Interleukin 6 Inhibits Proliferation and, in Cooperation with an Epidermal Growth Factor Receptor Autocrine Loop, Increases Migration of T47D Breast Cancer Cells

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ABSTRACT

Interleukin (IL)-6, a multifunctional regulator of immune response, hematopoiesis, and acute phase reactions, has also been shown to regulate cancer cell proliferation. We have investigated IL-6 signaling pathways and cellular responses in the T47D breast carcinoma cell line. The IL-6-type cytokines, IL-6 and oncostatin M, simultaneously inhibited cell proliferation and increased cell migration. In T47D cells, IL-6 stimulated the activation of Janus-activated kinase 1 tyrosine kinase and signal transducers and activators of transcription (STAT) 3 and 5 transcription factors. Expression of dominant negative STAT3 in the cells strongly reduced IL-6-mediated growth inhibition but did not prevent IL-6-induced cell migration. IL-6 treatment led to activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3′-kinase (PI3K) pathways. Inhibition of MAPK or PI3K activity reversed IL-6- and oncostatin M-stimulated migration. Because cross-talk between cytokine receptors and members of the ErbB family of receptor tyrosine kinases has been described previously, we have examined their interaction in T47D cells. Down-regulation of ErbB receptor activity, through the use of specific pharmacological inhibitors or dominant negative receptor constructs, revealed that IL-6-induced MAPK activation was largely dependent on epidermal growth factor (EGF) receptor activity, but not on ErbB-2 activity. Using a monoclonal antibody that interferes with EGF receptor-ligand interaction, we have shown that in T47D cells, IL-6 cooperates with an EGF receptor autocrine activity loop for signaling through the MAPK and PI3K pathways and for cell migration. Both the tyrosine phosphatase SHP-2 and the multisubstrate docking molecule Gab1, which are potential links between IL-6 and the MAPK/PI3K pathways, were constitutively associated with the active EGF receptor. On IL-6 stimulation, SHP-2 and Gab1 were recruited to the gp130 subunit of the IL-6 receptor and tyrosine phosphorylated, allowing downstream signaling to the MAPK and PI3K pathways. Thus, in T47D breast carcinoma cells, IL-6 acts in synergy with EGF receptor autocrine activity to signal through the MAPK/PI3K pathways. Cooperation between IL-6 and the EGF receptor in T47D breast carcinoma cells illustrates how a combination of multiple stimuli, either exogenous or endogenous, may result in synergistic cellular responses.

INTRODUCTION

IL-6 is a pleiotropic cytokine that is implicated in a variety of cellular functions in immune, hematopoietic, neural, and hepatic systems (1). IL-6 has also been shown to influence the proliferation of normal and tumor-derived cells. IL-6 promotes proliferation of hematopoietic progenitors, keratinocytes, myeloma/plastocytoma, and Kaposi's sarcoma cells, whereas it inhibits the proliferation of M1 myeloid leukemia cells, early-stage melanoma cells, and lung and breast tumor cells. Thus, depending on the target cell, IL-6 induces various and sometimes contrasting biological responses.

IL-6 is a member of the IL-6-type cytokine family, which comprises OSM, LIF, IL-11, ciliary neurotrophic factor, and cardiotoxin-1 (1, 2). These peptides promote similar biological responses in various tissues and cells. This redundancy in biological actions can be explained at the molecular level because the different members of this cytokine family share signaling molecules. Indeed, IL-6-type cytokines bind to multimeric receptors comprising an α chain, which confers ligand specificity and a signal-transducing β subunit (gp130) common to all IL-6-type cytokines.

On ligand binding, the gp130-associated intracellular tyrosine kinases Jak1, Jak2, and Tyk2 become activated and phosphorylate the gp130 cytoplasmic tail on specific tyrosine residues. These phosphotyrosines serve as docking sites for signaling molecules, including STATs and molecules mediating activation of the Ras/MAPK pathway, such as the tyrosine phosphatase SHP-2 and the Shc adapter molecule. STATs, in turn, are phosphorylated on tyrosine residues, allowing their dimerization and translocation to the nucleus, where they regulate gene transcription. Shc and SHP-2 are also tyrosine phosphorylated by the Jaks, initially leading to the recruitment of adapter molecules, such as Grb2, and ultimately leading to the activation of MAPK. More recently, the multisubstrate docking molecule, Gab1, was shown to act as an adapter linking gp130 to both the MAPK and the PI3K pathways (3).

Recent studies have shown that cytokine-induced activation of the Ras/MAPK pathway is, in some instances, dependent on cross-talk with ErbB receptor tyrosine kinases. Aberrant expression of members of the ErbB receptor tyrosine kinase family, which is observed in a variety of human tumors (4, 5), has been linked to abnormal cell growth and transformation. The ErbB family includes four members: (a) the EGF receptor/ErbB-1; (b) ErbB-2/Neu; (c) ErbB-3; and (d) ErbB-4. ErbB receptor activity is regulated by EGF-related peptides, which bind to ErbB-1 and ErbB-4, and neuregulins, which are ligands for ErbB-3 and ErbB-4. ErbB-2 has no known ligand, but it is the preferred dimerization partner of all ErbB receptors and plays a central role in ErbB signaling (6, 7). In prostate carcinoma cells, IL-6 appears to induce activation of ErbB-2 and ErbB-3 receptors, forming a gp130/ErbB-2/ErbB-3 complex and leading to MAPK activation and cell proliferation (8). GH-induced activation of the Ras/MAPK pathway has also been shown to be dependent on the EGF receptor. In this case, a mechanism involving Jak2-mediated tyrosine phosphorylation of a Grb2 binding site in the cytoplasmic domain of the EGF receptor was proposed (9).

IL-6-type cytokines have diverse actions on breast cancer cell lines, including changes in morphology, decreased cell-cell association, and inhibition of cell proliferation (10–12). Interestingly, expression of gp130 and the α-specific subunits of IL-6-type cytokine receptors has been found by PCR analyses in a large majority of breast cancer cell lines and in primary malignant breast tissue (12). In addition, IL-6 and other IL-6-type cytokines are expressed in many primary breast tu-
mors (13–15). However, IL-6 expression is reduced in invasive breast carcinoma relative to normal mammary tissue and appears to be inversely associated with histological tumor grade (15, 16). Although little is known about IL-6-induced signal transduction in breast cancer, these observations suggest that this cytokine might be involved in regulating the growth of these cancer cells.

We have investigated IL-6-induced signaling in the T47D breast carcinoma cell line. Our results show that IL-6-type cytokines inhibit proliferation and increase cell migration. We demonstrate that IL-6-induced growth inhibition is dependent on activation of the Jak/STAT pathway, whereas activation of the MAPK and PI3K pathways is required for IL-6-stimulated cell motility. Finally, we show that in T47D cells, contrary to what has been described in some prostate carcinoma cell lines, IL-6-induced MAPK activation does not involve ErbB-2 activation but depends on the cooperation of an EGF receptor autocrine loop.

MATERIALS AND METHODS

Cell Culture and Cell Transfection. T47D, SKBr3, and MCF7 breast carcinoma cells were maintained in DMEM supplemented with 10% FCS (Life Technologies, Inc., Grand Island, NY) and 5 μg/ml insulin. T47D-5R cells were obtained by infection of T47D cells with a pBabe-based retrovirus expressing the scFv-5R DNA as described previously (17). Infected cells were selected in 2 μg/ml puromycin, and pools of resistant cells were analyzed in all experiments. pCAGGS-NeoSTAT3wt and pCAGGS-NeoSTAT3F (kindly provided by T. Hirano; Osaka University, Osaka, Japan) were introduced into T47D cells using Superfect transfection reagent (Qiagen). A HA-tagged MAPK (Erk2) construct (kindly provided by M. El-Shemely and Y. Nagamine; Friedrich Miescher Institute, Basel, Switzerland) was cotransfected with a DN EGF receptor construct lacking 533 COOH-terminal amino acids (kindly provided by A. Ullrich; Max Planck Institut, Martinsried, Germany) according to the manufacturer’s protocol. Cells were selected in 1 mg/ml G418, and several clones were picked, expanded in the presence of G418, and analyzed for STAT3wt or STAT3F expression. Similar results were obtained with two independent STAT3F-expressing T47D clones.

For transient transfections, plasmids were introduced into the cells using Superfect transfection reagent (Qiagen). A HA-tagged MAPK (Erk2) construct (kindly provided by M. El-Shemely and Y. Nagamine; Friedrich Miescher Institute, Basel, Switzerland) was cotransfected with a DN EGF receptor construct lacking 533 COOH-terminal amino acids (kindly provided by A. Ullrich; Max Planck Institut, Martinsried, Germany), a DN ErbB-2 construct (18), or a DN Jak1 construct (kindly provided by O. Silvernöien; University of Tampere, Tampere, Finland). Cells were allowed to grow for 24 h before starvation and cytokine treatment.

Cells were starved for 16 h in serum-free medium before treatment with recombinant human IL-6 (R&D Systems, Minneapolis, MN) or ProteoTechEC (London, United Kingdom), recombinant human OSM and LIF (PeproTech EC, London, United Kingdom). Cells were selected in 1 mg/ml G418, and several clones were picked, expanded in the presence of G418, and analyzed for STAT3wt or STAT3F expression. Similar results were obtained with two independent STAT3F-expressing T47D clones.

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RESULTS

IL-6-type Cytokines Inhibit the Proliferation of the T47D Breast Carcinoma Cell Line. IL-6 and other IL-6-type cytokines have been shown to inhibit the proliferation of some breast cancer cell lines. We have evaluated the effect of IL-6, OSM, and LIF on T47D breast tumor cells in an anchorage-dependent colony formation assay. T47D cells seeded at low density were allowed to grow in the presence of various amounts of recombinant human IL-6, and the number and size of colonies formed after 10 days were evaluated relative to untreated cells. IL-6 inhibited the proliferation of T47D cells in a dose-dependent manner and was effective at doses as low as 1 ng/ml (Fig. 1A). The effect on the formation of large colonies was more pronounced, indicating that IL-6 retards proliferation. Flow cytometry analysis of T47D cells grown in the presence of IL-6 for 24 h showed a reproducible 10–15% increase in the number of cells in the G1 phase of the cell cycle (data not shown). Whereas OSM was even more potent than IL-6 in inhibiting T47D cell proliferation, LIF had a more moderate effect, as seen in the colony growth assay (Fig. 1B) or by cell cycle analysis (data not shown).

IL-6-induced Activation of the Jak/STAT Pathway. The Jak/STAT pathway is one of the major mediators of IL-6 signal transduction. We first verified that IL-6 is capable of inducing tyrosine phosphorylation of the gp130 receptor subunit, a primary event in IL-6-induced intracellular signaling (Fig. 2A). We then investigated which particular Jak and STAT were activated by IL-6. Although Jak1, Jak2, and Tyk2 were expressed in T47D cells, only Jak1 was

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significantly tyrosine phosphorylated on IL-6 stimulation (Fig. 2A). Differential activation of the Jak family members in different cell lines has been described previously (23, 24). IL-6 also induced the phosphorylation of STAT3 and, to a lesser extent, STAT1 (Fig. 2B). Neither STAT5a nor STAT5b, which can be activated in response to IL-6 in some cell lines, was tyrosine phosphorylated in IL-6-stimulated T47D cells.

IL-6-induced MAPK and PI3K Activation: Role of ErbB Receptors. Activation of MAPK by cytokines and, in particular, by IL-6 has been shown, in some instances, to be dependent on cross-talk with ErbB receptor tyrosine kinases (8, 9). In T47D cells, IL-6 stimulated both the MAPK pathway and the PI3K pathway, as monitored by the phosphorylation of MAPK and PKB, a PI3K downstream effector (Fig. 3A). These cells are also known to express moderate levels of all ErbB receptors. To evaluate the role of these receptors in IL-6 signaling, we tested the effect of ErbB-specific pharmacological inhibitors on IL-6 induction of the MAPK and PI3K pathways. T47D cells were pretreated with the EGF receptor-specific inhibitor CGP59326 (25) or with the bispecific EGF receptor/ErbB-2 inhibitor PD153035 (26) before stimulation with IL-6, EGF, or heregulin β, a member of the neuregulin family. Heregulin β signals through ErbB-2-containing heterodimers, whereas EGF signaling involves EGF receptor- and ErbB-2-containing dimers (7). As expected, whereas both the CGP59326 and the PD153035 inhibitors completely blocked EGF-induced MAPK phosphorylation, only the PD153035 inhibitor effectively inhibited heregulin β-induced MAPK activation (Fig. 3A). Interestingly, both inhibitors strongly diminished IL-6-induced MAPK activation (Fig. 3A). The PI3K pathway was affected by the ErbB inhibitors in the same manner (Fig. 3A). In contrast, IL-6-induced STAT3 phosphorylation was not influenced by the presence of the ErbB inhibitors (Fig. 3B). These results indicate that EGF receptor activity might be involved in IL-6 induction of the MAPK and PI3K pathways, but not the STAT pathway.

To confirm the specific involvement of EGF receptor in IL-6 signaling, we tested the effect of a DN signaling-defective EGF receptor, as well as DN ErbB-2 and DN Jak1 constructs, on IL-6-induced activation of the MAPK pathway. T47D cells were cotransfected with a HA-tagged MAPK construct and DN ErbB-1, DN ErbB-2, or DN Jak1 constructs before stimulation with IL-6 and analysis of MAPK phosphorylation. In the presence of DN ErbB-1, IL-6-induced MAPK phosphorylation was significantly reduced relative to the control, whereas the presence of DN ErbB-2 did not affect the level of MAPK phosphorylation (Fig. 3C). This result confirms that IL-6 requires the presence of active EGF receptor for full activation of MAPK. Interestingly, DN Jak1 also interfered with IL-6-induced MAPK activation (Fig. 3C), indicating that Jak1 is required for MAPK activation in response to IL-6.

ErbB-2 Is Not Required for IL-6-induced Activation of MAPK and PI3K. The above-mentioned results indicate that ErbB-2 activity is not required for MAPK activation in response to IL-6. It is possible, however, that ErbB-2 may still be used as a scaffold by IL-6 to activate MAPK, in the same manner as the GH receptor uses EGF receptor (9). To more definitively establish the role of ErbB-2 in IL-6-induced MAPK activation, we have functionally inactivated the ErbB-2 receptor through intracellular expression of an ErbB-2-specific single chain antibody, scFv5R, which traps ErbB-2 in the endoplasmic reticulum (17). T47D-5R cells expressed levels of ErbB-2 similar to those of the control T47D cells (Fig. 4A), but intracellular retention of ErbB-2 resulted in faster electrophoretic mobility because of impaired glycosylation (17, 27). Treatment of T47D cells with heregulin β strongly stimulated ErbB-2 tyrosine phosphorylation (Fig. 4B). Interestingly, both inhibitors strongly diminished IL-6-induced MAPK phosphorylation (Fig. 3C). This result confirms that IL-6 requires the presence of active EGF receptor for full activation of MAPK. Interestingly, DN Jak1 also interfered with IL-6-induced MAPK activation (Fig. 3C), indicating that Jak1 is required for MAPK activation in response to IL-6.

IL-6 Signaling in Breast Cancer Cells. T47D cells were seeded at low density and allowed to grow in the presence of the indicated cytokine for 10 days. A, colonies formed in the presence of the indicated concentrations of IL-6 were fixed and stained, and size distribution was evaluated. Data are expressed relative to cells grown in the absence of IL-6. B, colonies formed in the presence of LIF, IL-6, or OSM were fixed and stained, and colonies over 200 nm in diameter were counted.

**Fig. 1.** IL-6-type cytokines inhibit the proliferation of T47D breast carcinoma cells. T47D cells were seeded at low density and allowed to grow in the presence of the indicated cytokine for 10 days. A, colonies formed in the presence of the indicated concentrations of IL-6 were fixed and stained, and size distribution was evaluated. Data are expressed relative to cells grown in the absence of IL-6. B, colonies formed in the presence of LIF, IL-6, or OSM were fixed and stained, and colonies over 200 nm in diameter were counted.

**Fig. 2.** IL-6-induced activation of the Jak/STAT pathway in T47D cells. Cells were incubated in presence of 100 ng/ml IL-6 for 15 min, before lysis, immunoprecipitation with the indicated antibodies (IP), and Western blotting (WB) with a phosphotyrosine-specific antibody (P-Tyr, top panels). Membranes were stripped and reprobed with specific antibodies (bottom panels).
phosphorylation and activation of intracellular signaling pathways, including the PI3K and MAPK pathways (Fig. 4B). IL-6, although inducing MAPK and PKB phosphorylation (Fig. 4B), did not significantly increase ErbB-2 tyrosine phosphorylation (Fig. 4A). As shown previously (27), T47D-5R cells treated with heregulin β displayed decreased ErbB-2 tyrosine phosphorylation and, consequently, decreased MAPK and PI3K activation relative to control cells (Fig. 4). Moreover, heregulin β-stimulated proliferation of T47D-5R cells was strongly impaired (data not shown). Despite the fact that ErbB-2 was functionally inactive, IL-6 was still capable of activating the MAPK and PI3K pathways to levels comparable to those in control cells. Therefore, ErbB-2 does not appear to be involved in IL-6-induced activation of the MAPK and PI3K pathways in the T47D cell line. Similar results were obtained using the scFv-5R-expressing MCF7 and SKBr3 breast carcinoma cell lines, which express moderate and high levels of ErbB-2, respectively (data not shown). Thus, in breast carcinoma cells, ErbB-2 does not appear to be a critical element in IL-6-induced MAPK activation.

IL-6-induced MAPK Activation: Role of an EGF Receptor Autocrine Loop. We further explored the mechanism of EGF receptor contribution to IL-6 signaling. Contrary to what has been observed for GH (9) or agonists of GPCRs (28, 29), IL-6 treatment did not cause a detectable increase in EGF receptor phosphotyrosine content.

However, we observed that the EGF receptor displayed low but significant levels of tyrosine phosphorylation, even in the absence of an exogenous stimulus. This observation raised the possibility that in T47D cells, the EGF receptor may be active due to autocrine stimulation by endogenous EGF-related peptides and that this could contribute to IL-6 induction of the MAPK pathway. In fact, several EGF receptor ligands, including EGF, HB-EGF, amphiregulin, and transforming growth factor α are expressed in T47D cells (5). To test this hypothesis, T47D cells were pretreated with the EGF receptor-specific mAb 528, which interferes with ligand binding to the EGF receptor, before being stimulated with IL-6. As a control, cells were pretreated with mAb R1, which also binds the EGF receptor extracellular domain but does not interfere with ligand binding. Treatment with mAb 528 decreased the basal level of MAPK activation, indicating the presence of an EGF-like ligand/EGF receptor autocrine loop (Fig. 5A).

Interestingly, IL-6-induced MAPK activation was also strongly decreased in the presence of the EGF receptor blocking antibody (Fig. 5A). IL-6-induced PKB phosphorylation was similarly decreased by the EGF receptor blocking antibody (data not shown). This effect was due to the blocking ability of mAb 528 because the control mAb R1 did not inhibit IL-6-induced MAPK phosphorylation and even increased its activation slightly, probably through EGF receptor clustering (Fig. 5B). Importantly, densitometric analysis indicated that, when combined, IL-6 treatment and EGF receptor autocrine activity (Fig. 5A, 4 D. Salomon, personal communication.)
Tyrosine Phosphorylation of SHP-2 and Gab1, but not Their Association with the EGF Receptor, Is IL-6 Dependent. To better understand the role of the EGF receptor autocrine loop in IL-6-induced MAPK activation, we investigated several adapter molecules that might link IL-6 receptor to the Ras/MAPK pathway. The Shc adapter molecule has been implicated in IL-6 signaling to the MAPK pathway (30–32). However, in T47D cells, IL-6 failed to significantly increase Shc tyrosine phosphorylation. Shc association with the EGF receptor, which can be seen as a coimmunoprecipitating tyrosine-phosphorylated band (Fig. 6A), or the Shc-Grb2 association (data not shown). However, inhibition of EGF receptor autocrine activity using the blocking antibody mAb 528 or the EGF receptor-specific inhibitor CGP59326 resulted in decreased Shc phosphorylation and association with the EGF receptor (Fig. 6A). Thus, Shc contributes to basal but not IL-6-induced MAPK activation in T47D cells.

The tyrosine phosphatase SHP-2 and the multisubstrate docking molecule Gab1 have also been involved in the transmission of gp130 signals to the MAPK and PI3K pathways (3, 33). Indeed, IL-6 strongly increased tyrosine phosphorylation of SHP-2 and Gab1, as observed after their immunoprecipitation (Fig. 6B). Interestingly, analysis of coimmunoprecipitated proteins revealed that IL-6 induced the formation of a complex of SHP-2-associated tyrosine-phosphorylated molecules (Fig. 6B, Lane 4) identified as Gab1 and the p85 subunit of PI3K. Similarly, on IL-6 stimulation, Gab1 associated with SHP-2 and p85 (visible only at longer exposure of Fig. 6B; data not shown). It is noteworthy that although no phosphotyrosine was detected on Gab1 or SHP-2, they were both associated with the tyrosine-phosphorylated EGF receptor, even in the absence of IL-6 (Fig. 6B, Lanes 1 and 7). Pretreating the cells with mAb 528 or with the EGF receptor-specific inhibitor CGP59326 resulted in decreased phosphorylation of SHP-2 and Gab1 (Fig. 6B), and decreased association with the other members of the immune complex, including the EGF receptors (data not shown). Thus, IL-6-induced activation of Gab1 and SHP-2 is largely dependent on EGF receptor autocrine activation.

SPH-2 complexes were further examined. SHP-2 association with gp130 and Gab1 was significantly increased in the presence of IL-6, whereas its interaction with EGF receptor was not dependent on IL-6 (Fig. 6C). Thus, in the absence of IL-6 stimulation, SHP-2 and Gab1 are present in a complex comprising the EGF receptor and gp130 (data not shown). On IL-6 treatment, there is increased tyrosine phosphorylation and recruitment to gp130 of SHP-2 and Gab1 (Fig. 6C).

IL-6/OSM-induced Inhibition of T47D Cell Proliferation Is STAT3 Dependent. The biological responses elicited by IL-6 treatment of T47D cells are 2-fold: (a) decreased proliferation; and (b) increased cell motility, as shown below. In the following experiments, we probed the contribution of the cytoplasmic pathways activated by IL-6, namely, the Jak/STAT, MAPK, and PI3K pathways, to IL-6-mediated biological responses.

To analyze the contribution of STAT3 activity, we generated T47D clones expressing a DN form of STAT3 (STAT3F) and selected clones expressing similar amounts of wild-type STAT3 (STAT3wt) as controls. STAT3F is mutated at tyrosine 705 and therefore cannot be phosphorylated on docking to gp130 (34). OSM- and IL-6-induced
STAT3 DNA binding activity to a specific probe was found to be strongly decreased in the T47D-STAT3F clones (data not shown). In contrast, OSM- and IL-6-induced MAPK activity was not significantly altered in T47D-STAT3F relative to T47D cells or T47D-STAT3wt cells (data not shown).

In a colony outgrowth assay, IL-6 and OSM inhibited the proliferation of T47D-STAT3wt cells as efficiently as that of the parental T47D cells (Fig. 7A). In comparison, T47D-STAT3F cells were less sensitive to the effect of IL-6 or OSM. Indeed, the IL-6 inhibitory effect was about 75% less for T47D-STAT3F cells relative to control cells, and OSM efficiency was reduced by more than 60% (Fig. 7A). Furthermore, cell cycle analysis indicated that, contrary to what was observed with T47D or T47D-STAT3wt cells, IL-6/OSM treatment did not increase the percentage of T47D-STAT3F cells in the G1 phase of the cell cycle (data not shown). Thus, the inhibition of T47D cell proliferation induced by IL-6 and OSM requires STAT3 activity. In contrast, inhibition of the EGF receptor or the MAPK and PI3K pathways with specific inhibitors resulted in an increased percentage of the cells in the G1 phase of the cell cycle, showing that the MAPK and the PI3K pathways positively affect proliferation (data not shown).

IL-6/OSM-induced Cell Migration Requires MAPK/PI3K Activation. IL-6 induced morphological changes and scattering of T47D cells (data not shown). To evaluate whether the scattering effect corresponded to increased cell motility and not merely to decreased cell-cell adhesion, we set up a migration assay in Boyden-like chambers, using IL-6 or OSM as a chemoattractant. IL-6 induced a moderate but significant increase in T47D cell migration, whereas OSM was even more efficient in stimulating cell motility (Fig. 7B). IL-6 and OSM effects on cell migration were completely reversed in the presence of the MAPK kinase inhibitors PD98059 (data not shown) and UO126 or in the presence of the PI3K inhibitor LY294002 (Fig. 7B). Thus, IL-6-type cytokines stimulate T47D cell migration through a MAPK- and PI3K-dependent mechanism. Because IL-6-induced MAPK and PI3K activation is dependent on EGF receptor activity, we evaluated the contribution of the EGF receptor to T47D cell migration. IL-6-induced migration was inhibited in the presence of the EGF receptor blocking antibody (mAb 528) or the specific inhibitor CGP59326 (Fig. 7C). Finally, IL-6 and OSM stimulated migration of T47D-STAT3F to a similar extent as control cells (Fig. 7D), indicating that cell migration is independent of STAT3 activity.

DISCUSSION

There is increasing evidence pointing to a role for IL-6 as a regulator of cancer cell proliferation. IL-6 treatment results in different biological responses, depending on the target cell type. Indeed, whereas IL-6 stimulates proliferation of myeloma/plasmacytoma, renal cell carcinoma, or Kaposi’s sarcoma cells, it inhibits the growth of cells derived from melanomas and lung or breast carcinomas. Understanding the mechanisms that can lead in some instances to proliferation and in other instances to growth inhibition will require knowledge of the intracellular events triggered by IL-6 in the different cell types. In this study, we have delineated IL-6-induced signaling pathways in the T47D breast carcinoma cell line and analyzed their respective contribution to IL-6-induced biological responses. We found that IL-6-type cytokines inhibit T47D cell proliferation but stimulate cell migration. The two biological effects are mediated by independent pathways involving STAT3 activity for the former and MAPK/PI3K activation for the latter. We show here that IL-6-induced MAPK/PI3K activation depends on the intactness of an EGF receptor autocrine loop. The tyrosine phosphatase SHP-2 and the multisubstrate docking molecule Gab1, which are constitutively associated with the EGF receptor in T47D cells and recruited to the gp130 transducing subunit and tyrosine phosphorylated on IL-6 stimulation, appear to play pivotal roles in the mechanism by which the combined
actions of IL-6 and EGF receptor autocrine activity promote a synergistic increase in MAPK and PI3K activation. Most breast tumors coexpress ErbB1 and one of its ligands, along with IL-6 signaling components. Our results suggest that in primary tumors, activation of both ErbB receptor tyrosine kinases and cytokine receptors might synergize to potently activate intracellular signaling pathways. Moreover, our results also imply that ErbB1, even when expressed at low levels, can play an important role in tumor cell biology and should therefore be considered as a potential therapeutic target.

We have shown here that STAT3 plays a crucial role in IL-6/OSM-induced growth inhibition of T47D cells. Studies using DN forms of STATs demonstrated that growth inhibition of M1 leukemic cells and the A375 melanoma cells in response to IL-6-type cytokines is also dependent on STAT3 activation (34–36). On the other hand, the mitogenic effect of IL-6 for some cell types was shown to involve SHP-2 and MAPK activation (34, 37). Thus, in some cells, the biological effect of IL-6 depends on the balance of a growth-inhibitory STAT3-dependent pathway and a growth-promoting MAPK/PI3K-dependent pathway (38). Whereas we could not observe increased proliferation in STAT3F-expressing T47D cells, blocking MAPK/PI3K in IL-6-stimulated T47D cells did lead to a higher percentage of cells in the G1 phase of the cell cycle. However, such a simplistic model for IL-6 action, involving two main opposing pathways to control cell proliferation, may not apply to all cell systems. Indeed, several reports demonstrate that STAT3 itself can promote cell growth (39) and even behave as an oncogene when constitutively active (40). These observations indicate that there may be several unknown cell-specific mechanisms that dictate the biological outcome to STAT3 activation.

In T47D cells, basal MAPK and PI3K signaling is due mainly to an EGF receptor autocrine loop, which is very likely kept active by one or more of the EGF-related peptides expressed in the cells. Whereas Shc is not activated on IL-6 treatment, low levels of Shc are associated with the constitutively active EGF receptor and are tyrosine phosphorylated and associated with Grb2 in the absence of exogenous stimuli. Furthermore, on treatment with the EGF receptor blocking antibody 528, we observed less Shc complexed to the EGF receptor and decreased Shc tyrosine phosphorylation and association to Grb2, which was concomitant with the decrease in MAPK activation. Thus, our results indicate that Shc mediates basal activation of MAPK.

In addition to Shc, SHP-2 and Gab1 are also present in the autocrine-activated EGF receptor complex. However, in the absence of IL-6, tyrosine phosphorylation of SHP-2 and Gab1 was not observed. This indicates that the relatively low levels of EGF receptor activity in T47D cells are sufficient for recruitment but not for phosphorylation of the two molecules. On IL-6 stimulation, SHP-2 and Gab1 show significantly increased levels of phosphorylation and increased association with gp130, but not with the EGF receptor. Moreover, SHP-2 and Gab1 have been reported to be direct substrates for the Jaks (33, 41), and expression of DN Jak1 blocked IL-6-induced MAPK activation. Thus, it is likely that SHP-2 and Gab1 are phosphorylated by Jak1. Whereas the EGF receptor might not be directly responsible for their phosphorylation, it is intriguing that inhibition of EGF receptor activity results in a diminished association of SHP-2 and Gab1 with the EGF receptor and in decreased IL-6-induced SHP-2 and Gab1 tyrosine phosphorylation. We have also observed that gp130 is part of the EGF receptor complex (data not shown). Taken together, we envision that the EGF receptor, by recruiting SHP-2 and Gab1 to a complex that includes gp130 and its associated Jak1, increases the amount of SHP-2 and Gab1 locally available for phosphorylation by Jak1. Tyrosine-phosphorylated Gab1 can then serve as a docking molecule for PI3K (through p85), Grb2, and even SHP-2. SHP-2 associated with gp130 or Gab1 can interact via its phosphotyrosine molecule for PI3K (through p85), Grb2, and even SHP-2. SHP-2 associated with gp130 or Gab1 can interact via its phosphotyrosine

The role EGF receptor plays in IL-6-induced signaling is quite different from its role in GPCR- or GH-mediated signaling to the Ras/MAPK pathway. Indeed, GPCR agonists stimulate MAPK via transactivation of the EGF receptor (reviewed in Refs. 28 and 29). A recent study indicates that this may occur, in some instances, through processing of membrane-anchored EGF receptor ligands (42). In contrast, GH, via Jak2, induces phosphorylation of the EGF receptor on a specific tyrosine residue, thereby creating a docking site for
Grb2, which leads to activation of the Ras/MAPK pathway (9). Our results show that in T47D cells, IL-6, which does not activate Jak2, does not increase EGFR tyrosine phosphorylation. Instead, IL-6 induction of Jak1 activates signaling molecules that are already associated with the autocrine-activated EGFR receptor. Therefore, IL-6 does require basal EGFR receptor activity in T47D cells to strongly activate the MAPK pathway.

Other ErbB family members were previously shown to mediate the effects of IL-6 on the MAPK pathway. In LNCaP prostate carcinoma cells, IL-6-induced MAPK activation was dependent on transactivation of ErbB-2/ErbB-3 (8). In T47D, MCF7, and SKBr3 breast carcinoma cells, IL-6 does not lead to activation of ErbB-2/ErbB-3, and the suppression of ErbB-2 activity does not affect IL-6-induced MAPK activation. However, in our hands, IL-6 also failed to stimulate ErbB-2 activity in prostate carcinoma cell lines, suggesting that further experimentation is required to clarify the role of ErbB-2 in IL-6 signaling.

IL-6 was previously shown to decrease cell-cell association and to stimulate scattering of breast carcinoma cells (10). We show here that IL-6 and OSM act as chemoattractants for T47D breast carcinoma cells, and IL-6 signaling.

further experimentation is required to clarify the role of ErbB-2 in IL-6 signaling.

5 Unpublished observations.

REFERENCES


Interleukin 6 Inhibits Proliferation and, in Cooperation with an Epidermal Growth Factor Receptor Autocrine Loop, Increases Migration of T47D Breast Cancer Cells

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