Therapeutics, Targets, and Chemical Biology

IL-6 Trans-Signaling in Formation and Progression of Malignant Ascites in Ovarian Cancer

Chi-Wen Lo1, Min-Wei Chen2, Michael Hsiiao3, Shiuian Wang1, Chi-An Chen4, Sheng-Mou Hsiao5, Jeng-Shou Chang3, Tsung-Ching Lai3, Stefan Rose-John6, Min-Liang Kuo2, and Lin-Hung Wei1,4

Abstract

Classic signaling by the proinflammatory cytokine interleukin 6 (IL-6) involves its binding to target cells that express the membrane-bound IL-6 receptor α. However, an alternate signaling pathway exists in which soluble IL-6 receptor (sIL-6Rα) can bind IL-6 and activate target cells that lack mIL-6Rα, such as endothelial cells. This alternate pathway, also termed trans-signaling, serves as the major IL-6 signaling pathway in various pathologic proinflammatory conditions including cancer. Here we report that sIL-6Rα is elevated in malignant ascites from ovarian cancer patients, where it is associated with poor prognosis. IL-6 trans-signaling on endothelial cells prevented chemotherapy-induced apoptosis, induced endothelial hyperpermeability, and increased transendothelial migration of ovarian cancer cells. Selective blockade of the MAPK pathway with ERK inhibitor PD98059 reduced IL-6/sIL-6Rα-mediated endothelial hyperpermeability. ERK activation by the IL-6/sIL-6Rα complex increased endothelial integrity via Src kinase activation and Y685 phosphorylation of VE-cadherin. Selective targeting of IL-6 trans-signaling in vivo reduced ascites formation and enhanced the taxane sensitivity of intraperitoneal human ovarian tumor xenografts in mice. Collectively, our results show that increased levels of sIL-6Rα found in ovarian cancer ascites drive IL-6 trans-signaling on endothelial cells, thereby contributing to cancer progression. Selective blockade of IL-6 trans-signaling may offer a promising therapeutic strategy to improve the management of patients with advanced ovarian cancer. Cancer Res; 71(2); 424–34. ©2010 AACR.

Introduction

Interleukin 6 (IL-6) is one of the major immunoregulatory cytokines present in the ovarian cancer microenvironment. Ovarian cancer cells and tumor-associated macrophages produce IL-6 (1, 2), and a high level of serum IL-6 is associated with poor overall and progression-free survival for patients with ovarian cancer (3, 4). IL-6 target cells express a low affinity receptor (IL-6Rα) devoid of transducing activity on their surface. The complex of IL-6 and IL-6Rα associates with the signal transducing membrane protein gp130, thereby inducing its dimerization and the initiation of signaling (5). In the context of unfavorable prognoses, IL-6 signaling in ovarian cancer cells can function as a regulator of tumor cell proliferation and invasion (6, 7).

A soluble form of the cognate IL-6 receptor-α (sIL-6Rα) can bind IL-6 with the same affinity as the membrane-bound form (mIL-6Rα). Cells that are deficient in or that lack IL-6Rα can respond to IL-6 when it is associated with sIL-6Rα in a process called trans-signaling (8, 9). This is relevant for endothelial function in vivo because endothelial cells, which play key roles in inflammation, lack IL-6Rα expression. The endothelial cells respond to IL-6/sIL-6Rα signaling with the selective induction of chemokine production and intracellular adhesion molecule 1 expression, through which it regulates leukocyte recruitment and promotes transition from acute to chronic inflammation (10–12). Increasing evidence has suggested that IL-6 trans-signaling serves as the major proinflammatory paradigm of IL-6 signaling under various pathophysiologic conditions, such as chronic inflammatory diseases, as well as cancers (13–15).

Malignant ascites is a distressing complication of ovarian cancer, which may arise from both the tumor surface and the non–cancer-bearing peritoneal surface by increasing capillary permeability and angiogenesis (16). It has become clear that factors produced by tumor cells and that induce angiogenesis and disrupt the vascular barrier not only contribute to ascites formation but also potentiate tumor cell intravasation, leading to widespread metastatic disease (17). Vascular endothelial growth factor (VEGF), for example, has been identified as one of the most potent and specific angiogenic factors involved in this process (17, 18). A thorough understanding of the cellular and molecular...
mechanisms that regulate endothelial integrity in ovarian cancer is still lacking and would be essential for developing novel effective therapeutic approaches.

Tumor angiogenesis plays an essential role in cancer progression and metastasis, and IL-6 has been shown to be involved in tumor angiogenesis of ovarian cancer (19). An increased understanding of the regulatory properties of sIL-6Rα has prompted us to investigate the functional role of IL-6 trans-signaling on endothelial cells and its significance in ovarian cancer progression. In this article, we show that sIL-6Rα levels are significantly elevated in malignant ascites from ovarian cancer patients and are associated with poor clinical outcome. Selectively blocking IL-6 trans-signaling with sgp130Fc reduced ascites formation and enhanced tumor sensitivity to Taxol (paclitaxel; Bristol-Myers Squibb) in a mouse model of intraperitoneal ovarian cancer. We further clarified the molecular mechanisms underlying these observations.

Materials and Methods

Reagents and cell lines

Human umbilical vein endothelial cells (HUVEC), endothelial cell growth medium, trypsin-EDTA, and trypsin-neutralizing solutions were purchased from Clonetics. Human ovarian cancer cell lines, SKOV-3, Caov-3, and OVCAR-3 were obtained from American Type Culture Collection. All cell cultures were cultured according to the suppliers’ recommendations. The production of designer cytokine Hyper IL-6 and sgp130Fc has been described previously (20, 21).

Preparation of ascites samples, collection of conditioned medium, and detection of sIL-6R concentration

Ascitic fluid was collected from patients with epithelial ovarian cancer. The National Taiwan University Hospital Institutional Review Board approved the project, and written informed consent was obtained from all patients and controls before their inclusion. Conditioned medium was collected 24 hours after 10^6 cells mL^{-1} were seeded. sIL-6Rα was detected by the ELISA kit (R&D Systems).

Immunoblotting

Immunoblotting was performed with primary antibodies for anti–phospho-STAT3, anti-PARP, anti–phospho-Src (Cell Signaling Technology), anti-STAT3, anti-ERK, anti–phospho-ERK, anti–VE-cad, anti-Src, anti-β-actin (Santa Cruz Biotechnology), anti–α-tubulin (Sigma), and anti–phospho-VE-cad (Tyr 685; ECM Biosciences). Cytoplasmic VE-cadherin protein was prepared by the CNMCS compartmental protein extraction kit (BioChain).

Cytotoxicity assay

Cell survival was determined using the MTT assay (Sigma).

Assay of chemotactic endothelial cell migration

HUVECs (10^5) were seeded onto 8.00-µm pore Transwell inserts in a 24-well plate (Costar). HUVEC migration was examined 16 hours after Hyper IL-6 and/or Taxol were stained with 0.5% crystal violet.

In vitro permeability assay

HUVECs (2 × 10^5) were seeded onto collagen-coated Transwells (0.4-µm pore; Costar). After the endothelial monolayer formed, Hyper IL-6 or VEGF-A (R&D Systems) was treated. Horseradish peroxidase (HRP) was added to the upper chamber (0.1 mg/mL), and the presence of HRP in the lower chamber after 15 minutes was determined by a colorimetric assay with 2,2’-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] (Sigma). The absorbance was read using a spectrophotometer at 405 nm.

Vascular permeability in vivo

Vascular permeability in vivo assay (Miles assay) has been described (22). In brief, the mice received i.v. injections of 200 µL of Evans blue dye (0.5%) via the tail vein. Ten minutes later, the mice received intradermal injections of indicated agents. The subdermis was harvested and photographed 30 minutes later to document any leakage of the dye into the dermal tissue. For quantification, wheals (5 mm in diameter) were resected and incubated in 500 µL of formamide at 37°C for 48 hours to extract the Evans blue dye. The absorbance of the extracts was read at 630 nm in a spectrophotometer.

Transendothelial migration assay

HUVEC monolayers were formed on the collagen-coated 8-µm Transwells (Costar). A complete M199 with the indicated agents was then treated for 24 hours. SKOV-3 cells stained with 5-µmol/L CellTracker (Invitrogen) were seeded on an endothelial monolayer. After 16 hours, cells were fixed with 4% ice-cold paraformaldehyde and transmigrated cells in the lower chamber were counted.

Intravasation assay in the chick embryo metastasis model

Intravasation was evaluated in the lower chorioallantoic membrane (CAM; ref. 23). In short, fertilized chick eggs were incubated in a humidified atmosphere at 37°C for 9 days. On day 10, tumor cells were seeded onto the upper CAM at 1 × 10^6 cells through a 1-cm² window cut into the eggshell. PBS or Hyper IL-6 was applied to the upper CAM following tumor cell seeding. Tissue samples in the lower CAM were collected, and genomic DNA was extracted using a DNA extraction kit (Qiagen). Specific primers for human Alu and chick GAPDH sequences were tabulated in Supplementary Table S1.

Immunofluorescence

HUVECs grown on coverslips were fixed immediately using 4% paraformaldehyde for 5 minutes, and processing, blocking, and washing were performed. Staining using an anti–VE-cadherin antibody (Santa Cruz) was performed. Confocal images were obtained with a laser confocal scanning microscopy (Zeiss Inverted LSM410; Zeiss Optical Company).

Animal studies

All animal work was done in accordance with a protocol approved by the National Taiwan University College of
Medicine and National Taiwan University College of Public Health Institutional Animal Care and Use Committee. Age-matched NOD/SCID female mice (6–8 weeks old) were used. Luciferase-expressing cells (SKOV3-LG) were established by infecting with lentivirus expressing pWPI-Luc-ires-GFP vector. Mice were intraperitoneally (i.p.) injected with $3 \times 10^6$ SKOV3-LG cells and separated into 4 treatment groups. Tumor progression was monitored and quantified using non-invasive bioluminescence (IVIS Spectrum; Caliper LifeSciences). Ascites fluid and tissue samples were collected at 42 days after injection for pathologic analysis.

**Statistical analysis**

Progression-free survival was estimated using the Kaplan-Meier method. Univariate analysis of survival was done by the log-rank test. Cox proportional hazards model was used to find the prognostic factors affecting progression-free survival. Covariates included sIL-6Rα levels, age, stage (1–2 vs. 3–4), cell type (serous vs. nonserous), and the presence of secondary debulking surgery. Comparisons of the results of the sIL-6Rα levels in clinical samples and ascites volume in animal studies were done using the Wilcoxon signed-rank test. For all other experiments, ANOVA and Duncan’s multiple range tests were used to assess the statistical significance of the differences between groups. Differences were considered significant at $P < 0.05$.

**Results**

**Soluble IL-6Rα levels are increased in malignant ascites of ovarian cancer and are associated with poor prognosis in advanced ovarian cancer**

Although the elevated levels of IL-6 in the sera and ascites of ovarian cancer patients have been previously described (24), the clinical significance of sIL-6Rα levels in malignant ascites has not been evaluated. Our first objective was to analyze sIL-6Rα expression in malignant ovarian ascites. The clinical characteristics of these 24 ovarian cancer patients are listed in Supplementary Table S2. A remarkable increase in sIL-6Rα was observed in malignant ovarian ascites as compared with those in peritoneal fluid from benign ovarian tumors [Fig. 1A; median (range), 2,645 (940–4,760) vs. 1,950 (800–2,380); $P = 0.0046$]. To evaluate the in vitro expression of sIL-6Rα in ovarian cancer cells, ELISA analysis confirmed that a substantial amount of sIL-6Rα was produced by most ovarian cancer cell lines growing in culture (Fig. 1B). Receiver operating characteristic (ROC) curve analysis of sIL-6Rα levels identified that a sIL-6Rα cutoff point of 2,455 pg/mL discriminated malignant ovarian tumors from benign ovarian tumors well (Fig. 1C). The area under the curve was 0.78 (0.53–0.92). The sensitivity and specificity at a sIL-6Rα concentration of 2,455 pg/mL were 0.60 (0.39–0.79) and 1.00 (0.75–1.00), respectively. Using Cox proportional hazards model, sIL-6Rα level (HR $= 1.001$, 95% CI $= 1.0003–1.002$, $P = 0.01$) and advanced stage (1–2 vs. 3–4; HR $= 24.3$, 95% CI $= 1.3–465.9$, $P = 0.03$) were independent factors affecting progression-free survival. These results indicated that sIL-6Rα in the malignant ascites of ovarian cancer may contribute to ovarian cancer progression.

**IL-6/sIL-6Rα activates endothelial cells and antagonizes Taxol-induced antiangiogenesis**

The direct role of IL-6 in tumor angiogenesis is controversial, owing to the argument for IL-6Rα expression on endothelial cells from different anatomic regions. We next addressed
the question of whether sIL-6Rα, when added together with IL-6 to endothelial cells, would elicit a response through trans-signaling by engaging gp130. To test this, we treated HUVEC cells with the designer cytokine Hyper IL-6 (the IL-6Rα/IL-6 complex) and examined its effects against Taxol-induced antiangiogenesis (25). Western blot analysis evaluated the levels of STAT3 and ERK phosphorylation, confirming the cytokine response of endothelial cells (Fig. 2A). Similar to its effects on tumor cell lines (data not shown), Hyper IL-6 signaling on endothelial cells increased cell viability under cytotoxic concentrations of Taxol treatment (Fig. 2B) and prevented cell death by apoptosis (Fig. 2C). Moreover, Taxol dose-dependently inhibited endothelial cell migration, and Hyper IL-6 remarkably enhanced endothelial migration and antagonized Taxol effects on endothelial cell migration (Fig. 2D). The IL-6 responsiveness of endothelial cells through trans-signaling could, therefore, be involved in the tumor progression of patients with advanced ovarian cancer.

**IL-6/sIL-6α signaling induces vascular leakage and potentiates tumor cell intravasation**

To examine whether IL-6/sIL-6Rα signaling directly induced endothelial barrier dysfunction, we performed an in vitro permeability assay. Confluent monolayers of HUVECs plated in transwell plates had minimal HRP flux across the monolayer under nonstimulated conditions, whereas administration of Hyper IL-6 greatly increased HRP flux into the lower chamber in a dose- and time-dependent manner (Fig. 3A). Additionally, local administration of Hyper IL-6 dose-dependently increased Evans blue dye extravasation in C57BL mice (Fig. 3B, left panel). The amount of dye extravasation caused by local administration of Hyper IL-6 or VEGF substantially increased compared with the nontreated control (Fig. 3B, right panel). These results suggest that IL-6/sIL-6α signaling directly mediates vascular hyperpermeability. Furthermore, breakdown of the vascular barrier may potentiate tumor cell intravasation and enhance metastasis. As shown in Figure 3C, SKOV-3 invasion drastically increased when the endothelial monolayer was activated by Hyper IL-6. The influence on the invasive ability of the tumor cell by Hyper IL-6 was negligible (Fig. 3C), suggesting that disruption of endothelial barriers by Hyper IL-6 played the major role in this process. The consequence of the increased transendothelial migration ability of tumor cells should make them capable of escaping from the primary tumor and entering the vasculature. The arrival of intravasated human tumor cells in the lower CAM of tumor-bearing chick embryos was readily observed after 24 hours in the Hyper IL-6-treated embryos, and they continued to arrive everyday thereafter (Fig. 3D), as assessed using the human alu PCR-based assay. In contrast, there was no detectable intravasation until day 3 in PBS-treated embryos.

**IL-6/sIL-6α modulates cell–cell adherens junctions of endothelial cells through tyrosine phosphorylation of VE-cadherin**

The integrity of endothelial cell–cell junctions and vascular barrier functions is regulated by tight junctions and adherens

![Figure 2](image-url)
junctions, which are functionally and structurally linked (26). VE-cadherin expression and organization at adherens junctions are crucial determinants for vascular stabilization (27). To determine if IL-6/sIL-6α modulates VE-cadherin expression at cell–cell junctions, hence interfering with gap formation, we examined the VE-cadherin staining of HUVEC monolayers with both confocal immunofluorescence microscopy (Fig. 4A) and Western blots (Fig. 4B). Compared with vehicle control, exposure of HUVECs to Hyper IL-6 caused redistribution of VE-cadherin from intercellular junctions, resulting in monolayer disruption (Fig. 4A). The decrease of surface VE-cadherin appeared to result from an increasing endocytosis of cell surface VE-cadherin (Fig. 4A, bottom panel). The effect of Hyper IL-6 on the cytoplasmic level of VE-cadherin expression was quantified by Western blots. Remarkably, Hyper IL-6 caused, at best, a 2.3-fold increase in cytosolic VE-cadherin expression compared to vehicle control (Fig. 4B). Because tyrosine phosphorylation of VE-cadherin is intimately linked to VE-cadherin redistribution and, thus, is a functional change of intercellular adhesion, we examined the effect of Hyper IL-6 on VE-cadherin tyrosine phosphorylation. Hyper IL-6 induced substantial phosphorylation of tyrosine 685 in VE-cadherin, which was selectively prevented by PD98059 (Fig. 4C).
monolayers to PD98059 before Hyper IL-6 exposure effectively prevented Hyper IL-6–induced VE-cadherin tyrosine phosphorylation (Fig. 4C). In contrast, AG490 and LY-294002 had no effect, suggesting that Hyper IL-6 reduced VE-cadherin proteins at cell junctions via a MAPK-dependent pathway. In agreement with these findings, PD98059 prevented Hyper IL-6–augmented tumor transendothelial migration, whereas AG490 and LY-294002 had no such effect (Fig. 4D).

**Activation of MAPK by IL-6/sIL-6R phosphorylates VE-cadherin via an Src-dependent pathway**

An earlier study showed that VE-cadherin is a direct substrate for Src kinase and that Y685 is a unique phosphorylation site in the VE-cadherin cytoplasmic domain (28). To examine whether tyrosine phosphorylation of VE-cadherin by Hyper IL-6 signaling is dependent on active Src in HUVECs, the activation of Src by Hyper IL-6 was analyzed by Western blotting. As shown in Fig. 5A, exposure of Hyper IL-6 substantially induced tyrosine phosphorylation of Src and VE-cadherin in HUVECs. The inhibition of Src kinase activity by PP2 or dominant-negative (DN) forms of c-Src (K295R) substantially reduced Hyper IL-6–induced tyrosine phosphorylation of VE-cadherin. These results suggest that Src kinase is critically involved in the Hyper IL-6–mediated dissociation of the VE-cadherin/β-catenin complex that contributes to increased transendothelial permeability and migration of cancer cells. Considering that Hyper IL-6–induced tyrosine phosphorylation of VE-cadherin occurs via a MAPK-dependent pathway, we ascertained whether it was the MAPK that controlled the Src activity in response to Hyper IL-6. As shown in Fig. 5B, we found that PD98059 as well as the Src kinase inhibitor PP2 inhibited the activation of Src kinases in response to
Hyper IL-6, whereas PP2 did not change the Hyper IL-6–mediated ERK phosphorylation. Similarly, transfection with lentivirus containing dominant-negative mutants of ERK (LV-DN-ERK) abrogated the Hyper-IL-6–induced Src phosphorylation, whereas transfection with LV-DN-Src did not change the Hyper IL-6–mediated ERK phosphorylation (Fig. 5C). These findings suggest that Hyper IL-6–mediated activation of ERK is upstream of Src-dependent regulation of the VE-cadherin/β-catenin complex integrity. Furthermore, we validated the effect of IL-6/ERK/Src pathway in vivo using a Miles assay. Mice were pretreated with PD98059 (5 mg/kg), PP2 (1 mg/kg), or vehicle control 1 hour prior to Evans blue dye injection. The vascular permeability was quantified using Miles assay. Mice were treated weekly with PBS or sgp130Fc (100 μg/mouse), alone or in combination with Taxol (10 mg/kg). In all animals, i.p. injections were initiated 14 days after SKOV-3-LG cell inoculation and continued for 4 weeks. Intraperitoneal tumor growth was quantified weekly by an in vivo bioluminescence imaging system. All PBS-treated animals displayed increasing intraperitoneal tumor burden and signs of ascites formation following SKOV-3 inoculation (Fig. 6A and C). Significant tumor suppression by Taxol was observed 3 weeks after initiation of treatment. The mean tumor burden in the Taxol-treated group (2.2 × 10^9 photons/s) was reduced by approximately 67% compared with the control group (6.7 × 10^9 photons/s) at 42 days after injection, whereas sgp130Fc alone only displayed modest inhibition of tumor growth (Fig. 6A). Notably, the combination of sgp130Fc with Taxol markedly reduced tumor burden (2.6 × 10^8 photons/s) compared with Taxol alone (P < 0.05). In the postmortem examination, tumors were found on the surface of the peritoneum, the uterus, the mesentery, and the diaphragm in the control group, whereas visible tumors over the diaphragm in vivo

Effects of sgp130Fc in an intraperitoneal ovarian cancer model: inhibition of ascites formation and potentiating of chemotherapeutic effects

Based on clinical and in vitro results, we hypothesized that the combination of IL-6 and sIL-6Rα is present in the tumor microenvironment and facilitates IL-6 trans-signaling, which in turn has consequences for ovarian cancer progression. To test this hypothesis, we studied the effects of sgp130Fc treatment, which selectively inhibits IL-6 trans-signaling (29), on the progression of intraperitoneal SKOV-3 tumors. Mice were treated weekly with PBS or sgp130Fc (100 μg/mouse), alone or in combination with Taxol (10 mg/kg). In all animals, i.p. injections were initiated 14 days after SKOV-3-LG cell inoculation and continued for 4 weeks. Intraperitoneal tumor growth was quantified weekly by an in vivo bioluminescence imaging system. All PBS-treated animals displayed increasing intraperitoneal tumor burden and signs of ascites formation following SKOV-3 inoculation (Fig. 6A and C). Significant tumor suppression by Taxol was observed 3 weeks after initiation of treatment. The mean tumor burden in the Taxol-treated group (2.2 × 10^9 photons/s) was reduced by approximately 67% compared with the control group (6.7 × 10^9 photons/s) at 42 days after injection, whereas sgp130Fc alone only displayed modest inhibition of tumor growth (Fig. 6A). Notably, the combination of sgp130Fc with Taxol markedly reduced tumor burden (2.6 × 10^8 photons/s) compared with Taxol alone (P < 0.05). In the postmortem examination, tumors were found on the surface of the peritoneum, the uterus, the mesentery, and the diaphragm in the control group, whereas visible tumors over the diaphragm in vivo.
mesentry were substantially reduced in the treatment groups (Fig. S2). We verified the in vivo sgp130Fc effect by analyzing p-STAT3 expression in tumors using Western blots (B) and ascites quantification (C). D, pathologic angiogenesis and vascular maturation were shown by immunohistochemical staining for von Willebrand factor (vWF) and smooth muscle actin (α-SMA) using serial sections from tumor tissues.

Discussion

Chronic inflammation is considered to be one of the most important environmental factors that participate in the neoplastic process, fostering tumor progression (30). In this article, we showed that sIL-6Rα is present in high amounts in the ascites of patients with ovarian cancer. Patients with ovarian cancer who have elevated levels of sIL-6Rα at diagnosis also have features of high-risk disease and unfavorable clinical outcome. Finding whether the sIL-6Rα status is independent of the current overall risk stratification system of ovarian cancer requires further investigation. Nonetheless, the presence of sIL-6Rα renders all cells responsive to IL-6 in the ovarian cancer tumor environment. Here, we found that endothelial cells of the gp130+/IL-6Rα−low phenotype respond to the designer cytokine Hyper IL-6. The IL-6/sIL-6Rα complex

Figure 6. Effect of sgp130Fc on intraperitoneal SKOV-3 tumor development and ascites production in mice inoculated with SKOV3-LG cells. A, top, representative images of mice bearing SKOV-3-LG cells 42 days after i.p. injection. Bottom, primary tumor growth upon i.p. injection of SKOV-3-LG cells. Photon flux was quantified in a fixed region of interest at the abdominal region. n = 8 per group per time-point. The data are presented as the mean ± SD. Four weeks after initial treatment, the experiment was ended. The in vivo sgp130Fc effect was verified by the p-STAT3 expression in tumors using Western blots (B) and ascites quantification (C). D, pathologic angiogenesis and vascular maturation were shown by immunohistochemical staining for von Willebrand factor (vWF) and smooth muscle actin (α-SMA) using serial sections from tumor tissues.
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