Translational Regulation of X-Linked Inhibitor of Apoptosis Protein by Interleukin-6: A Novel Mechanism of Tumor Cell Survival

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ABSTRACT

Interleukin-6 (IL-6) is a pleiotropic cytokine with diverse biological effects. IL-6 has been implicated in autocrine signaling pathways promoting tumor progression and chemoresistance in some human tumors. However, the mechanisms by which IL-6 modulates these responses are unknown. Aberrant apoptosis has been implicated as a fundamental mechanism of chemotherapeutic resistance. Thus, we investigated whether IL-6 alters the expression of apoptosis regulatory proteins as a mechanism of drug resistance. We provide evidence that IL-6 rapidly phosphorylates the translation initiation factor eukaryotic initiation factor-4E and triggers antiapoptotic responses in cholangiocarcinoma cells. Reduction of cellular eukaryotic initiation factor-4E by RNA interference decreases IL-6-induced effects on cytotoxic drug-induced caspase activation and apoptosis. Furthermore, IL-6 increases expression of the endogenous X-linked inhibitor of apoptosis protein expression by translation at an internal ribosome entry site. Our findings that IL-6 translationally regulates X-linked inhibitor of apoptosis protein expression reveal a novel mechanism by which IL-6 mediates tumor cell survival that may be targeted therapeutically to decrease tumor progression and chemoresistance.

INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic cytokine with diverse biological effects (1). In addition to regulating inflammatory responses, IL-6 modulates the growth of many tumor cells. Autocrine IL-6-mediated signaling pathways have been implicated in tumor progression and chemoresistance in epithelial (e.g., cholangiocarcinoma) and hematopoietic (e.g., multiple myeloma) human tumors (2–5). Dysregulation of apoptosis is a fundamental phenomenon in human cancers and may promote tumor cell survival in response to environmental perturbations, such as cytotoxic drugs that may otherwise be lethal (6). Thus, modulation of tumor growth may involve direct effects on tumor cell survival as well as mitogenic signaling mechanisms. Although mitogenic signaling by IL-6 is well characterized, the role of its survival signaling is poorly understood. Cell survival signaling mechanisms may involve modulation of classical apoptosis-inducing pathways, such as those mediated by death receptor ligation or involving mitochondrial dysfunction or by aberrant expression of endogenous downstream apoptosis inhibitory proteins.

IL-6 is a well-characterized autocrine cytokine promoting growth of cholangiocarcinoma (4, 7–9). These tumors are uniformly fatal and highly refractory to conventional chemotherapeutic agents (10). Although modulation of survival pathways by IL-6 may contribute to chemoresistance, it is unknown if IL-6 signaling can modulate apoptosis in cholangiocarcinoma. IL-6 activates intracellular p38 mitogen-activated protein kinase (MAPK) signaling in malignant but not normal cholangiocytes (8, 11). We have also shown that p38 MAPK signaling activates a dominant cell survival pathway in malignant cholangiocytes in response to double-stranded RNA (12). Furthermore, p38 MAPK signaling promotes tumor growth and is involved in regulation of the initiation of translation in malignant cholangiocytes (8, 13). These observations suggest that IL-6 may promote cell survival pathways in malignant cholangiocytes by translational regulation of gene expression.

Although many critical genes involved in the tumoral response to environmental changes can be translationally regulated, the role of translational dysregulation in mediating chemoresistance is poorly understood (14). Our overall objective was to investigate whether IL-6 alters the expression of apoptosis regulatory proteins as a mechanism of drug resistance. Our specific aims were to answer the following questions using a human cholangiocarcinoma cell line: (a) does IL-6 inhibit apoptosis in response to chemotherapeutic drugs?; (b) can IL-6 modulate translation in malignant cholangiocytes?; (c) are translationally regulated genes involved in the IL-6 response to chemotherapy; and (d) if so, how does IL-6 regulate their expression? Our results suggest that IL-6 inhibits apoptosis by regulating the translational expression of the X-linked inhibitor of apoptosis (XIAP), an endogenous inhibitor of apoptosis.

MATERIALS AND METHODS

Cells. KMCH-1 human malignant cholangiocytes were obtained as described previously and cultured in DMEM with 10% fetal bovine serum (8). H69 cells, immortalized human nonmalignant cholangiocytes, were obtained and cultured as described previously (15). Mz-ChA-1 cells, malignant human cholangiocytes (kindly provided by Dr. J. G. Fitz, University of Colorado, Denver, CO), and TFK-1 cells (kindly provided by Dr. Y. Ueno, Tohoku University, Sendai, Japan) were cultured in Connaught Medical Research Laboratories 1066 media with 10% fetal bovine serum, 1% L-glutamine, and 1% antimycotic antibiotic mix.

Apoptosis Assay. Cells with morphological changes indicative of cell death by apoptosis were identified and quantitated by fluorescence microscopy after staining with 4′,6-diamidino-2-phenylindole as described previously (8). Fluorescence was visualized using an Olympus BX40 upright fluorescence microscope (Olympus America, Inc., Melville, NY). Apoptotic nuclei were identified by condensed chromatin as well as nuclear fragmentation. At least 300 nuclei in four high-power fields were counted.

Caspase-3 Activation Assay. Cells were harvested and permeabilized and fixed using the Cytofix/Cytoperm kit (BD Biosciences). The cells were stained with anti-caspase-3 monoclonal antibody (BD Biosciences), washed, and then stained with Cy5-anti-IgG (Jackson ImmunoResearch Laboratories). The cells were also stained with the nucleic acid dye SYTO-16 (Molecular Probes, Eugene, OR). Stained cells were suspended in an isobuoyant cell buffer at 2 × 10^6 cells/ml. Ten microliters of the cell suspension were applied to sample wells of the cell assay chip and assayed on the Agilent 2100 Bioanalyzer microfluidic system (Agilent, Palo Alto, CA). A range of cell events (500–1000) was collected per sample. Fluorescence emission from the cells is detected with photodiodes at 510–540 and 674–696 nm. Cell events in the SYTO-16-positive population were cross-gated onto the caspase-3 histogram to determine the percentage of apoptotic cells.

Translational Activity. Translational activity was assessed by sucrose gradient fractionation and polysomal RNA analysis as we have described previously (13). Approximately 1 × 10^7 cells were grown to 50–70% confluence on 100-mm plates. Before cell harvesting, 100 μg/ml cycloheximide were
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added for 5 min to arrest ribosome movement on polysomes. Cellular cytosolic extracts were obtained by lysis using buffer containing 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 100 mM MgCl2, 10% NP40, and 30 units/ml RNase inhibitor at 4°C and removal of nuclei by centrifugation. The supernatant was then layered onto a 10-mL linear 10–40% (w/v) sucrose gradient supplemented with 10 mM Tris-HCl (pH 7.5), 100 mM ammonium chloride, and 2 mM 2-mercaptoethanol and centrifuged in a SW41 rotor without brake (Beckman, Palo Alto, CA) at 35,000 rpm, for 15 min, and at 4°C. Fractions (500 μl) were collected, and absorbance at 260 nm was recorded. The polysome: monosome ratio, an index of translational efficiency, was determined as the ratio of the areas of 2–4mer polysomes (actively translated mRNA) to the areas of 80S monosomes (untranslated or under-translated mRNA) measured using NIH image.

**Immunoblot Analysis.** Cell lysis and immunoblotting were performed as described previously using the respective antihuman primary antibody (1:1000 dilution), and a polyclonal goat antirabbit secondary antibody (1:2000 dilution), and visualized using an enhanced chemiluminescence method (Lumi-GLO; Cell Signaling, Beverly, MA; Ref. 12).

**Double-Stranded RNA Design and Synthesis and Transfection.** RNA interference for gene silencing was performed using small interfering 21-bp oligonucleotides (synthesized previously (17)). The cells were then treated with IL-6 (10 ng/ml) for varying times >24 h. Cell extracts were obtained using the M-PER extraction reagent (Pierce, Rockford, IL). β-galactosidase enzymatic activity was determined in cell extracts by a spectrophotometric assay using chloroformolcohol-β-D-galactopyranoside monosodium as we have reported previously (18). CAT activity was assessed using a commercially available ELISA assay (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions. IRES-mediated translation was assessed as the relative CAT activity calculated by normalizing CAT activity with β-galactosidase activity and expressed relative to activity in untreated cells which were set as 100%.

**Materials.** Fetal bovine serum and Bradford reagent were obtained from Sigma (St. Louis, MO). All other cell culture reagents and media were from Life Technologies, Inc. (Grand Island, NY). Monoclonal antibodies to eIF-4E, XIAP, and actin were from Sigma. All phospho-specific antibodies used were obtained from Cell Signaling. The protease inhibitor cocktail tablets were obtained from Roche. Gemicitabine was provided by Eli Lilly (Indianapolis, IN). All other reagents were of analytical grade from the usual commercial sources.

**Statistical Analysis.** Data are expressed as the mean ± SE from at least three separate experiments performed in triplicate, unless otherwise noted. The differences between groups were analyzed using a double sided Student t test when only two groups were present. Statistical significance was considered as P < 0.05. Statistical analyses were performed with the GB-STAT statistical software program (Dynamic Microsystems, Inc., Silver Spring, MD).

**RESULTS**

IL-6 Decreases Chemotherapy-Induced Apoptosis in Malignant Cholangiocytes. IL-6 has been shown to decrease the susceptibility of human cholangiocytes to undergo apoptosis (19). To assess the role of IL-6 in mediating cell survival in malignant cholangiocytes, we began by determining IL-6 receptor expression. All cell lines were shown to express the IL-6 receptor by immunoblot analysis (data not shown).

**Translational Inhibitors Block the Antiapoptotic Effects of IL-6 and Decrease Tumor Cell Resistance to Chemotherapy-Induced Apoptosis.** We have shown previously that IL-6 aberrantly activates the p38 MAPK signaling pathway and that p38 MAPK signaling mediates diverse tumoral characteristics (8, 11, 13). Furthermore, we have shown that p38 MAPK signaling modulates the initiation of protein translation (13). To assess the role of translational mechanisms during cell death induced by chemotherapy, we first determined the effects of the translation inhibitors cycloheximide or rapamycin on the protective effect of IL-6 on camptothecin-induced apoptosis. Both cycloheximide and rapamycin increased apoptosis during incubation with camptothecin (Fig. 2). Furthermore, both of these inhibitors of translation decreased the antiapoptotic effects of IL-6 (Fig. 2). Col-

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lectively, these data support the involvement of translationally modulated mechanisms in IL-6 modulation of camptothecin-induced apoptosis.

**IL-6 Increases eIF-4E Phosphorylation and Translation in KMCH Cells.** Translational regulation of gene expression mainly occurs by modulation of the initiation stage of protein translation. The main mechanisms for regulating the initiation of translation involve the formation of active eIF4F and the eIF2-GTP-Met-tRNA ternary complexes. The initiation of translation is highly regulated, and the assembly and functional activity of these initiation complexes can be manipulated by phosphorylation of specific subunits or their interacting proteins. The phosphorylation state of specific translational regulatory factors was assessed in serum-starved KMCH cells incubated with IL-6 for varying periods of time (Fig. 3). Although expression of eIF-4E was not increased, IL-6 markedly increased eIF-4E (Ser209) phosphorylation. Relatively minor changes were seen in the phosphorylation of eIF2A (Ser51), p70 S6 kinase (Thr389), or ribosomal protein S6 (Ser235/236), suggesting a role for direct modulation of eIF4E by IL-6 as a mechanism for translational regulation. eIF-4E is a rate-limiting translation initiation factor that binds to the cap structure at the 5'-end of mRNAs of eukaryotic mRNAs as a component of the cap-binding complex eIF-4F that mediates the recruitment of ribosomes to mRNA. Alterations in initiation will result in altered
EIF4E Expression Modulates Camptothecin Sensitivity. EIF4E is an important regulator of mRNA translation and protein synthesis and is up-regulated in malignant or proliferating cholangiocytes (20). To directly assess the role of translational regulation in the response to camptothecin, we used siRNA to functionally decrease EIF4E expression. Translational dysregulation by siRNA to EIF4E increased camptothecin-induced cell death compared with scrambled nucleotide RNA controls (Fig. 4). Thus, resistance to camptothecin-induced apoptosis is dependent on translationally regulated gene expression.

IL-6 Increases XIAP Translation in an EIF4E-Dependent Manner. We next sought to define translationally regulated survival mechanisms downstream of IL-6 receptor activation. Cells were transiently transfected with either siRNA to EIF4E or scrambled nucleotide control before incubation with IL-6 (10 ng/ml) for 60 min. Using a commercial antibody microarray (Clontech antibody microarray), we compared relative changes in expression levels of 378 proteins in cellular extracts between the two groups. Several proteins were identified whose expression was induced or repressed by ≥1.5-fold. This protein-profiling screen revealed alterations in the XIAP, an endogenous inhibitor of apoptosis that has been associated with chemoresistance in other cell types. XIAP expression was reduced in cells transfected with siRNA to EIF4E compared with controls. This raised the possibility that XIAP may represent an effector of the antiapoptotic effects of IL-6. We first confirmed our antibody microarray data by Western blot analysis using commercial antibodies to XIAP after IL-6 treatment in KMCH cells. An increase in XIAP protein was observed during incubation with IL-6 (Fig. 5). Furthermore, we assessed the effect of IL-6 on mRNA expression by reverse transcription-PCR. No significant changes in XIAP mRNA were detected, suggesting that the observed increase in XIAP protein levels is attributable to translational up-regulation.

XIAP Mediates Resistance to Camptothecin. To assess the role of XIAP in mediating resistance to camptothecin, two sets of studies were performed. First, we used siRNA to decrease cellular XIAP expression. Compared with a scrambled nucleotide control, siRNA to XIAP decreased cell viability during incubation with camptothecin (Fig. 6). The reduction in cell viability was, however, less than that observed during incubation with siRNA to EIF4E or with the translational inhibitors cycloheximide or rapamycin. These results suggest the presence of other translationally regulated mediators of resistance to camptothecin, in addition to XIAP. Next, we assessed the potential functional effects of increased cellular XIAP expression. Cells were transiently transfected with pCl-IRES-XIAP to increase XIAP. Compared with control cells transfected with pCl-lacZ, there was a decrease in camptothecin cytotoxicity in cells overexpressing XIAP (Fig. 7). In combination, these data suggest that modulation of distribution of rRNA between monosome and polysome-associated fractions. Incubation of serum-starved KMCH cells with IL-6 (10 ng/ml) altered global rRNA distribution with an increase in the polysome:monosome ratio to 112 ± 4% of controls after 1 h and 129 ± 7% after 3 h, consistent with an increase in initiation of translation. Collectively, these data support the involvement of EIF4E in translational regulation by IL-6.
XIAP expression can inhibit the effects of IL-6 on sensitivity to camptothecin.

**IL-6 Increases Internal Initiation of XIAP at an IRES Site.** XIAP expression is regulated primarily at the translational level (17, 21, 22). XIAP is one of only a few mRNA whose expression can be translationally regulated by initiation of translation at an IRES site located in the 5′ untranslated region (23). To determine the mechanism of regulation of XIAP expression, we assessed the effect of IL-6 on a bicistronic construct comprising of constructs for β-gal and CAT separated by the XIAP IRES. Cap-dependent translation results in β-gal expression, whereas IRES activation results in increased CAT expression. Because both reporter genes are transcribed on the same mRNA, the relative amounts of cap-dependent versus cap-independent, IRES-mediated translation can be determined by the expression of CAT relative to that of β-gal. IL-6 increased relative CAT activity, and, hence, XIAP IRES activity, in a time-dependent manner (Fig. 8). These results suggest that IL-6 regulation of XIAP expression occurs by increased translation at the XIAP IRES site.

**DISCUSSION**

The principal findings of this study are that human cholangiocarcinoma cells respond to IL-6 by increased IRES-mediated translation of the endogenous apoptosis inhibitor XIAP. Dysregulation of protein synthesis and translation by the inhibitors cycloheximide and rapamycin or siRNA-targeted reduction in expression of the rate-limiting translation initiation factor eIF-4E decrease resistance to apoptosis. These studies thus describe a translationally regulated mechanism by which IL-6 can modulate chemotherapy-induced apoptosis.

Abundant evidence implicates translational dysregulation in promoting tumor growth (14, 24). Aberrant expression of translationally regulated apoptosis regulatory genes could promote cell survival under otherwise detrimental environmental conditions. This could result in the survival of cells with inheritable genetic defects that may have otherwise caused cell death. eIF-4E is overexpressed in several diverse malignancies, including cholangiocarcinoma, and modulation of eIF-4E decreases tumor cell growth (20, 25). Consistent with several other observations, eIF-4E appears to translationally regulate proteins that may be involved in the early cellular response to otherwise detrimental environmental changes. We have extended these observations by demonstrating a role for translational regulation of the response of malignant cholangiocytes to chemotherapy-induced apoptosis.

Suppression of apoptosis is a critical feature in human malignancies. XIAP is a member of a multigene family that suppresses apoptosis, has been implicated in chemoresistance, and is overexpressed in many human cancers (26–28). Indeed, inhibition of XIAP is being explored as a strategy for cancer treatment (29, 30). Our studies have identified XIAP as an IL-6-regulated gene. IL-6-mediated autocrine signaling has been implicated in cholangiocarcinoma growth and progression. Thus, modulation of XIAP expression by autocrine IL-6 signaling may contribute to chemoresistance as well as to the pathogenesis and progression of cholangiocarcinoma. Thus, targeting XIAP expression and the cellular mechanisms involved in IL-6 regulation of XIAP expression are attractive targets for improving chemosensitivity and the treatment of cholangiocarcinoma.

Although IRES-mediated translation was initially described for viral RNAs as a mechanism for translation of uncapped viral RNAs, IRES elements have been described in some eukaryotic mRNAs, such as XIAP (23). The presence of IRES sites allows for a rapid response to environmental changes independent of de novo transcription. Furthermore, these IRES sites may function by allowing cap-independent translation of mRNAs under conditions in which cap-dependent translation is inhibited. An emerging paradigm is that selective translation of mRNAs based on internal initiation IRES sites facilitates translation of stress–response proteins under conditions, such as apoptosis, that are associated with inhibition of cap-dependent protein synthesis (31). Our findings are consistent with this concept and support other
studies demonstrating IRES-mediated translation of XIAP during cellular stresses, such as exposure to ionizing radiation-induced stress or apoptosis (17, 21).

Translational regulation of survival proteins such as XIAP, that are capable of modulating response to chemotherapy, is significant given the intense interest in using mRNA microarray analysis to identify predictors of chemosensitivity in human cancers. Such strategies will be limited to identifying transcriptionally regulated genes but will fail to identify those genes capable of modulating responses to chemotherapy that are predominantly regulated at a translational level. Complementary proteomic profiling may enhance the validity and applicability of these strategies.

In summary, we have shown that chemoresistance of malignant human cholangiocytes can be mediated by a mechanism involving translational regulation of XIAP. Furthermore, increased XIAP expression in response to autocrine IL-6 stimulation may contribute to the refractoriness of human cholangiocarcinoma to conventional chemotherapy. Thus, manipulation of XIAP expression or inhibition of the antiapoptotic effects of XIAP is an attractive strategy to improve cholangiocarcinoma responses to chemotherapy.

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