 Activity and Induces Apoptosis in the Human Leukemia Virus Type 1-Transformed Cell Line MT-2

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ABSTRACT

T cells expressing human leukemia virus (HTLV) type 1, the etiological agent of adult T-cell leukemia, are remarkably resistant to conventional chemotherapy, and the need for drugs that effectively kill these cells is apparent. Here we show that roscovitine, an inhibitor of cyclin-dependent kinases (CDKs), induces the apoptosis of the HTLV-1-transformed T-cell line MT-2. Roscovitine prevented the tyrosine phosphorylation and consequent activation of the transcription factor signal transducer and activator of transcription (STAT) 5 when presented to MT-2 cells in the presence or absence of a caspase-3 inhibitor, and ectopic expression of a dominant-negative form of STAT5 in MT-2 cells induced apoptosis. Roscovitine and dominant-negative STAT5 also reduced the expression of the antiapoptotic protein XIAP, and STAT5 was associated with the XIAP promoter in vivo. Antibody to platelet-derived growth factor (PDGF) α receptors coprecipitated STAT5 from extracts of untreated but not roscovitine-treated cells. The tyrosine phosphatase inhibitor sodium orthovanadate ablated the inhibitory effects of roscovitine on STAT5/PDGF α receptor interaction, STAT5 activity, and cell survival. We suggest that roscovitine reduces the abundance of tyrosine-phosphorylated PDGF α receptors; as a result, STAT5 does not become active, and STAT5 gene products required for cell survival are not expressed.

INTRODUCTION

ATL\(^1\) is an aggressive lymphoproliferative disorder that occurs in individuals infected with HTLV-1 (1, 2). The poor prognosis of patients with ATL results in part from the innate resistance of HTLV-1-infected cells to apoptosis and thus to conventional chemotherapy regimens (3). HTLV-1 encodes Tax, which is the primary mediator of its transforming actions, and Tax promotes T-cell survival in multiple ways (1, 2). For example, Tax activates the transcription factor NF-κB (4–6), which up-regulates the expression of genes encoding antiapoptotic proteins such as Bcl-X\(_L\) and the IAP (7–9). Tax-independent events also enhance the survival of HTLV-1-infected T cells. Such events include increased production of the IAP-related protein survivin (10).

The need for agents that kill HTLV-1-infected cells is obvious, and cultured T cells that express HTLV-1 (or Tax in the absence of virus) have been used as tools for drug screening. Many routinely used antiapoptotic drugs or treatments had little or no effect on the survival of HTLV-1-infected cells; these include Taxol, tumor necrosis factor α, Fas antibody, and UV irradiation (11, 12). In some instances, all-trans retinoic acid reduced the survival of HTLV-1-infected cells (13), whereas in others, it did not (14). More promising results were obtained using drug combinations such as arsenic trioxide and either all-trans retinoic acid or IFN-α (14–16). In both cases, apoptosis correlated with reductions in abundance of Tax and NF-κB activity. Attempts to induce apoptosis by NF-κB inhibitors, most of which were nonspecific, were successful in some, but not all, studies (17–19). Thus, despite progress, the search for drugs that efficiently kill HTLV-1-infected T cells, either alone or in combination, is far from complete.

Anti-ATL drug targets other than Tax and NF-κB include the JAKs and the STAT proteins. In normal T cells, cytokines activated JAKs and activated JAKs phosphorylate cytosolic STAT monomers at specific tyrosine residues (20). Tyrosine-phosphorylated STATs dimerize, translocate to the nucleus, and interact with specific DNA response elements to induce or repress transcription. In cultured T cells infected with HTLV-1, JAK3 and STAT5 are often active in the absence of exogenously added cytokines (21–25). Constitutive activation of JAK3 and STAT5 occurs several months after infection and coincides with cell transformation and the switch from cytokine-dependent to cytokine-independent cell proliferation. JAK3 and STAT5 are also active in T cells derived from some ATL patients (24).

The mechanism responsible for JAK3/STAT5 activation in HTLV-1-infected cells is not known, but apparently does not involve Tax or the autocrine production of interleukin 2 or interleukin 15 (21–24). Like NF-κB, STAT5 induces the expression of Bcl-X\(_L\) (26, 27), and ablation of STAT5 function increases the spontaneous or drug-initiated apoptosis of hematopoietic cells (26, 28–30). Thus, STAT5 is an attractive target for drug intervention. STAT5 is also constitutively active in leukemias other than ATL because of activation of JAKs or other tyrosine kinases such as Bcr-Abl or the PDGF receptor (31).

Roscovitine is a purine analogue that inhibits the activity of the CDKs that mediate the progression of cells into S phase (CDK2) and mitosis (cdk2) (32, 33). As a result of or in addition to CDK inactivation, roscovitine is apoptotic for numerous cell types. These include adenosine-resistant mouse leukemia cells (34), breast carcinoma cells (35, 36), pancreatic and gastric cancer cells (37, 38), Jurkat T-cell leukemia cells (39), and head and neck squamous cell carcinomas (40). Roscovitine also potentiates apoptosis induced by farnesyltransferase inhibitors and E2F-1 (41–43). The mechanism by which roscovitine induces apoptosis is not known, although an involvement of the apoptotic protein Bcl-X\(_L\) and cytochrome c (which activates the apoptotic enzyme caspase-3) has been suggested (40, 41).

Here we show that roscovitine induces the apoptosis of the HTLV-1-transformed T-cell line MT-2. As a mechanism of action, we suggest that roscovitine reduces the survival of MT-2 cells by inhibiting the activity of STAT5. Rather than JAK-dependent STAT5 activation, we propose a model of apoptosis in which roscovitine targets the interaction of STAT5 with the PDGF α receptor.

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The abbreviations used are: ATL, adult T-cell leukemia; HTLV, human leukemia virus type; STAT, signal transducer and activator of transcription; PDGF, platelet-derived growth factor; NF-κB, nuclear factor κB; JAK, Janus kinases; CDK, cyclin-dependent kinase; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; PARP, poly(ADP-ribose) polymerase; IAP, inhibitor of apoptosis protein; XIAP, X-linked IAP.
MATERIALS AND METHODS

Cell Culture. MT-2 cells were grown in suspension in RPMI 1640 containing 2 mM l-glutamine, 50 units/ml penicillin, and 10% FCS. All experiments were done on exponentially proliferating cells.

Annexin Binding Assay. Cells were rinsed with PBS and resuspended in binding buffer [1 mM HEPES (pH 7.5), 140 mM NaCl, and 0.25 mM CaCl₂] at a concentration of 10⁶ cells/ml. Aliquots (100 µl) were incubated with 5 µl of phycoerythrin-conjugated annexin V and 5 µl of 7-amino-actinomycin D for 15 min at room temperature. Aliquots were brought to 400 µl with binding buffer and analyzed by flow cytometry.

Immunoprecipitation and Western Blotting. Cells were lysed in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.5% NP-40, 1 mM DTT, 0.1 mM PMSF, 2.5 µg/ml leupeptin, 0.5 mM NaF, and 0.1 mM NaVO₄ (lysis buffer). After a 30-min incubation, insoluble material was removed by centrifugation. For immunoprecipitations, cell extracts (100 µg of protein) were incubated with antibody overnight at 4°C. Immune complexes were recovered with protein A-agarose beads (1:2 h, 4°C) and washed three times with lysis buffer. For Western blotting, cell extracts (100 µg of protein) or immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked in PBS containing 0.05% Tween and 5% instant milk and incubated with antibody in PBS containing 0.05% Tween 2 h at room temperature. Proteins recognized by the antibody were detected by enhanced chemiluminescence using a horseradish peroxidase-coupled secondary antibody as specified by the manufacturer (Pierce).

mRNA Analysis. Total mRNA was isolated using Trizol, and Northern blotting was performed as described previously (44). Cells were resuspended in a hypotonic buffer containing 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na₃VO₄, 1 mM Na₃P₂O₅, 1 mM DTT, 0.5 mM PMSF, 0.1 µM aprotinin, 1 µM leupeptin, and 1 µM antipain. Nuclei were collected by centrifugation and extracted in a hypotonic buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 20 mM NaF, 1 mM Na₃VO₄, 1 mM Na₃P₂O₅, 1 mM DTT, 0.5 mM PMSF, 0.1 µM aprotinin, 1 µM leupeptin, and 1 µM antipain. Nuclear extracts (10 µg) were incubated with double-stranded 32P-labeled oligonucleotide probes for 30 min at room temperature, and protein-DNA complexes were resolved on 5% nondenaturing polyacrylamide gels and visualized by autoradiography. The probes used were as follows: STAT5 EMSAs, 5'-AGATTTCAGGAAATTCGCA, which contains the mammary gland factor element of the β-casein promoter; and NF-κB EMSAs, 5'-TGACACAGGCGATTTTCGCAGAGGC.

ChIP Assay. ChIP assays were performed using the ChIP assay kit from Upstate Biotechnology. Growing MT-2 cells (3 × 10⁷) were treated with formaldehyde to cross-link DNA and associated proteins. Cross-linked chromatin was extracted, sheared by sonication, and incubated with antibody overnight at 4°C and subsequently incubated with protein A-agarose beads. After washing, immune complexes were eluted from the beads, heated to reverse the cross-links, and treated with proteinase K and RNase A to remove proteins and any contaminating RNA. DNA was analyzed by PCR using primers that generate a 106-bp product that corresponds to a region (~271 to ~375) of the human XIAP promoter. The XIAP sequences used were as follows: forward primer, 5'-TGCCCTGTTAAATATTACCTTCTCTCTAAA-3'; and reverse primer, 5'-ACTACAGCCGCTAAGAAACATCT-3'. As a negative control, PCR reactions were performed using primers specific for the human actin promoter. The actin sequences used were as follows: forward primer, 5'-GGGCTCTGCCCACTCAGC-3'; and reverse primer, 5'-CTGGAGCTGCTGCTTTCAT-3'. PCR products were detected on 2.5% agarose gels.

Vaccinia Virus Infection. Recombinant vaccinia virus encoding dominant-negative STAT5 or CD56 was constructed using the pSP11 vector in recombination with the WR strain of vaccinia. CD56 is a large granular lymphocyte-specific surface marker. Vaccinia virus was generated as described previously (45). For infection, MT-2 cells (5 × 10⁶) were incubated with the virus for 2 h at 37°C in serum-free medium at a multiplicity of infection of 2. Cells were washed and incubated in medium containing 10% serum for 12-18 h at 37°C.

Antibodies. Antibodies were obtained from the following sources: PARP, Cell Signaling; XIAP and Bcl-2, BD Transduction Laboratories; c-IAP-1 and c-IAP-2, R&D; STAT5α, STAT5α/b, STAT1α, and phosphotyrosine, Santa Cruz Biotechnology; survivin, Alpha Diagnostic; and Tax, NIH. Polyclonal antibody to caspase-3 was provided by Dr. Hong-Gang Wang. Polyclonal antibody to PDGF-α receptor was prepared as described previously (46). The caspase-3 inhibitor Z-DEVD-FMK was purchased from Alexis Biochemicals.

RESULTS

Roscovitine Induces the Apoptosis of HTLV-1-Transformed T Cells. Using three different assays, we examined the effects of roscovitine on the survival of MT-2 cells. In the first set of experiments, cells were treated with 12.5 or 25 µM roscovitine for 15 h, and their capacity to bind annexin V was determined by flow cytometry. In cultures receiving 25 µM roscovitine, ~30% of the cells bound annexin V as compared with ~10% of the cells in control cultures (Fig. 1A). In the second set of experiments, cells were treated with 30 µM roscovitine for 10 or 22 h, and cell lysates were assayed for caspase-3 activity. Caspase-3 activity was ~6-fold higher in roscovitine-treated cells than in control cells at 10 h and ~10-fold higher at 22 h (Fig. 1B). In the third set of experiments, cells received 10, 20, or 30 µM roscovitine for 12 h, and cleavage of the caspase-3 substrate PARP was determined by Western blotting. PARP was cleaved to a small extent in cells receiving 20 µM roscovitine and to a larger extent in cells receiving 30 µM roscovitine (Fig. 1C). Collectively, the data in Fig. 1 show that roscovitine induces the apoptosis of MT-2 cells.

Fig. 1. Effects of roscovitine on annexin V binding, caspase-3 activity, and PARP cleavage in MT-2 cells. A, MT-2 cells were treated with DMSO (vehicle control) or 12.5 or 25 µM roscovitine for 15 h. Cells were assayed for annexin V binding as described in “Materials and Methods.” The data show the percentage of cells that bound annexin V. The error bars represent SD. B, MT-2 cells were exposed to 30 µM roscovitine for 10 or 22 h. Cell lysates were analyzed for caspase-3 activity using a colorimetry kit from BioVision. The error bars represent SD. C, MT-2 cells received DMSO or 10, 20, or 30 µM roscovitine for 12 h. PARP cleavage was determined by Western blotting.
At an early time point at which most cells were viable, roscovitine inhibited cell cycle progression, as evidenced by a reduction in the percentage of S phase cells. The percentages of untreated MT-2 cells in G1, S, and G2/M were 39, 29, and 33, respectively. Six h after addition of roscovitine, the percentages of cells in G1, S, and G2/M were 45, 17, and 37, respectively. Thus, roscovitine is both anti-proliferative and pro-apoptotic for MT-2 cells.

As a means of addressing the mechanism by which roscovitine kills MT-2 cells, we assessed its effects on the expression of several antiapoptotic proteins. Exposure of MT-2 cells to 10, 20, or 30 μM roscovitine for 12 h had no effect on the abundance of c-IAP-1, c-IAP-2, survivin, Bcl-XL, and XIAP (Fig. 2A). Similarily, amounts of Bcl-2 and Tax remained constant in MT-2 cells treated with 30 μM roscovitine for times up to 22 h (Fig. 2B). On the other hand, amounts of XIAP fell between 5 and 10 h after addition of 30 μM roscovitine to cells, and XIAP was barely detectable at 22 h. Roscovitine-induced decreases in XIAP abundance were paralleled by decreases in the amounts of XIAP mRNA (Fig. 2A) and increases in the amounts of cleaved PARP (Fig. 2B). These findings show that roscovitine selectively down-regulates XIAP expression at the mRNA level in MT-2 cells.

**Roscovitine Inhibits the Activity of STAT5 in MT-2 Cells.**

There are two closely related forms of STAT5, STAT5a and STAT5b (47). To identify molecular targets of roscovitine in MT-2 cells, we examined the effects of roscovitine on the activity of STAT5a. MT-2 cells received various concentrations of roscovitine for 15 h. Cell extracts were immunoprecipitated with antibody to STAT5a and immunoblotted with antibody to STAT5a or phosphorytrosine. STAT5a was phosphorylated on tyrosine (referred to as P-Tyr-STAT5a) and thus was active in control cells (Fig. 3A). Roscovitine reduced the abundance of P-Tyr-STAT5a without affecting the overall abundance of STAT5a. An approximate 50% decrease in the amount of P-Tyr-STAT5a was seen in cells receiving 10 μM roscovitine, and in cells receiving 30 μM roscovitine, P-Tyr-STAT5a was barely detectable. Thus, when added to MT-2 cells, roscovitine converts active, tyrosine-phosphorylated STAT5a to an inactive, non-tyrosine-phosphorylated form. Roscovitine also reduced the amounts of P-Tyr-STAT5b without affecting total amounts of STAT5b (data not shown).

Decreases in the abundance of P-Tyr-STAT5 in roscovitine-treated MT-2 cells were accompanied by decreases in the amounts of STAT5 DNA binding activity (Fig. 3B). In these experiments, MT-2 cells received various concentrations of roscovitine for 15 h. Nuclear extracts were examined for STAT5 DNA binding activity by EMSA using a radiolabeled oligonucleotide probe containing a STAT5 DNA binding element. Untreated MT-2 cells contained a DNA binding activity that was supershifted by antibodies to STAT5 but not STAT1α. Compared with control cells, amounts of STAT5 DNA binding activity were somewhat lower in cells receiving 20 μM roscovitine and substantially lower in cells receiving 30 μM roscovitine.

**Fig. 2. Effects of roscovitine on the expression of antiapoptotic proteins.** A, MT-2 cells received the indicated concentrations of roscovitine for 12 h. Amounts of c-IAP-1, c-IAP-2, survivin, Bcl-XL, and XIAP were determined by Western blotting, and amounts of XIAP mRNA were determined by Northern blotting. Amounts of 28S and 18S rRNA are shown as loading controls. B, MT-2 cells were exposed to 30 μM roscovitine for the indicated times. PARP cleavage and amounts of Bcl-2, Tax, and XIAP were determined by Western blotting.

**Fig. 3. Inhibition of STAT5 activity by roscovitine.** A, MT-2 cells were treated with DMSO or the indicated concentrations of roscovitine for 15 h. Cell extracts were immunoprecipitated with antibody to STAT5a and immunoblotted with antibody to phosphorytrosine (P-Tyr-STAT5a). The blot was stripped and reprobed with antibody to STAT5a. B, MT-2 cells received DMSO (Lanes 1–4) or the indicated concentrations of roscovitine for 15 h (Lanes 5–7). Nuclear extracts were prepared, and STAT5 DNA binding activity was determined by EMSA using the β-casein probe. For supershifts (Lanes 1–3), nuclear extracts of control cells were incubated with antibody to STAT5a, STAT5ab, or STAT1α (negative control) for 30 min before probe addition. The STAT5ab antibody recognizes both STAT5a and STAT5b.
Roscovitine Inhibited STAT5a Activity and Induced PARP Cleavage with Similar Kinetics in MT-2 Cells (Fig. 4A). Amounts of P-Tyr-STAT5a fell slightly within 3 h of addition of roscovitine (30 μM) to cells and were significantly reduced at 9 and 12 h. PARP cleavage products were detectable 3–6 h after roscovitine addition and increased in abundance thereafter. Thus, whether one event precedes the other is unclear. To determine whether decreases in P-Tyr-STAT5a abundance result from caspase activation and consequent cell death, we cotreated MT-2 cells with roscovitine and the caspase-3 inhibitor Z-DEVD-FMK for 12 h. Cleavage of procaspase-3 by upstream caspases or other proteases generates an intermediate form that is partially active but not apoptotic, and autocleavage of the intermediate form produces fully active, apoptotic caspase-3 (referred to as the active form; Ref. 48).

The active form of caspase-3 was present in roscovitine-treated but not untreated MT-2 cells (Fig. 4B). When added with roscovitine, Z-DEVD-FMK reduced the abundance of the active form and concomitantly increased the abundance of the intermediate form. Decreases in the amounts of active caspase-3 were paralleled by decreases in the amounts of cleaved PARP. These observations show that Z-DEVD-FMK effectively inhibits caspase-3 activity in roscovitine-treated MT-2 cells. Amounts of P-Tyr-STAT5a were substantially reduced by roscovitine in both the absence and presence of Z-DEVD-FMK, as were amounts of XIAP. These findings exclude cell death as a cause of STAT5a inactivation and XIAP down-regulation in roscovitine-treated MT-2 cells.

Fig. 4. Occurrence of STAT5 activation in roscovitine-treated MT-2 cells in the absence of caspase-3 activity. A, MT-2 cells received 30 μM roscovitine for the indicated times, and cell extracts were immunoprecipitated with antibody to STAT5a and immunoblotted with antibody to STAT5a or phosphotyrosine (P-Tyr-STAT5a). PARP cleavage was determined by Western blotting. B, MT-2 cells were pretreated with 20 or 80 μM Z-DEVD-FMK for 2 h. Cells then received DMSO (control) or 30 μM roscovitine for 12 h. Cell extracts were immunoprecipitated with antibody to STAT5 and immunoblotted with antibody to STAT5 or phosphotyrosine (P-Tyr-STAT5). Cells extracts were also Western blotted with antibody to caspase-3, PARP, and XIAP.

Collectively, the data in Fig. 3 show that roscovitine blocks STAT5 activation when added to MT-2 cells.

Dominant-Negative STAT5 Induces Apoptosis when Expressed in MT-2 Cells. To determine whether ablation of STAT5 activity results in the death of MT-2 cells, we infected them with vaccinia virus encoding a dominant-negative form of STAT5 (referred to henceforth as STAT5-dn). STAT5-dn contains a dimerization and DNA-binding domain but lacks the COOH-terminal transactivation domain (49). As controls, cells were infected with virus expressing an unrelated protein (CD56) or were mock-infected; cells were harvested 12 and/or 18 h after infection. High-level expression of STAT5-dn was confirmed by EMSA (Fig. 5A). The percentage of apoptotic cells was determined by annexin V binding, and the percentages of annexin-bound cells in mock-infected cultures, cultures overexpressing CD56, and cultures overexpressing STAT5-dn were 8%, 7%, and 40%, respectively (Fig. 5B). Thus, considerably more cells were committed to apoptosis in the STAT5-dn-infected populations than in the control populations. The capacity of STAT5-dn to induce apoptosis in MT-2 cells is consistent with the premise that roscovitine kills MT-2 cells by inactivating STAT5. Like roscovitine, STAT5-dn also reduced the amounts of XIAP mRNA and protein (Fig. 5C).

STAT5 Interacts with the XIAP Promoter. Because XIAP is a NF-κB gene product (7), inhibition of XIAP expression by STAT5-dn was unexpected. NF-κB consists of two subunits, p65 and p50, and becomes active and binds DNA after dissociation from members of the IκB family (50). Tax activates NF-κB, and as a result, NF-κB is constitutively active in HTLV-1-transformed cells (4–6). However, as monitored by EMSA, NF-κB activity was similar in control and roscovitine-treated MT-2 cells (Fig. 6A). This finding suggests that roscovitine regulates XIAP expression by destabilizing the XIAP transcript or that transcription of the XIAP gene requires factors other than or in addition to NF-κB (e.g., STAT5).

To determine whether STAT5 interacts with the XIAP promoter,
ChIP assays were performed. MT-2 cells were treated with formaldehyde to cross-link chromatin to associated proteins. Chromatin was immunoprecipitated with antibody to STAT5, STAT1α, or the p50 subunit of NF-κB, and recovered DNA was analyzed by PCR using primers corresponding to a region of the XIAP promoter or the actin promoter. As shown in Fig. 6B, STAT5 antibody coprecipitated XIAP promoter DNA, as did NF-κB antibody. Negative results were obtained using STAT1α antibody and in mock immunoprecipitations (no antibody), and none of the antibodies coprecipitated actin promoter DNA. These findings show that both STAT5 and NF-κB interact specifically with the XIAP promoter. However, XIAP expression in control and roscovitine-treated MT-2 cells correlates with STAT5 activity but not with NF-κB activity.

**Roscovitine Inhibits the Interaction of the PDGF α Receptor with STAT5.** Although JAK3 activates STAT5 and is often active in HTLV-1-infected T cells, the JAK inhibitor AG490 had no effect on STAT5 activity when added to MT-2 cells (data not shown). Thus, kinases other than or in addition to JAKs activate STAT5 in these cells. PDGF receptors (PDGF α and PDGF β) are tyrosine kinases whose substrates include the STAT proteins (51), and HTLV-1-infected T cells express PDGF receptors and secrete the ligands for these receptors (52–55). Consistent with the premise that PDGF receptors account (at least in part) for STAT5 activation in MT-2 cells, we found that antibody to the PDGF α receptor coprecipitated STAT5a from MT-2 cell extracts (Fig. 7A). To determine whether roscovitine affects PDGF α receptor/STAT5a interaction, MT-2 cells were exposed to 30 μM roscovitine for 15 h, and cell lysates were immunoprecipitated with antibody to the PDGF α receptor and immunoblotted with antibody to STAT5a. Although roscovitine did not affect the overall abundance of the PDGF α receptor, antibody to the PDGF α receptor did not coprecipitate STAT5a from lysates of roscovitine-treated cells. Thus, PDGF α receptors do not bind STAT5a in MT-2 cells exposed to roscovitine.

Association of PDGF receptors with their substrates typically requires autophosphorylation of intracellular tyrosines that serve as substrate recognition sites, and the extent of autophosphorylation reflects two opposing processes: (a) receptor activity; and (b) tyrosine phosphatase activity (51). Sodium orthovanadate inhibits tyrosine phosphatase activity, and its capacity to override the inhibitory effects of roscovitine on PDGF α receptor/STAT5a interaction in MT-2 cells was determined. Although not detectable in extracts of cells exposed to roscovitine alone, PDGF α receptor/STAT5a complexes were readily apparent in cells cotreated with roscovitine and sodium orthovanadate (Fig. 7B). Moreover, PARP was not cleaved, and amounts of P-Tyr-STAT5a and XIAP were not reduced in cells receiving both agents. These findings suggest that roscovitine inhibits STAT5a activity by preventing the autophosphorylation of PDGF α receptors and their consequent interaction with STAT5a. Importantly, negation of the inhibitory effects of roscovitine on STAT5a activity prevented PARP cleavage and thus resulted in cell survival.

**DISCUSSION**

The data presented here show that roscovitine is apoptotic for the HTLV-1-transformed cell line MT-2. Apoptosis occurred within 12 h of addition of roscovitine to cells, and by 22 h, >30% of the cells in
the roscovitine-treated population were apoptotic. Our studies also addressed the mechanism by which roscovitine kills MT-2 cells. We show that roscovitine inhibits the activity of STAT5 and that STAT5 activity is required for the survival of MT-2 cells. We suggest that PDGF α receptors activate STAT5 in MT-2 cells and that roscovitine induces apoptosis by eliciting events that prevent STAT5/PDG F α receptor interaction.

Inhibition of STAT5 activity by roscovitine was demonstrated by Western blotting using an antibody that recognizes the tyrosine-phosphorylated (and thus active) form of STAT5 and by EMSA using a probe that contains a STAT5 DNA binding element. The inhibitory effects of roscovitine on STAT5 activity were not reversed by the caspase-3 inhibitor Z-DEVDFMK and a dominant-negative form of STAT5 induced apoptosis when ectopically expressed in MT-2 cells. Thus, STAT5 inactivation causes rather than results from cell death.

Arsenic trioxide (with or without IFN-α) also inhibited STAT5 activity and induced apoptosis when presented to MT-2 cells (data not shown). These findings suggest that STAT5 is a survival factor for MT-2 cells, as it is for other types of leukemic cells (26, 28, 31, 56–58). Roscovitine did not inhibit STAT5 activity in chronic myelogenous leukemia cells that express Bcr-Abl (data not shown), thus suggesting that roscovitine targets some but not all of the signaling pathways that lead to STAT5 activation.

The presence of active JAK3 in HTLV-1-transformed T cells suggests that JAK3 phosphorylates and activates STAT5 in these cells (21, 22). In support, Kirken et al. (59) demonstrated reduced STAT5 activity in MT-2 cells exposed to the JAK inhibitor AG490. In our studies, however, AG490 had no effect on amounts of tyrosine-phosphorylated STAT5 or STAT5 DNA binding activity in MT-2 cells (data not shown). The reason for the difference between our observations and those of Kirken et al. (59) is not known. We show that antibody to the PDGF α receptor coprecipitates STAT5α from extracts of MT-2 cells and thus suggest that autocrine activation of PDGF α receptors, which occurs in HTLV-1-transformed cell lines (52), accounts (at least in part) for the constitutive activation of STAT5 in MT-2 cells. Whether PDGF α receptors directly phosphorylate STAT5, as do PDGF β receptors (60, 61), or simply act as scaffolds that bring STAT5 and other tyrosine kinase into proximity is not known. Although the non-receptor tyrosine kinase Src phosphorylates STAT3 in a PDGF-dependent manner (62, 63), the Src inhibitor PD166285 had no effect on either PDGF α receptor/STAT5 interaction or STAT5 tyrosine phosphorylation (data not shown).

STAT5α did not associate with PDGF α receptors in roscovitine-treated MT-2 cells, and tyrosine phosphatase activity was required for ablation of PDGF α receptor/STAT5 interaction by roscovitine. This finding suggests that PDGF α receptors are not tyrosine phosphorylated in roscovitine-treated cells and thus do not contain docking sites for STAT5 (or for an intermediary protein that binds STAT5). When added to MT-2 cells in combination with the tyrosine phosphatase inhibitor sodium orthovanadate, roscovitine did not reduce amounts of P-Tyr-STAT5 or induce PARP cleavage. This finding suggests that these events require STAT5/PDG F α receptor interaction. Interestingly, activating mutations in PDGF α receptors have been shown to activate STAT3 in gastrointestinal stromal tumors (64).

Roscovitine does not function as a tyrosine kinase inhibitor (32), and a direct effect of roscovitine on PDGF α receptor activity is unlikely. Alternatively, roscovitine may in some way increase the expression or activity of a tyrosine phosphatase that targets the PDGF α receptor. Such phosphatases include low molecular weight tyrosine phosphatase (LMW-PTP) which inhibits the STAT activation by PDGF (65), and SHP-1 whose expression is greatly reduced in HTLV-1-transformed T cells (66, 67).

Consistent with potential effects of roscovitine on the expression of a phosphatase, the roscovitine target CDK2 is capable of indirectly modulating protein expression. For example, we have shown that CDK2 regulates the translation of the α subunit of the interleukin-2 receptor in primary splenocytes (68). Whether roscovitine induces the apoptosis of MT-2 cells by inactivating CDK2, however, has yet to be established.

An obvious question arising from our studies concerns the identity of the STAT5 gene products that promote the survival of MT-2 cells. Roscovitine and STAT5α-dn reduced the abundance of the antiapoptotic protein XIAP and its transcript in MT-2 cells, whereas roscovitine plus sodium orthovanadate did not. Thus, XIAP expression correlates with STAT5 activity in MT-2 cells. Although NF-κB up-regulates XIAP expression in a number of systems (7, 69–72), our data suggest that NF-κB activity is insufficient for XIAP expression. Roscovitine did not inhibit NF-κB activity in MT-2 cells, and the XIAP promoter interacted with both STAT5 and NF-κB in vivo. Decreases in XIAP expression occurred in cells cotreated with roscovitine and Z-DEVDFMK and thus were not secondary consequences of cell death. Although additional studies are required, XIAP is a potential mediator of the apoptotic effects of roscovitine in MT-2 cells.

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