Interleukin-10 Activation of the Interleukin-10E1 Pathway and Tissue Inhibitor of Metalloproteinase-1 Expression Is Enhanced by Proteasome Inhibitors in Primary Prostate Tumor Lines

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Abstract
The interleukin-10 (IL-10) activation of Janus kinase (JAK) family members (JAK1/TYK2) and IL-10E1 is subsequently inactivated by ~3–4 h in primary prostate tumor lines. We examined the effect of proteasome inhibition on IL-10 activation of the IL-10E1 pathway following stimulation of HPCA-10a cells. Treatment of HPCA-10a cells with the proteasome inhibitor, N-acetyl-L-leucinyl-L-leucinyl-norleucinal (LLnL), led to stable tyrosine phosphorylation of the IL-10 receptor and IL-10E1 following stimulation. Further investigation showed that these stable phosphorylation events were the result of prolonged activation of JAK1 and TYK2 plus IL-10E1. IL-10E1 signaling normally induced the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) and LLnL treatment of the HPCA-10a and HPCA-10c cells significantly enhanced IL-10 induction of TIMP-1 levels to block tumor cell invasion in modified Boyden chamber invasion assays. These observations were confirmed using pharmacologic inhibitors by Western blot and ELISAs. In the presence of LLnL, stable phosphorylation of IL-10E1 and induction of TIMP-1 was abrogated if the tyrosine kinase inhibitor, staurosporine, was added. The effect of staurosporine on IL-10E1 phosphorylation and TIMP-1 could be overcome if the phosphatase inhibitor, vanadate, was also added, suggesting that phosphorylated IL-10E1 could be stabilized by phosphatase, but not by proteasome inhibition. These observations are consistent with the hypothesis that proteasome-mediated protein degradation can modulate the activity of the IL-10E1 pathway and TIMP-1 induction by regulating the deactivation of JAK1/TYK2.

Introduction
The role(s) of ubiquitination and/or proteolytic degradation of proteins by the 20S and 26S proteasomes have received increased attention during recent years, and it is now apparent that these two processes provide an additional point of regulation for many fundamental biological functions (1, 2). Ubiquitin-dependent proteolysis has been shown to be integral in the following: the degradation of cyclins and cell-cycle progression (3–7); the generation of peptides presented on the cell surface by MHC class I molecules (8); and the modulation of several transcriptional regulators, including c-Jun (9) and IκB (10–14), as well as the modulation of the Rel family member, NF-κB (14). Recently, ubiquitination has also been shown to signal receptor-mediated endocytosis of the yeast G-protein receptor, Ste2p (15), and has been implicated in down-modulating c-kit receptor expression (16). Consequently, it is conceivable to expect many biochemical pathways to be affected by ubiquitin-dependent proteolysis, including the signaling cascades of cytokine receptors.

Interleukin-10 (IL-10) signaling pathways have been well characterized and constitute a useful model for growth factor signaling (17). One of the first signaling events, following IL-10 stimulation, is the increased tyrosine phosphorylation of and subsequent activation of the receptor-associated protein tyrosine kinase, Janus kinase-1 (JAK1) and TYK2 (17–19). The newly activated JAK1/TYK2 mediates the subsequent phosphorylation of tyrosine residues within the β-chain of the IL-10 receptor (19–21). The phosphotyrosine residues of the β subunit provide docking sites for signal transducers and activators of transcription (STATs) (20, 21). STATs bind to the activated receptors via their SH2 domains on which they are phosphorylated on a single tyrosine residue COOH-terminal to its SH2 domain by JAK (22, 23). Phosphorylated STATs dissociate from the receptor, homodimerize or heterodimerize, and translocate to the nucleus, where they bind specific DNA elements to activate transcription of target genes (24). IL-10 stimulation predominantly leads to the activation of STATs (25–28) and induces the expression of several genes, including cytokine-inducible SH2-containing protein (CIS), pim-1, osm, and c-fos (26). Although the activation of STATs is well understood, this is not so for their inactivation. The most likely model is that STATs are negatively regulated by dephosphorylation, a process that probably occurs within the nucleus (29). Recently, Bernard et al. (30) have investigated the effect of proteasome inhibition on IL-3 activation of the JAK/STAT pathway following stimulation of Ba/F3 cells. Treatment of Ba/F3 cells with the proteasome inhibitor, N-acetyl-L-leucinyl-L-leucinyl-norleucinal (LLnL), led to stable tyrosine phosphorylation of the IL-3 receptor, β common (βc), and STAT5. The effects of LLnL were not restricted to the JAK/STAT pathway,
as Shc and mitogen-activated protein kinase (MAPK) phosphorylation were also prolonged in LLaLtreated cells as the result of prolonged activation of JAK2 and JAK1. These observations were confirmed using pharmacologic inhibitors (staurosporine and vanadate). Taken together, these observations suggest that proteasome-mediated protein degradation can modulate the activity of the JAK/STAT pathway by regulating the deactivation of JAK.

Recently, another model for the negative regulation of STATs has been proposed. Using specific inhibitors of the proteasome, active or phosphorylated STAT1 has been shown to be stabilized following IFN-γ stimulation (31). This study also identified ubiquitinated forms of phospho-STAT1, suggesting that active STAT1 was inactivated by ubiquitin-mediated proteolysis within the 26S proteasome. Because the proteasome has been shown to degrade several phosphorylated proteins, including IκB (10–13), cyclin G1 (6, 7), and SHP-1 (32), via an ubiquitination-dependent pathway, proteasome-dependent degradation of signal molecules provides an alternate mechanism by which the cell could down-regulate STAT activity or the activity of other signal molecules.

We originally reported that IL-10 signaling stimulated activation of a specific enhancer element, termed HTE-1, to promote tissue inhibitor of metalloproteinase-1 (TIMP-1) expression in human prostate PC-3 ML cells (33). Recently, we have identified a protein with 22,000 daltons, termed IL-10E1, as one of the signal molecules that bind the HTE enhancer element to activate TIMP-1 expression in human prostate cancer cells (34). IL-10 binding to the IL-10R was found to induce tyrosine phosphorylation of the JAK1/TYK2 kinases, tyrosine phosphorylation of Y57 and Y62 tyrosines in the “NH2-terminal” domain of the IL-10E1 protein, and the rapid transport to the nucleus of the IL-10E1 protein (34) in both primary and established malignant human prostate cell lines (34). In a recent paper (35), we demonstrated that two tyrosine residues (Tyr446 and Tyr496) located in the cytoplasmic domain of the IL-10R α chain were required for receptor function and for phosphorylation and activation of IL-10E1. Immunoprecipitation studies revealed that 12 amino acid peptides encompassing either of these two tyrosine residues in phosphorylated form co-precipitated IL-10E1 and blocked ligand-dependent IL-10E1 phosphorylation in a cell-free system. The data demonstrate that IL-10E1 is directly recruited to the ligand-activated IL-10R by binding to specific phosphotyrosine groups that control tyrosine phosphorylation of the LIM domain of the IL-10E1 protein [i.e., Y57/Y62 groups (35)].

To determine whether phosphatase- or proteasome-mediated degradation pathways play a role in IL-10E1 regulation, we investigated IL-10E1 inactivation in the IL-10-dependent primary human prostate cancer cell line, HPCA-10a, derived from a high-grade Gleason score 10 tumor (36, 37). Previous studies have shown that IL-10E1 is rapidly activated by IL-10 and that accumulation of the activated protein reaches a maximum within 30–60 min of stimulation and then declines to baseline levels within 2–4 h (36). The relatively short half-life of activated IL-10E1 indicates that the transcription factor’s activity is tightly regulated and reduces the likelihood of the cell accumulating harmful levels of gene products. To examine the effect on IL-10-induced activation of the IL-10E1 pathway and whether IL-10E1 might also be proteolytically degraded, we treated HPCA-10a cells with the proteasome-specific inhibitor, LLaL (8), and investigated the effect of proteasome inhibition on IL-10E1 activation, as well as tyrosine phosphorylation of JAK1 following IL-10 stimulation. The results presented here show that treatment of HPCA-10a cells with LLaL resulted in prolonged activation of IL-10E1 as a consequence of prolonged JAK1 phosphorylation/activation.

**Results**

**IL-10E1p Antibody Characterization**

Monoclonal antibodies were raised against tyrosine-phosphorylated IL-10E1, termed IL-10E1p. Western blots of crude cell extracts demonstrated that the anti-IL-10E1p failed to recognize non-phosphorylated IL-10E1 protein from untreated cells (Fig. 1, lane 1), but specifically recognized the phosphorylated IL-10E1 protein from IL-10-treated HPCA-10a cells (IL-10 at 15 ng/ml for 10 min) (Fig. 1, lane 2). Competition assays showed that preincubation of the anti-IL-10E1p antibodies with excess phosphorylated IL-10E1 in crude cell extracts specifically competes out antibody binding to IL-10E1 (Fig. 1, lane 3). Preincubation of the anti-IL-10E1p antibodies with excess non-phosphorylated IL-10E1 failed to compete out antibody binding to IL-10E1 isolated from IL-10-treated cells (Fig. 1, lane 4). The blots were stripped and reblotted with IL-10E1 antibodies that recognized non-phosphorylated IL-10E1 (36) to confirm that sample loading was not a contributing factor in these experiments (Fig. 1, lanes 1–4, lower band).

**LLaL Inhibition Studies**

Previous studies by others have shown that when Jurkat cells are stimulated, the IκB molecules undergo site-specific phosphorylation and ubiquitination and then are degraded by the 26S proteasome (38). The released dimeric NF-κB enters the nucleus and induces expression of the target genes. They

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**FIGURE 1.** Western blots with anti-IL-10E1p antibodies (1:600 dilution) of non-phosphorylated IL-10E1 (lane 1) and phosphorylated IL-10E1 (lane 2) proteins. In control experiments, IL-10E1p antibodies were preabsorbed with excess phosphorylated IL-10E1 (lane 3) or non-phosphorylated IL-10E1 (lane 4) isolated from untreated and IL-10-treated HPCA-10a cells, respectively. Lanes 1–4 (lower band), membranes were stripped and reblotted with IL-10E1 antibodies. Lanes loaded with 2 mg protein/lane isolated from crude whole cell extracts of untreated (lane 1) and IL-10-treated (lanes 2–4) HPCA-10a cells.
found that in addition to IκB molecules, the transcription factor c-Rel was also regulated by the Ub-Pr pathway (38). However, when Jurkat cells were pretreated with the proteasome inhibitors (i.e., LLnL), Ub-c-Rel conjugates accumulated, indicating that the basal turnover of c-Rel was mediated by the Ub-Pr pathway (38). Preliminary studies by our lab revealed that in the presence of optimal dosages of LLnL (50 μM), c-Rel expression was stabilized and enhanced in cytoplasmic extracts of HPCA-10a cells. Western blot analysis of cytoplasmic protein extracts combined with densitometric scans of the gels showed that after prolonged LLnL treatment of 60–120 min, the levels of c-Rel increased by ~3-fold compared to shorter treatments of 5–30 min (Fig. 2). Note that ubiquitination of c-Rel was detectable (top bands) in the preparations where cells were exposed to LLnL for 60–120 min.

Additional studies were carried out to determine the optimal dilution of LLnL for inhibition of phosphatase activity. Total phosphatase activity in 20-μl aliquots was assayed according to the manufacturer’s protocol over 15-min intervals at 37°C. To examine the effect of proteasome inhibition on IL-10 signaling, the tyrosine phosphorylation pattern of the IL-10E1 was determined following IL-10 stimulation of IL-10-depleted HPCA-10a cultures in both the presence and absence of LLnL (Fig. 3A). In the absence of IL-10, a phosphorylated protein was not detected in IL-10E1 immunoprecipitates blotted with PY 20 antibodies (data not shown). The addition of IL-10 induced the appearance of a band by ~10 min (Fig. 3A, lane 1), indicating that IL-10E1 was being converted into a phosphorylated form. IL-10E1 phosphorylation was transiently induced by IL-10 and was maximal within 30 min of stimulation (Fig. 3A, lane 2). The band disappeared by longer intervals of induction of 2 and 4 h (Fig. 3A, lanes 3 and 4, respectively). In contrast, the presence of LLnL stabilized the transient nature of phosphorylated IL-10E1 observed after 10 and 30 min (Fig. 3A, lanes 6 and 7, respectively) for 2–4 h in response to IL-10 stimulation (Fig. 3A, lanes 8 and 9, respectively). Reblotting with an anti-IL-10E1 antibody showed an equivalent amount of immunoreactive protein in all samples, ruling out the possibility that LLnL had affected the stability of IL-10E1 (Fig. 3A, lanes 1–9, lower band). Thus, one effect of LLnL is to stabilize the tyrosine phosphorylation of IL-10E1.

**EMSAs**

We then examined the effect of LLnL on IL-10E1 binding to the HTE-1 DNA sequence. In the presence of LLnL (top panel), IL-10 still induced a rapid accumulation in IL-10E1 DNA-binding activity by 10–30 min (Fig. 3B, lanes 1 and 2). However, the subsequent decrease in DNA-binding activity was not observed by 60 and 120 min (Fig. 3B, lanes 3 and 4, respectively). In the absence of proteasome inhibitor, IL-10 induced a rapid nuclear accumulation of IL-10E1 DNA-binding activity, which was maximal within 30–60 min of stimulation (Fig. 3B, lanes 3 and 4). DNA-binding activity then declined to undetectable levels by 120 min (Fig. 3B, lane 4). EMSAs in Fig. 3C further show that with LLnL-treated cells, IL-10E1 is expressed in nuclear protein extracts after 0, 30, 60, 120, and 240 min, respectively (Fig. 3C, lanes 2–5). Note that IL-10E1 was not expressed in the nuclear protein extracts in cells treated with IL-10 for 0 min (untreated cells) (Fig. 3C, lane 1).

**Pharmacologic Inhibitor Studies**

Using the pharmacologic inhibitors, staurosporine and orthovanadate, we examined whether IL-10E1 was a target of LLnL. HPCA-10a cells were stimulated with IL-10 in the presence of LLnL for 0, 10, 30, and 60 min (Fig. 4A, lanes 1–4). After 60 min stimulation, either staurosporine, vanadate, or both were added to the culture and incubation...
continued for an additional 60 or 90 min (Fig. 4A, lanes 5–13). In these experiments, we found that the addition of LLnL stabilized the DNA-binding activity of IL-10E1 at both 10, 30, and 60 min treatment before adding staurosporine or vanadate or both (Fig. 4A, lanes 2–4, respectively). When staurosporine was added in the continued presence of IL-10, it abolished the LLnL-induced stabilization of IL-10E1 DNA-binding activity after 60 and 90 min (Fig. 4A, lanes 5 and 6, lanes 9–10). The addition of vanadate in the presence of IL-10 gave rise to an enhanced level of IL-10E1 DNA-binding activity (compared with LLnL alone) after 60 and 90 min (Fig. 4A, lanes 7 and 8), while the addition of both vanadate and staurosporine (plus IL-10) gave rise to intermediate levels of DNA-binding activity compared with either agent alone (Fig. 4A, lanes 11–13). Identical results were obtained by Western analysis using an anti-phosphotyrosine (Fig. 4B) and anti-IL-10E1p antibodies (Fig. 4C).

To eliminate the possibility that staurosporine was toxic, cytosolic extracts from the previous experiment were separated by SDS-PAGE and anti-phosphotyrosine and total IL-10E1 immunoreactivity determined. Total anti-phosphotyrosine immunoreactivity was considerably reduced in the staurosporine-treated sample, whereas the amount of immunoreactive IL-10E1 was comparable with untreated samples (data not shown). This result demonstrated that, over the indicated time course, the effect of staurosporine was due to the inhibition of tyrosine kinase activity and not to the accelerated loss of protein from the cell arising from toxic effects. Cell viability was >95% in all these experiments as determined by MTT assays (39) of metabolic activity.

**JAK1/TYK2 Experiments**

Both JAK1 and TYK2 have been reported to be activated by IL-10. Preliminary studies showed that JAK1 was the principal kinase responsible for IL-10E1 phosphorylation by IL-10 (35). The phosphorylation of both TYK2 and JAK1 was transient with both being induced within 5–10 min of stimulation and declining to basal levels by 30 min (data not shown). Following the treatment of cells with LLnL, JAK1 was phosphorylated by ~10 min (Fig. 5, lane 7). LLnL prevented the dephosphorylation of JAK1 after 30, 60, and 120 min (Fig. 5, lanes 8–10, respectively). In comparison, the dephosphorylation of TYK1 observed by 10 min (Fig. 5, lane 2) was not blocked by LLnL after 30, 60, and 120 min (Fig. 5, lanes 3–5). It was possible that LLnL affected the stability of TYK2 and JAK1, however. See Fig. 5, TYK2 (lanes 1–5, lower band) and JAK1 (lanes 6–10, 10) gave rise to intermediate levels of DNA-binding activity while the addition of both vanadate and staurosporine (plus IL-10) gave rise to intermediate levels of DNA-binding activity compared with either agent alone (Fig. 4A, lanes 11–13). Identical results were obtained by Western analysis using an anti-phosphotyrosine (Fig. 4B) and anti-IL-10E1p antibodies (Fig. 4C).

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**Cytosolic Extracts from the Previous Experiment**

A. Nuclear extracts were prepared and the binding to 32P-labeled HTE oligonucleotide probe determined. B. Nuclear extracts from A (25 μg) were separated by SDS-PAGE (7% gel), transferred, and immunoblotted with anti-phosphotyrosine. C. The blots in B were stripped and rebotted with IL-10E1p antibodies.

**FIGURE 3.** A. LLnL stabilizes tyrosine phosphorylation of IL-10E1. IL-10-deprived HPCA-10a cells (4×10⁷ cells) were treated with vehicle or LLnL (i.e., +LLnL or −LLnL) for 1 h and then stimulated with IL-10 for 10 min, 30 min, and 2 and 4 h, respectively. Nuclear protein extracts from the cells were immunoblotted with PY20 anti-phosphotyrosine (top band) and IL-10E1 antibodies (bottom band). Left panel, −LLnL + IL-10 treatment for 10 min (lane 1), 30 min (lane 2), 2 h (lane 3), and 4 h (lane 4). Right panel, +LLnL + IL-10 treatment for 0 min (lane 5), 10 min (lane 6), 30 min (lane 7), 2 h (lane 8), and 4 h (lane 9), respectively. Note that lanes 1–4 were loaded with 20 μg protein/well and lanes 5–9 were loaded with 10 μg protein/well. B. Electrophoretic mobility shift assays (EMSAs) showing DNA-binding activity of IL-10E1 in nuclear protein extracts from HPCA-10a cells treated with LLnL (i.e., +LLnL) or without LLnL (i.e., −LLnL) for 1 h and then stimulated with IL-10 for 10 (lane 1), 30 (lane 2), 60 (lane 3), and 120 min (lane 4), respectively. C. EMSAs showing DNA-binding activity of IL-10E1 in nuclear protein extracts from HPCA-10a cells treated with LLnL for 1 h and then stimulated with IL-10 for 0 (lane 1), 90 (lane 2), 60 (lane 3), 120 (lane 4), and 240 min (lane 5).

**FIGURE 4.** Staurosporine prevents LLnL-induced stabilization of IL-10E1 phosphorylation. IL-10-depleted HPCA-10a cells were treated with LLnL for 1 h and then stimulated with IL-10 for 0, 10, 30, and 60 min (lanes 1–4). After 30 min stimulation, the cells were exposed to IL-10 plus staurosporine for 60 and 90 min (lanes 5 and 6), or staurosporine plus vanadate for 60, 90, and 120 min (lanes 11–13). A. Nuclear extracts were prepared and the binding to 32P-labeled HTE oligonucleotide probe determined. B. Nuclear extracts from A (25 μg) were separated by SDS-PAGE (7% gel), transferred, and immunoblotted with anti-phosphotyrosine. C. The blots in B were stripped and rebotted with IL-10E1p antibodies.
Together, these results show that the effect of LLnL on IL-10 signaling is to prolong the activity of JAK1, presumably through its stabilizing effect on JAK1 kinase tyrosine phosphorylation.

**Tyrosine Phosphatase Activity Studies**

To eliminate the possibility that LLnL can directly inhibit overall tyrosine phosphatase activity, the following experiment was performed. Tyrosine phosphatase assays were performed in vitro on NP40 extracts from untreated HPCA-10a cells. As clearly demonstrated in Fig. 6, neither DMSO nor LLnL affected total tyrosine phosphatase activity in NP40 extracts after 30 or 60 min incubations. When orthovanadate was added to the lysate, only background levels of activity were detected. Control extracts that were treated with vehicle exhibited phosphatase activities close to 100%. This result demonstrates that LLnL is not functioning directly as a tyrosine phosphatase inhibitor. MTT assays (39) confirmed that cell viability was >95% in all these studies.

**ELISA Studies**

ELISAs were carried out with antibodies specific to IL-10E1 and IL-10E1p to compare the relative levels of antigen expression in untreated and LLnL-treated HPCA-10a cells which were subsequently exposed to IL-10 for 10–80 min. Preliminary studies showed that ELISAs with IL-10E1 (1:6000 dilution) and IL-10E1p (1:4000 dilution) antibodies each yielded linear standard curves in assays with increased amounts of crude protein extracts (1–16 μg protein) from HPCA-10a cells treated with IL-10 (15 ng/ml for 30 min) (Fig. 7A). These antibody dilutions and standard curves were employed in subsequent assays of IL-10E1 and IL-10E1p levels in untreated and LLnL-treated HPCA-10a cells. Fig. 7, B and C shows that the levels of IL-10E1p (Fig. 7B) and IL-10E1 (Fig. 7C) in crude nuclear extracts (10 μg/ml) increased in a relatively linear manner after pretreatment of the cells with LLnL followed by exposure to IL-10 for 10–80 min (●). In comparison, in untreated cells exposed to IL-10, the levels of IL-10E1p increased from 10 to 50 min and then declined rapidly to near zero from 50 to 70 min stimulation (●). Negative control experiments showed that the LLnL-treated cells exposed to IL-10 in the presence of IL-10 receptor (▲) or IL-10 (●) antibodies failed to express IL-10E1p in the nuclear protein extracts over the 10- to 80-min time period (Fig. 7B). In Fig. 7D, the cytoplasmic protein extracts were assayed for IL-10E1 expression in untreated and LLnL-treated cells followed by IL-10 treatment for 10–80 min. These data correlated well with the results in Fig. 7C and showed that in both untreated (▲) and LLnL-treated (●) cells, the levels of IL-10E1 declined to near zero levels from 10 to 40 min and remained near zero up to 60 min. The cytoplasmic levels of

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**FIGURE 5.** LLnL stabilizes tyrosine phosphorylation of JAK1, but not TYK2. IL-10-depleted HPCA-10a cells were treated with DMSO or with LLnL for 1 h and stimulated with IL-10 (15 ng/ml) for 0 (lanes 1 and 6), 10 (lanes 2 and 7), 30 (lanes 3 and 8), 60 (lanes 4 and 9), and 120 min (lanes 5 and 10), respectively. Extracts from 5 × 10⁷ cells were immunoprecipitated with either anti-TYK2 (left panel) or anti-JAK1 (right panel) antibodies. TYK2 and JAK1 immunoprecipitates were divided and electrophoresed on duplicate 7% SDS-PAGE gels, transferred, and immunoblotted with either PY 20 (top band) or anti-TYK2 and JAK1 (bottom bands) antibodies, respectively.

**FIGURE 6.** LLnL does not inhibit tyrosine phosphatase activity. HPCA-10a cells were washed and lysed with NP40 buffer in the absence of vanadate. The lysate from 5 × 10⁶ cells was assayed for total tyrosine phosphatase activity. Either buffer only (control), LLnL (50 μM), or DMSO as carrier was added to the reaction at a final concentration identical to that used when added to cell cultures. As a positive control, orthovanadate (1 mM) was also added to inhibit phosphatase activity. Data were normalized to activity obtained from control reactions (no additions) and each bar is the average of triplicate samples taken from a single experiment terminated at 30- and 60-min intervals, respectively. Similar results were obtained from several experiments using dilutions of lysate at 1:5 and 1:10 in the reaction buffer.
IL-10E1 began to recover after 60–80 min, presumably as a result of protein synthesis (Fig. 7D). In cells exposed to LLnL and then exposed to IL-10 in the presence of IL-10 receptor (▲) or IL-10 (●) antibodies for 10–80 min, the levels of IL-10E1 in the cytoplasmic fractions remained relatively constant at ~15 ng/ml.

**TIMP-1 Studies**

Because TIMP-1 production and secretion is stimulated by IL-10E1 activation, we have measured TIMP-1 levels secreted in the media of HPCA-10a (Fig. 8A) and HPCA-10c (Fig. 8B) cells. TIMP-1 secretion was measured by ELISAs at different time points over a 12-h interval. The treatment of HPCA-10a and HPCA-10c cells with LLnL for 60 (●) or 120 min (■) stabilized and prolonged TIMP-1 production over a 3- to 12-h interval (Fig. 8, A and B, respectively). In comparison, DMSO (●) had little or no effect on TIMP-1 production compared to cells which were exposed to IL-10 plus vehicle alone (○) where TIMP-1 production was elevated after ~4 h stimulation until ~8 h. At this juncture, the production of TIMP-1 diminished gradually back to baseline levels after 10–12 h treatment. The implication is that LLnL sustains TIMP-1 production for prolonged interval (up to 24 h in some experiments) as a direct result of IL-10-activated IL-10E1p signaling mechanisms. Fig. 8C further shows that the TIMP-1 levels produced by HPCA-10a (▲, ×, −) and HPCA-10c (●, ■, −) cells were near zero in cells exposed to staurosporine following pretreatment with LLnL and IL-10. In comparison, staurosporine plus vanadate partially inhibited TIMP-1 production in HPCA-10a (×) and HPCA-10c (■) cells. Vanadate alone had little effect on the production in either HPCA-10a (▲) or HPCA-10c (●).

**FIGURE 7.** ELISAs with IL-10E1 and IL-10E1p. A. Standard curves with IL-10E1 (●, 1:6000 dilution) and IL-10E1p (○, 1:4000 dilution) antibodies with increased amounts of crude nuclear protein extracts (1–16 μg/ml) from IL-10-treated HPCA-10a cells. Measurements of IL-10E1p (B) and IL-10E1 (C) in nuclear protein extracts (2 mg protein/assay) from −LLnL (●) and +LLnL (■) treated cells (1 h) which were then exposed to IL-10 for increased time intervals of 10–80 min. Negative controls included cells exposed to IL-10 in the presence of excess IL-10 receptor antibodies (▲) and IL-10 antibodies (●). D. Measurements of IL-10E1 levels in crude cytoplasmic protein extracts (5 mg protein/assay) from −LLnL (●) and +LLnL (■) treated cells (1 h) which were then exposed to IL-10 for increased time intervals of 10–80 min. Negative controls included cells exposed to IL-10 in the presence of excess IL-10 receptor antibodies (▲) and IL-10 antibodies (●).
Boyden Chamber Invasion Assays

Modified Boyden chambers (40) coated with Matrigel (30 μg/well) were employed to examine the relative influence of IL-10 alone and in combination with LLnL on the invasive activity of HPCA-10a cells. For these experiments, the HPCA-10a cells were plated with Matrigel on the bottom side of the membranes (for 1 h) and conditioned medium from HPCA-10a cells placed on the top well as the chemoattractant. IL-10 and/or LLnL were added to the medium (DMEM + 1% fetal bovine serum) in the lower well and the chambers placed in a CO2 incubator for 6 h before removing the cells from the top chamber for counting. Fig. 9A shows the relative influence of IL-10 vehicle, IL-10 at increased dosages of 1, 3, 7, 12, 17, and 22 ng/ml, and IL-10 at increased dosages in the presence of LLnL (50 μM). IL-10 at dosages of 7–20 ng/ml inhibited cell invasion of Matrigel in a dosage-dependent manner. At 10 ng/ml IL-10, invasion was inhibited by ~50% compared to untreated control cells. In the presence of LLnL (50 μM), invasion was inhibited at much lower dosages of IL-10 (3–7 ng/ml), indicating that LLnL significantly enhanced the influence of IL-10. Similarly, Fig. 9B shows the relative influence of LLnL alone, IL-10 at 5 ng/ml, and IL-10 at 5 ng/ml in the presence of increased concentrations of LLnL at 1, 5, 10, 20, 30, and 60 μM. LLnL alone at increased concentrations of 1–60 μg/l or IL-10 alone at 5 ng/ml failed to block tumor cell penetration of Matrigel. However, IL-10 at 5 ng/ml in the presence of LLnL blocked invasion by 50%, 80%, 90%, and 100% at LLnL dosages of 10, 20, 30, and 60 μM, respectively.

In the Boyden chamber assays, TIMP-1 levels in the medium (bottom chamber) (~0.2 ml) were measured at the end of the invasion assay (after an ~6 h interval). IL-10 at dosages of 3–22 ng/ml stimulated TIMP-1 secretion in the absence or presence of a constant amount of LLnL (50 μM). In the presence of vehicle alone (i.e., no IL-10), TIMP-1 production was maintained at basal levels (Fig. 10). Taken together, these data indicated that LLnL has a synergistic effect on TIMP-1 production and that this effect is manifested in the blockade of tumor cell invasion.

Discussion and Conclusions

The results presented in this study revealed that pretreatment of human HPCA-10a cells with the proteasome inhibitor, LLnL, resulted in the sustained tyrosine phosphorylation of both the IL-10 receptor subunit, JAK1, and IL-10E1 as opposed to their
normal transient nature of induced phosphorylation following stimulation. TYK2 was also activated by IL-10, but in the presence or absence of LLnL, it was only temporarily phosphorylated after ~10 min. Because IL-10E1 is phosphorylated by JAK1, we believe the sustained phosphorylation of IL-10E1 is most likely due to the prolonged activation of JAK1. Note that LLnL required a relatively short preincubation period of 30–60 min to elicit its full effects on IL-10E1 phosphorylation, suggesting that its effect is specific and unlikely to be due to general effects on cell viability.

When staurosporine was used to inhibit JAK1 activity, the addition of staurosporine in the presence of LLnL almost completely abrogated the stabilizing effect of LLnL on IL-10E1 phosphorylation. These observations suggest two possibilities. First, although JAK1 mediates IL-10E1 phosphorylation, it may be that the prolonged activation of JAK1, in the presence of LLnL, accounts for the sustained phosphorylation of IL-10E1. However, LLnL may induce other effects that contribute to prolonged receptor phosphorylation. For example, in yeast, ubiquitination of the Ste2p receptor signals its endocytosis (15), and a recent study has demonstrated a similar role for ubiquitin in growth hormone receptor internalization (41). It is also possible that LLnL could affect signaling. Future pulse-chase experiments using radiolabeled ligand will help to confirm or reject this possibility.

How is IL-10E1 signaling inactivated? Studies of the STATs proteins indicate that the STATs may be inactivated by proteolytic degradation by the 26S proteasome. For example, ubiquitinated forms of phosphorylated STAT1 have been identified in response to IFN stimulation (31). However, we have found no evidence to indicate that IL-10E1 is inactivated via a degradative pathway involving the 26S proteasome.

However, LLnL may stabilize phosphotyrosine-IL-10E1 by preventing its degradation. The data indicate that LLnL failed to stabilize IL-10E1 activity in the presence of staurosporine. Rather, it appeared that LLnL induced its effects by preventing the signal for IL-10E1 phosphorylation from being down-regulated. In accordance, the presence of orthovanadate alone resulted in an enhanced stabilization of IL-10E1 activity.

Several points in the IL-10E1 pathway could be affected by phosphatase inhibition, leading to increased IL-10E1 activity, including the dephosphorylation of either JAK1 or IL-10E1. The presence of vanadate resulted in the persistence of IL-10E1 activity and offset the effect of staurosporine; vanadate alone had an even more pronounced effect. Thus, phosphorylated IL-10E1 could be stabilized by phosphatase inhibition, but not by proteasome inhibition per se. These results support the conclusion that the accumulation of active IL-10E1 in the presence of LLnL requires the persistent phosphorylation of IL-10E1 by JAK1.

The transient nature of IL-10E1 activity observed in this study supports a mechanistic model where its activity is up-regulated by phosphorylation and down-regulated principally by dephosphorylation. Furthermore, the loss of tyrosine-phosphorylated IL-10E1 in the combined presence of staurosporine and LLnL suggests that the activity of the IL-10E1-specific phosphatase is unaffected by proteasome inhibitors.

The normal inactivation of JAK1/TYK2 could be mediated by at least two possible mechanisms. First, dephosphorylation of JAK1 could lead to loss in activity. The SH2-containing protein tyrosine phosphatases, SHP-1 and SHP-2, have been implicated in the dephosphorylation of both TYK2 and JAK1 (42–46). Thus, these candidate phosphatases may require proteasomal processing for activation or their activity may be modulated by proteasome function. This process may occur by degrading an inhibitor complex similar to the degradation of IR-B and subsequent activation of NF-κB (10–14). In support of
this, SHP-1 has been shown to be degraded by ubiquitindependent proteolysis in mast cells expressing oncogenic c-kit (32), suggesting the proteasome regulates SHP-1 function. It is therefore possible that LlnL could nonspecifically inhibit phosphatase activity, including that of SHP-1 and SHP-2. However, this is unlikely to be a general effect of LlnL, because IL-10E1 was still dephosphorylated in the combined presence of LlnL and staurosporine. Furthermore, the data presented in Fig. 3 argue against the possibility that LlnL functions as a tyrosine phosphatase inhibitor.

An additional possibility involves the cytokine-induced expression of the newly identified, CIS-related, STAT-induced STAT-inhibitor (SSI) family of proteins (47–49). These proteins, once expressed, could negatively feedback and inhibit JAK1 activity by binding to and inactivating the kinase domain. Therefore, it is possible that LlnL's effect on JAK1 activity could be a combined result of modulation of SHP-1 or SHP-2 activity and inhibited expression of or function of SSI family proteins.

Recently, it has been shown that both JAK1 and JAK3 activities are stabilized by proteasome inhibition following IL-2 induction of T cells (50). The investigators attributed the effect on JAK to modulation of phosphatase activity by proteasome-mediated protein degradation. Similarly, the effect on JAK1 by LlnL may be mediated by a similar mechanism in HPCA-10a and HPCA-10c cells. How the proteasome modulates the deactivation of JAK1 is unknown and remains the focus of future studies.

We have shown that IL-10 in combination with LlnL more effectively blocked tumor cell invasion in modified Boyden chamber invasion assays. In these experiments, ELISA measurements of TIMP-1 levels in the medium at the conclusion of the experiment (i.e., after ~6 h incubation) were significantly elevated in the presence of IL-10 plus LlnL compared to IL-10 alone. The clinical relevance of these observations relates to the fact that IL-10 induction of TIMP-1 secretion serves to block tumor cell growth and metastases in human xenograph SCID model studies (51). Because LlnL sustains IL-10E1p phosphorylation and the induction of TIMP-1 production, the combined treatment of tumors with LlnL and IL-10 should sustain TIMP-1 production in vivo and greatly improve the efficacy of IL-10 as an anti-tumor agent.

Materials and Methods

Chemicals and Antibodies

Recombinant human IL-10 was a generous gift from S. Narula, Schering Plough, Kennilworth, NJ. LlnL, staurosporine, and sodium orthovanadate were obtained from Sigma (St. Louis, MO). LlnL and staurosporine were dissolved in DMSO and used at final concentrations of 50 µM and 500 nM, respectively. Sodium orthovanadate was prepared in PBS and used at a final concentration of 1 mM. Sulfo-NHS-LC-Biotin was purchased from Pierce (Rockford, IL).

Mouse antiphosphotyrosine-IL-10E1 antibodies were produced against IL-10E1 isolated from nuclear protein extracts of IL-10-treated HPCA-10a cells using protein A-Sepharose beads coupled to PY 20 antibodies according to methods previously described (52). The antibodies were characterized by Western blotting with crude protein extracts from untreated and IL-10-treated HPCA-10a cells. Anti-IL-10E1 antibody has been previously characterized by our laboratory (34, 35). The horseradish peroxidase-conjugated anti-phosphotyrosine antibody was purchased from Santa Cruz Antibodies (San Diego, CA). Immunoprecipitations of JAK1 and TYK2 were performed with antibodies from UBI (Lake Placid, NY), while Western blotting was performed with antibodies from UBI and Santa Cruz (HR-758), respectively.

Cell Culture

The HPCA-10a and HPCA-10c cell strains were isolated from Gleason score 10 prostate glands and maintained in

FIGURE 10. ELISA measurements of TIMP-1 levels secreted in the medium of the Boyden chambers after 6 h incubation (see Fig. 9, A and B). Effect of increased amounts of IL-10 (1–22 ng/ml; left panel), increased amounts of IL-10 + LlnL (50 µM; middle panel), and vehicle (right panel). Columns, means averaged from four experiments with triplicate wells per experiment for each dosage of IL-10; bars, SD.
culture at low passage (<5 passages) (36, 37). These epithelial cells and HPV-18 immortalized strains derived from the HPCA-10a and HPCA-10c strains have been previously characterized and found to express PSA and cytokeratins 8 and 18 (36, 37). HPCA-10a cells were found to be androgen dependent, whereas HPCA-10a cells were androgen independent when grown subcutaneously in SCID mice (37). Cultures were maintained in MGEM supplemented with putritary extract and 0.1% ITS according to Clonetics (San Diego, CA) according to published methods (36, 37). Unless otherwise stated, the cells were treated with IL-10 at 15 ng/ml. Cellular proliferation was assessed using a modified semi-automated MTT assay (39).

Cell Extracts, Immunoprecipitations, and SDS-PAGE

Cells were washed three times with PBS and cultured for 12 h in the absence of IL-10. Where appropriate, L LNl 5000 was added and the culture continued for an additional 1 h, unless otherwise indicated, before stimulation with IL-10 (15 ng/ml) for 0–2 h. Cells were washed with PBS and both cytosolic and nuclear extracts were prepared as described previously by our lab (33) and others (51).

Immune complexes were washed twice with NP40 lysis buffer and once with Tris-buffered saline (TBS) before addition of 2× Laemlli sample buffer. Bound proteins were eluted by boiling for 10 min and separated by SDS-PAGE. JAK1 and TYK2 immunoprecipitations were performed as described earlier using protein A-Sepharose (Pharmacia, Piscataway, NJ) and the manufacturer’s recommended dilution of JAK1 or TYK2 antisa per immunoprecipitation, respectively. For Western blots of IL-10E1 or phospho-IL-10E1, extracts were mixed with 2× sample buffer and resolved by SDS-PAGE.

Western Blotting

Electrophoresed proteins were transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA) and blocked with 3% BSA in TBST (TBS plus 0.05% Tween 20). Antiphospho-IL-10E1 or anti-IL-10E1 antisera were diluted 1:10,000 in 1% BSA/TBST and incubated for 1 h at room temperature. Membranes were washed four times with TBST and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated protein A (Amersham, Arlington Heights, IL) in 1% BSA/TBST for 30 min at room temperature. After four washes with TBST, proteins were detected using enhanced chemiluminescence (ECL) reagent (Amersham). JAK1 and anti-TYK2 blotting were performed with 1:5000 dilutions of antisa. For PY 20 blotting, membranes were incubated with a 1:5000 dilution of antibody in 1% BSA/TBST, washed four times with TBST, and developed as described earlier. Where appropriate, membranes were stripped with a solution containing 2% SDS, 62.5 mM Tris, and 0.7% β-mercaptoethanol for 30 min at 55°C, washed extensively with H2O and twice with TBST, and reblocked with 3% BSA/TBST before addition of primary antibody. For some experiments, phosphorylated IL-10E1 was partially purified from IL-10-treated PC-3 ML cells (1 × 10³ cells) using protein A-Sepharose beads coupled to PY 20 antibodies according to published methodologies (51).

EMSA

Samples (5 µg) of nuclear extracts (described earlier) were used for EMSA. EMSA was performed with a IL-10E1 oligonucleotide probe (HTC-1) from TIMP-1 promoter element as described previously (33). After a 30-min incubation on ice with 32P-labeled probe, samples were electrophoresed on 6% non-denaturing polyacrylamide gels in 0.5× Tris/borate/EDTA (TBE) buffer. Gels were dried and subjected to autoradiography.

Phosphatase Assay

A non-radioactive tyrosine phosphatase assay kit was purchased from Mannheim Boehringer (Indianapolis, IN). Cells were washed once with PBS before NP40 lysis [1% NP40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 50 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin C, and 0.5 µg/ml leupeptin]. After 30 min, extracts were centrifuged (4°C) for 15 min at 13,000 rpm. Supernatants were diluted with lysis buffer to the equivalent of 10⁵ cells in a 20-µl volume.

ELISAs

ELISAs were carried out as previously described using IL-10E1, IL-10E1p, and TIMP-1 antibodies (35, 36).

Invasion Through Matrigel

The ability of cells to degrade and cross tissue barriers was assessed by two in vitro invasion assays that use Matrigel, a reconstituted basement membrane (Collaborative Research, Walthan, MA). Quantitative analysis of invasion was performed as previously described (40), using a modified Boyden chamber containing a Matrigel-coated filter, with HPCA-10a conditioned medium (prepared from 1 × 10⁵ cells incubated overnight in DMEM plus 1% FBS) as chemotactrant. Briefly, cells were placed onto the bottom chamber membrane surface ( precoated with 30 µg Matrigel/well) at 3 × 10⁴ cells/well (6.5-cm-diameter wells), allowed to attach for 1 h and then the chambers inverted, chemoattractant added to the top compartment, and incubations carried out at 37°C in a 5% CO₂ incubator for 6 h. Filters were then removed, fixed with methanol, and stained with Giemsa. Cells attached to the upper side of membranes (i.e., upper compartment) were removed and the number of invading cells determined by microscopy.

Source of Reagents

IL-10 (Schering-Plough); IL-10 and IL-10 receptor antibodies (Schering Plough; Kevin Moore, DNAX Inc., San Diego, CA); rabbit antibodies against human JAK1, JAK2, TYK2 (UBI, Inc., Saranac Lake, NY); protein A-Sepharose and protein G-Sepharose beads (Pharmacia); polyvinylidene difluoride membranes (Immobilon-P, Millipore); Anti-phosphotyrosine antibody PY 20 (Sigma).

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Interleukin-10 Activation of the Interleukin-10E1 Pathway and Tissue Inhibitor of Metalloproteinase-1 Expression Is Enhanced by Proteasome Inhibitors in Primary Prostate Tumor Lines 1CA 76639 to M.E.S.


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