Interleukin-6/Soluble Interleukin-6 Receptor Signaling Attenuates Proliferation and Invasion, and Induces Morphological Changes of a Newly Established Pleomorphic Malignant Fibrous Histiocytoma Cell Line

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Pleomorphic malignant fibrous histiocytoma (MFH) is occasionally associated with inflammatory paraneoplastic syndrome (PNS). Recently, we reported that interleukin (IL)-6, one of the candidate cytokines, which induces such systemic inflammatory reaction, may be a tumor-associated factor involved in the pathogenesis and its clinical manifestations of MFH. In the local microenvironment, tumor-induced inflammatory reaction may play a role favoring tumor progression. To clarify the biological relevance of IL-6 in MFH, we established a human MFH cell line, named MIPS-2, derived from a resected specimen of a patient presenting with PNS. In this patient, the serum IL-6 level ran parallel to the disease course: elevated serum IL-6 concentration normalized immediately after radical surgery, and re-elevation occurred on tumor recurrence. MIPS-2 presented pleomorphic appearance, severe nuclear abnormalities with prominent nucleoli, and tumorigenesis in nude mice. MIPS-2 expressed IL-6, IL-6 receptor (IL-6R), and glycoprotein 130 (gp130) but lacked the soluble form of IL-6R (sIL-6R), as determined by flow cytometry and reverse transcriptase-polymerase chain reaction analyses. Stimulation of MIPS-2 with IL-6 combined with exogenous sIL-6R induced phosphorylation of both signal transducer and activator of transcription 3 (STAT3) and mitogen-activated protein kinase (MAPK), decreased cell proliferation, attenuated invasion, and induced morphological changes. Collectively, these data suggested that the IL-6/sIL-6R signaling pathway plays a pivotal role for proliferation,

invasion, and morphology of MFH via STAT3 and MAPK pathway as autocrine and/or paracrine manner, and proposed the therapeutic potential for the use of both anti-growth factor and proinflammatory cytokine-targeting strategies to combat devastating MFH. (Am J Pathol 2004, 165:471–480)

Malignant fibrous histiocytoma (MFH) is the most common soft tissue sarcoma of late adult life.¹ Despite extensive cytogenetic analyses, to date, no consistent chromosome abnormalities have been described in MFH. Furthermore, MFH does not show any cluster genes commonly highly expressed,^{2–5} suggesting that MFH may contain multiple pathogenetic tumors and provide no good candidate for the molecular target therapy from the genome-based analysis. Therefore, it is necessary to clarify the pathogenesis of MFH providing a better molecular target for the treatment of this devastating tumor. Histopathological appearance of the tumor cells shows the marked heterogeneity, so called pleomorphic appearance, accompanied with numerous infiltrating immunoinflammatory cells.¹ These features indicate that inflammation and/or immune response may be closely associated with the pathogenesis of MFH. Recently, we reported that the patients with pleomorphic MFH occasionally present inflammatory PNSs such as fever elevation, leukocytosis, and elevated serum C-reactive protein (CRP).⁶ Elevated serum CRP level, which appears to directly reflect interleukin (IL)-6 level, proved to be the most representative clinical manifestation. Therefore we speculate that IL-6 may be a critical tumor-associated factor in MFH pathogenesis and progression. IL-6 is a pleiotropic cytokine that plays a key regulator of host

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Table 1. Physiologic Parameters During Clinical Course

Molecules (normal range)	A	B	C	D
IL-6 (<8 pg/ml)	9.6	0.7	12.6	5.5
sIL-6R (45 to 145 ng/ml)	162	192	148	162
CRP (<0.3 mg/dl)	4.8	<0.3	4.9	<0.3
WBC counts (4000 to 8000/mm ³)	10240	4840	10080	4800

A, preoperation; B, postoperation; C, pulmonary metastasis; D, post-metastasectomy; IL-6, interleukin-6; sIL-6R, soluble IL-6 receptor; CRP, C-reactive protein; WBC, white blood cell.

immune and inflammatory responses.⁷ On target cells, IL-6 binds and acts via a receptor complex composed of a ligand binding 80-kd glycoprotein (IL-6R) and a 130-kd signal transducing receptor subunit (gp130).⁸ IL-6 can also bind to a sIL-6R to induce IL-6 signal transduction pathways. sIL-6R is generated by alternative splicing or shedding and acts as a potent agonist of IL-6 on several cells.⁹ Dimerization of gp130 leads to the phosphorylation of Janus kinases JAK1, JAK2, and TYK2, resulting in tyrosine phosphorylation of several downstream signaling molecules, including the STAT family of transcription factors. Phosphorylated STATs translocate to the nucleus and initiate several gene transcriptions.¹⁰ In addition, gp130 also engaged to the Src-homology tyrosine phosphatase (SHP2) and subsequently activation of MAPK pathway.¹¹ A variety of malignant tumors have been shown to contain or synthesize IL-6, and an autocrine growth stimulation has been suggested.¹² In addition, elevated IL-6 serum levels have been frequently detected in patients with renal and ovarian cancer and myeloma.¹³ It has also been shown that IL-6 and sIL-6R complex resulted in a marked enhancement of biological activities in several cells.^{14–18} Nevertheless, the potential roles of IL-6 on MFH cells have been poorly understood. To characterize the biological relevance of MFH and IL-6, we established a novel human MFH cell line, named MIPS-2, derived from a resected specimen of a patient presenting with inflammatory PNS. We measured the secretion of IL-6 and sIL-6R of conditioned medium and the cell surface expression of IL-6, IL-6R, sIL-6R, and gp130 in MIPS-2 cells. We investigated that combination of IL-6 and sIL-6R induces activation of STAT and MAPK pathways in MIPS-2 cells, and also uses the specific monoclonal antibody against human IL-6R and sIL-6R¹⁹ to examine the cellular signaling of this cytokine for MIPS-2 cells.

Materials and Methods

Reagents

Recombinant human IL-6, human soluble IL-6R, and human tumor necrosis factor- α were obtained from R&D Systems (Minneapolis, MN). Polyclonal antibodies against STAT3, p42/p44 MAPK, phospho-STAT3, and phospho-p44/p42 MAPK were from New England Biolabs Inc. (Beverly, MA). Monoclonal antibody against β -actin was from Chemicon Inc. (Temecula, CA). Anti-poly-ADP-ribose polymerase (PARP) polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-cytochrome c antibody was obtained

from BD Pharmingen (San Diego, CA). Anti-vinculin monoclonal antibody was obtained from Sigma (Tokyo, Japan). Rhodamine phalloidin, 4',6-diamidino-2-phenylindole (DAPI), and Alexa 488 anti-mouse IgG were from Molecular Probes (Eugene, OR). The WST-8 assay kit was obtained from Dojindo Laboratories (Kumamoto, Japan). Monoclonal antibody for IL-6R and sIL-6R (MRA) was a generous gift from Dr. Nishimoto and Chugai Pharmaceutical Co. (Tokyo, Japan).

Patient

A 44-year-old man presented with an enlarging right thigh mass on November 1999. He showed fever elevation and weight loss. Laboratory findings (summarized in Table 1) showed several inflammatory reactions including leukocytosis (10,240/mm³) and elevated CRP (4.8 mg/dl). A large mass (12 × 12 × 5 cm) was surgically resected on January 2000 followed by adjuvant chemotherapy consisting of doxorubicin and ifosfamide. CRP levels subsided to the normal range promptly. Other inflammatory symptoms and signs disappeared immediately after the surgical resection. Histologically, the tumor cells were positively immunoreactive for CD68, anti-chymotrypsin, whereas S-100, desmin, and smooth muscle actin antigens were negative. The histological appearances were compatible with a high-grade pleomorphic storiform MFH (Figure 1A). After wide local excision, the serum level of CRP gradually increased. Four months later, pulmonary metastasis appeared in right upper lobe. After metastasectomy was performed on December 2000, the CRP level subsided into normal range (<0.3 mg/dl). Further 2 months later, CRP re-elevated with subcutaneous lymph node metastasis. The normalization of CRP occurred followed by the tumor resection. He died of multiple metastases involving lung, retroperitoneum, and bone at 17 months after first presentation.

Measurement of Serum IL-6 and sIL-6R of Patient's Sera

Venous blood samples were collected at the time of clinical assessment in pyrogen-free tubes, allowed to clot at 4°C for 1 hour, and centrifuged at 2000 × *g* for 10 minutes. The serum obtained was divided into aliquots and stored at –20°C until assayed for IL-6 and sIL-6R. The serum concentrations were assayed by specific, commercially available, enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D Systems Inc.) ac-

Figure 1. The establishment and characterization of MIPS-2 cell line from the patient suffered MFH with PNS. **A:** Original tumor showing pleomorphic-storiform pattern characteristic of MFH. The tumor is composed of pleomorphic spindle, fibrous, oval, and multinucleated giant cells. Numerous inflammatory cell infiltration was seen in the background of tumor cells. **B:** Morphological features of cultured MIPS-2 cell line. Mixed population with spindle cells, polygonal cells, and bizarre giant cells. **C:** Transplantation of MIPS-2 cells in nude mice. **D:** Immunohistochemistry of IL-6 for cultured MIPS-2 human MFH cell line. IL-6 is strongly and diffusely positive in the cultured MFH cells. Scale bars: 50 μ m (**A**); 25 μ m (**B**, **D**).

cording to the manufacturer's recommendations at Genome Science, Ltd. In a series of 115 healthy patients, serum IL-6 and sIL-6R concentrations were also measured as a control (56 men and 59 women). Reference values for IL-6 and sIL-6R serum levels were established from a study of the healthy controls and cutoff values were as follows: IL-6, <8 pg/ml; and sIL-6R, 45 to 145 ng/ml.

Cell Lines

MFH cells were isolated from the surgically resected tissues of MFH at the metastasectomy with the patient's informed consent and under the guidelines of our institution's ethical committee. The tumor tissues were minced and incubated with 2 mg/ml of collagenase (Sigma) for 1 hour at 37°C. Cell suspensions were passed through a mesh and tumor cells were isolated with the aid of Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). The cells were then cultured in RPMI 1640 (Invitrogen, Japan) with 10% fetal calf serum (Equitech-Bio, Kerrville, TX), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen) in a culture flask, and the nonadherent cells were removed. MIPS-2 cells were maintained for 15 months in culture, and the cells passed more than 50 times, fulfilling the criteria of a cell line. Throughout this cell-line establishment, the cells exhibited typical spindle cell morphology at the initial passage and pleomorphic morphology at the confluent status (Figure 1B). Human osteoblastic osteosarcoma cell line, SaOS-2 was cultured in McCoy's 5A medium (Invitrogen) supplemented with 15% fetal calf serum.

Cytogenetic Analysis

The cells, which had undergone two to three passages, were cultured in 50-cm² flasks and transferred to the cytogenetic laboratory (Shionogi Biomedical Laboratories, Osaka, Japan). Chromosome preparations were made from primary culture of the cell line using trypsin-Giemsa banding techniques.²⁰ The modal chromosome number and karyotype for the cell lines were determined by counting the chromosomes of 20 metaphases detected.

Immunohistochemistry

MIPS-2 cells were fixed with 3.7% paraformaldehyde and then permeabilized with 0.2% Triton X-100. Primary antibodies were anti-IL-6 monoclonal antibodies (1:100) at 4°C overnight, and signals were detected with streptavidin kit (Vector Laboratories, Burlingame, CA.) The tissues were lightly counterstained with methylene blue.

Nude Mouse Transplantation

Five-week-old athymic nude mice (BALB/c nu/nu; SLC, Shizuoka, Japan) were housed at the animal maintenance unit of Osaka Medical Center for Cancer and

Cardiovascular Diseases, in accordance with the guidelines approved by the local animal ethics committee. Five were injected subcutaneously into the both sides of back with $\sim 1 \times 10^6$ cells.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared by using TRIzol (Invitrogen) according to the manufacturer's instructions. Five μ g of total RNA was reverse transcribed with 200 U of Super-Script III reverse transcriptase (Invitrogen) and 0.5 nmol of oligo (dT)₁₂₋₁₈ primer, then PCR was performed in 50 μ l of reaction solution containing cDNA derived from 100 ng of total RNA, 1.25 U of TaqDNA polymerase (Sigma), 10 nmol of each dNTP (Sigma), and 0.5 nmol of sense and anti-sense primer pairs shown below with a DNA thermal cycler. PCR cycles were operated on a regimen of 30 seconds of denaturation of 94°C; 30 seconds of primer annealing at the temperature as below; and 60 seconds extension/synthesis at 72°C for 30 cycles for IL-6, IL-6R, sIL-6R and 25 cycles for gp130. Integrity of the obtained cDNA was tested by amplification of GAPDH transcripts in a 25-cycle PCR reaction. The primers, annealing temperatures, and lengths of PCR products are as follows: IL-6-sense: 5'-TAGCCGCCCCACACAGACAG-3'; IL-6-anti-sense: 5'-GGCTGGCATTGTGGTTGGG-3', annealing temperature 70°C, size 408 bp, IL-6R-sense: 5'-CATTGC-CATTGTTCTGAGGTTC-3' (bases 1580 to 1601: because this primer annealed to the region splicing out from IL-6R, thus sIL-6R transcript could not be amplified); IL-6R-anti-sense: 5'-GTGCCACCCAGCCAGCTATC-3' (bases 1840 to 1859), annealing temperature 65°C, size 280 bp, sIL-6R-sense: 5'-GCGACAAGCCTCCAGGTTC-3' (bases 1488 to 1503 continued to 1598 to 1601: with this primer mRNA of IL-6R cannot be amplified); sIL-6-anti-sense: 5'-GTGCCAC-CCAGCCAGCTATC-3' (bases 1840 to 1859), annealing temperature 68°C, size 278 bp, gp130-sense: 5'-GCAA-GATGTTGACGTTGCAGAGACTTG-3'; gp130-anti-sense: 5'-GGGCATTCTCTGCTTCTACCCAGAC-3', annealing temperature 70°C, size 615 bp, GAPDH-sense: 5'-GGTGAAGGTCGGAGTCAACGG-3'; GAPDH-anti-sense: 5'-GGTCATGAGTCCTCCACGAT-3', annealing temperature 68°C, size 520 bp. The sequences of cDNA were referred to GenBank (accession no. NM.000600 for IL-6, X12830 and M20566 for IL-6R and sIL-6R, and NM.002184 for gp130). The annealing portions of the primers for IL-6R and sIL-6R are shown in Figure 2B. Eight μ l of PCR products were depicted by electrophoresis on 2.0% agarose gels containing ethidium bromide, visualized by UV light, and photographed.

Fluorescence-Activated Cell Sorter (FACS) Analysis

Expression of cell surface receptor molecules was measured by FACS analysis. Briefly, semiconfluent monolayer culture cells were harvested with 0.25% trypsin, followed by washing three times, suspension in FACS

Figure 2. MIPS-2 cells expressed and/or secreted large amounts of IL-6, but less IL-6R and sIL-6R. **A:** RT-PCR of IL-6, IL-6R, sIL-6R, and gp130 from SaOS-2 human osteoblastic osteosarcoma cell line, and MIPS-2 MFH cell line. PCR conditions were described in Materials and Methods. **B:** The arrows showed annealing portions of the primers for IL-6R and sIL-6R. Thus, these primer sets are specific for IL-6R and sIL-6R, respectively. **C:** FACS analysis of IL-6R and gp130 on SaOS-2 and MIPS-2. The antibody-specific staining patterns are shown as shadowed area. **D:** Detection of IL-6 in the culture medium of MIPS-2 cells by ELISA.

buffer [phosphate-buffered saline (PBS) containing 1% fetal calf serum, 0.1% NaN₃, 50 μ g/ml trypsin inhibitor (Wako Pure Chemical Industries, Osaka, Japan)], and staining for 30 minutes on ice with phycoerythrin-conjugated direct antibodies: for IL-6R α chain (CD126) (Immu-

notech, Marseille, France) and gp130 (CD130) (BD PharMingen). FACS analysis was performed on a FACS-Calibur System with Cell Quest software (BD Immunocytometry Systems, San Jose, CA).

ELISA of Culture Medium

Cells were cultured (1.5×10^4 cells/500 μ l/well) for 1 to 7 days. The culture medium was collected and filtered by a 0.22- μ m filter unit (Millipore, Bedford, MA), and IL-6 and sIL-6R concentrations were measured with ELISA kit (Genome Science Ltd., Osaka, Japan).

Immunoblotting

To analyze the phosphorylation level of STAT3 and MAPK, cells were starved in RPMI 1640 with 0.1% bovine serum albumin for 24 hours and subsequently treated with various stimuli at 37°C for 30 minutes. For MRA treatment, cells were placed in RPMI 1640 with 0.1% bovine serum albumin (Invitrogen) containing 2.5 μ g/ml of MRA for 24 hours and subsequently treated with IL-6 (100 ng/ml)/sIL-6R (100 ng/ml) at 37°C for 30 minutes. Medium was removed, and the cells were directly dissolved in Laemmli's sample buffer.²¹ Immunoblotting analysis were done as previously described.²²⁻²⁴ The blot membrane was scanned with a color flat scanner (Epson, Tokyo, Japan) and analyzed with NIH image software using Macintosh personal computer (Apple, Tokyo, Japan). The relative phosphorylation level of STAT3 or MAPK in MIPS-2 cells was estimated from the signal of P-STAT3 or P-MAPK normalized with the signal of STAT3 or MAPK.^{23,24}

Immunofluorescence and Detection of PARP

For the staining of F-actin and vinculin, cells were stimulated with various cytokines [IL-6 (100 ng/ml), IL-6 (100 ng/ml)/sIL-6R (100 ng/ml)] for 7 days. For MRA treatment, the cells were started to incubate in growth medium containing 2.5 μ g/ml of MRA for 24 hours at 1 day after plating. For cytochrome c staining, cells were starved in RPMI 1640 with 0.1% bovine serum albumin for 24 hours. Then cells were treated with tumor necrosis factor- α (50 ng/ml) or various cytokines (IL-6, IL-6/sIL-6R) and MRA (2.5 μ g/ml), and further cultured for 48 hours. After treatment, cells were fixed with 1% paraformaldehyde in PBS for 20 minutes, and permeabilized with 0.2% Triton X-100 in PBS for 10 minutes. Primary antibodies were anti-vinculin monoclonal antibodies (1:100) at 4°C overnight, and secondary antibodies Alexa 488 anti-mouse IgG (1:1000), and then stained with rhodamine phalloidin (1:100).²² Cell nuclei were stained with DAPI (300 nmol/L) for 1 minute followed by washing with PBS. For detection of PARP, after treatment, cells were directly dissolved in Laemmli's sample buffer.²¹ Immunoblotting analysis were done as previously described.²²⁻²⁴ Primary antibodies were a 1:2500 dilution of anti-PARP Antibody (Santa Cruz).²⁵

In Vitro Invasion Assay

The chemoinvasion assay was performed essentially as described.²⁶ Twenty μ g of Matrigel matrix (BD Biosciences, San Jose, CA) was dried onto polycarbonate membrane (12 mm diameter, 12 μ m pore size) of upper chamber of the Transwell (Corning Inc., Corning, NY). Serum-free conditioned medium from HT1080 cells (Health Science Research Resource Bank, Osaka, Japan) was used as a chemoattractant in the lower chamber. MIPS-2 cells were stimulated with various cytokines (IL-6, IL-6/sIL-6R) for 7 days. For MRA treatment, the cells were started to incubate in growth medium containing 2.5 μ g/ml of MRA for 24 hours at 1 day after plating. Various treated MIPS-2 cells (5×10^4 cells) were applied to the upper chamber. After 24 hours, the migrating cells on the lower side of the filter were fixed with 70% methanol and following the protocol described previously.²²

Cell Proliferation Assay

MIPS-2 cells (1×10^4 cells) were plated into 24-well plates and cultured in RPMI 1640 with 10% fetal calf serum for 24 hours. The cells were stimulated with various cytokines (IL-6, IL-6/sIL-6R) for the indicated period up to 7 days. For MRA treatment, the cells were started to incubate in growth medium containing 2.5 μ g/ml of MRA for 24 hours at 1 day after plating. The culture medium was changed every 2 days. Cell numbers were monitored every 24 hours by the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-tetrazolium, monosodium salt] assay.²⁷

Statistical Analysis

All of the statistical analysis was performed using Statistical Analysis System for Windows. All of the data, including standardized densitometric intensities from replicate immunoblots, were analyzed as a completely randomized design using standard analysis of variance procedures. Treatment differences were assessed with Student's *t*-test. All of the experiments were independently repeated at least three times, and data were summarized as means $\pm$ SE. Two-sided *P* values of *P* < 0.05 (*) or *P* < 0.01 (**) were considered statistically significant.

Results

Serum IL-6 and sIL-6R Concentrations During Clinical Course

The preoperative serum IL-6 concentration in the patient was high (9.6 pg/ml). The serum IL-6 level promptly declined and normalized (0.7 pg/ml) followed by wide local excision, and re-elevated when relapsed (12.6 pg/ml; Table 1). Thus the level of IL-6 exactly positively correlated with the serum CRP level and reflected the disease activity while the level of sIL-6R in the serum continued to

be relatively high (148 to 192 ng/ml) during the whole clinical course.

Characteristics of Human MFH Cell Line (MIPS-2)

As shown in Figure 1, A and B, the surgical specimen of MFH and the established MIPS-2 cells exhibited similar heterogeneous, mainly spindle-shaped aspect. The MIPS-2 cells grew in pleomorphic-storiform pattern characteristic of the loss of anchorage dependence. The population doubling time during the period of exponential growth was ~48 hours. The cells induced tumors in nude mice within 3 weeks after transplantation (Figure 1C). A tuberous tumor (1 cm in diameter) was formed with 4 weeks and grew gradually. Eventually, the mice died of the tumor, ~2 to 3 months, with severe cachexia. The cell lines were associated with a significant aneuploidy, such as multi nuclei and prominent nucleoli. The following chromosomal abnormalities were also observed: 65, add(X)(q13), Y, +add(1)(p11), +add(3)(p11), add(4)(q31), del(6)(p23), add(7)(q22), +add(8)(p23)x2, -9, add(9)(p13), add(12)(p11), add(13)(p11), add(14)(p11)x2, add(15)(p11), -16, -16, +17, +19, +20, +20, +20, add(21)(p11), add(21)(q22), -22, -22, +16mar. Immunohistochemical stains were negative for myogenic markers (desmin, α -smooth muscle actin), CD34, CD68, and S-100 protein. Cells proved to be positive for vimentin (data not shown). All of the cells showed consistent positivity for IL-6 with diffuse cytoplasmic staining (Figure 1D). RT-PCR results demonstrated that both MIPS-2 and the human osteoblastic osteosarcoma cell line, SaOS-2, as a positive control for IL-6 and IL-6R, expressed mRNAs for IL-6, but MIPS-2 expressed less IL-6R and sIL-6R compared to SaOS-2.^{28,29} Both MIPS-2 and SaOS-2 expressed signal transducing molecule gp130 in similar extent (Figure 2A). In addition, FACS analysis also revealed that IL-6R and gp130 were expressed on the cell surface of SaOS-2. By contrast, gp130 was expressed on MIPS-2 the same as SaOS-2, but expression of IL-6R was not detectable on MIPS-2 (Figure 2C). Figure 2D showed that the level of IL-6 was increased in a time-dependent manner in the culture medium of MIPS-2 cells, however we could not detect any sIL-6R protein using ELISA.

Effects of IL-6/sIL-6R on Tyrosine Phosphorylation of STAT3 and MAPK in MIPS-2 Cells

Activation of STAT3 is one of the essential steps in the IL-6 signaling pathway.^{10,11,30} Thus, the phosphorylation level of Tyr 705 in STAT3 of MIPS-2 cells was examined by immunoblotting using anti-P-STAT3 antibody (specific for phosphorylated Tyr705).⁸ The treatment of the cells with a combination of IL-6 (100 ng/ml) and sIL-6R (100 ng/ml) robustly increased the phosphorylation level of STAT3 in a time-dependent manner with a peak at 30 minutes (data not shown). Thus we compared the effect of various stimuli at this time point. The conditioned medium from MIPS-2 cells (containing significant amounts of IL-6; Figure 2D) and sIL-6R (100 ng/ml), or the treatment

Figure 3. Phosphorylation of STAT3 and MAP kinase by IL-6/sIL-6R stimulation. **A:** IL-6, sIL-6 + MIPS-2 culture medium, or IL-6/sIL-6R enhanced the phosphorylation of STAT3, and the pretreatment of the cells with MRA blocked this stimulatory effect. **Top lane,** phospho-STAT3; **middle lane,** total-STAT3; **bottom lane,** β -actin. **B:** IL-6, sIL-6 + MIPS-2 culture medium, or IL-6/sIL-6R enhanced the phosphorylation of MAPK, and the pretreatment of the cells with MRA blocked this stimulatory effect. **Top lane,** phospho-p44/p42 MAPK; **middle lane,** total-p44/p42 MAPK; **bottom lane,** β -actin.

with IL-6 (100 ng/ml) alone also induced phosphorylation of STAT3 (92-fold or 118-fold increase compared to vehicle, respectively; Figure 3A). The effect was most pronounced by the treatment with IL-6/sIL-6R complex (388-fold increase, Figure 3A). By contrast, treatment of MIPS-2 cells with sIL-6R alone or MRA (2.5 μ g/ml) and IL-6/sIL-6R failed to induce phosphorylation of STAT3 (Figure 3A). MRA has been reported to neutralize the effect of human IL-6R and sIL-6R.¹⁹ In addition to activation of STATs, IL-6 activates multiple signal transduction pathways, including the SHP2-Ras-MAPK signaling.^{10,11,30} Thus we next examined the phosphorylation level of p44/p42 MAPK in the cells stimulated with different cytokines by immunoblotting using anti-phospho-p44/p42 MAPK antibodies. The treatment of MIPS-2 cells with IL-6/sIL-6R increased the phosphorylation level of p44/p42 MAPK in a time-dependent manner peaking at 15 to 30 minutes in MIPS-2 cells (data not shown). The phosphorylation level of p44/p42 MAPK increased 8.5-fold in MIPS-2 cells treatment with IL-6/sIL-6R compared

to vehicle (Figure 3B), and this enhancement was also reversed by the pretreatment with MRA (2.5 $\mu\text{g/ml}$). Although addition of sIL-6R alone did not induce phosphorylation of STAT3 (Figure 3A), the same treatment activated p44/p42 MAPK (Figure 3B). Thus, in MIPS-2 cells, two distinct signaling pathways, JAK-STAT and MAPK, may be activated and involved in distinct nuclear events via IL-6 signaling.

Effect of IL-6 and sIL-6R on the Invasiveness and Morphology of MIPS-2 Cells

Cytoskeletal function such as increased cell motility and morphological change, is critical for tumor progression, invasion, and metastasis. To examine whether IL-6 signaling plays a role in invasiveness of MIPS-2 cells, we assessed the effect of various cytokines using an *in vitro* Matrigel invasion assay. MIPS-2 cells showed very low chemotaxis toward the conditioned medium from the same cells as a chemoattractant. By contrast, the conditioned medium from human fibrosarcoma HT1080 cells (Figure 4A; control) showed high potency as a chemoattractant, and exhibited a time-dependent increase in the number of cells migrating through Matrigel-coated filter.²² Pretreatment of MIPS-2 cells with IL-6 (100 ng/ml)/sIL-6R (100 ng/ml) or sIL-6R (100 ng/ml) alone for 7 days resulted in marked inhibition of their migration (70% or 52% inhibition compared with the control, respectively) (Figure 4A). Furthermore, the migration of the cells of pretreatment with MRA (2.5 $\mu\text{g/ml}$), IL-6/sIL-6R for 7 days was comparable to that of the nontreated MIPS-2 cells. Therefore, MRA could reverse the inhibitory effect of IL-6/sIL-6R on the cellular invasiveness. Immunofluorescence analysis of MIPS-2 cells treated with IL-6 (100 ng/ml)/sIL-6R (100 ng/ml) by staining with rhodamine phalloidin for F-actin and with anti-vinculin antibody demonstrated enhanced stress fiber formation and focal adhesion complexes (Figure 4B) as compared to the nontreated cells. sIL-6R (100 ng/ml) alone treated MIPS-2 cells showed modest morphological change (Figure 4B). MIPS-2 cells treated with both MRA (2.5 $\mu\text{g/ml}$) and IL-6/sIL-6R demonstrated reversed morphology to the control nontreated cells. Together, these results demonstrated that IL-6 signaling inhibited the invasive capacity and induced the morphological changes and MRA reversed these inhibitory effects by IL-6/sIL-6R.

IL-6/sIL-6R Inhibits MIPS-2 Cell Proliferation

We have also examined the dose and time dependence of effect on the cellular growth of IL-6, IL-6/sIL6R signaling in MIPS-2. Both IL-6 and sIL-6R demonstrated the growth inhibitory effects in a dose-dependent manner. The effect of sIL-6R was less pronounced but enhanced by the addition of IL-6 (Figure 5A). However, the IL-6 alone showed no effect on the cellular growth (data not shown). The growth curve became plateau after 7 days of culture, and the final cell density cultured with IL-6/sIL-6R was much lower (77%) compare to the control (Figure 5B). These inhibitory effects with IL-6/sIL-6R were almost

Figure 4. Effect of IL-6/sIL-6R and MRA on the invasiveness (**A**) and cellular morphology (**B**) of MIPS-2 cells. **A:** Pretreatment with IL-6/sIL-6R or sIL-6R alone dramatically inhibited their migration and the inhibitory effect of IL-6/sIL-6R was completely reversed by the treatment with MRA. Representative membrane fields from each pretreatment with IL-6/sIL-6R or sIL-6R are depicted on the top. **B:** Enhanced stress fiber and focal adhesion complexes were observed in cells with sIL-6R and/or IL-6 treatment. Morphological changes were completely reversed by the treatment with MRA. Cells cultured on collagen-coated chamber slides were fixed after 24 hours and co-stained for F-actin (red) and vinculin (green). Blue color corresponds to the DAPI-stained nuclei. Scale bars: 100 μm (**A**); 50 μm (**B**).

totally reversed by the pretreatment of the cells with MRA antibodies.

Induction of Cellular Apoptosis in MIPS-2 Cells with IL-6/sIL-6R Complex

To assess the mechanism of inhibitory effects of cell growth by IL-6/sIL-6R, we lastly examined the apoptotic signaling in MIPS-2 cells. As shown in Figure 5, C and D,

Figure 5. **A** and **B:** Anti-proliferative effect of IL-6/sIL-6R on MIPS-2 cells. IL-6 reduces tumor cell proliferation in the presence of sIL-6R. Results are representative of three different experiments. **C** and **D:** Effect of IL-6/sIL-6R and MRA on the PARP cleavage (**C**) and cytosolic cytochrome *c* (green) release (**D**) of MIPS-2 cells. Scale bar, 50 μ m.

the treatment of IL-6 (100 ng/ml)/sIL-6R (100 ng/ml) for 48 hours evoked the cleavage of the well-characterized caspase substrate PARP and the release of cytochrome *c* from mitochondria to the cytoplasm (shown in green fluorescent color), as similar degree to that induced by 50 ng/ml of tumor necrosis factor- α for 48 hours (Figure 5C, third lane; and data not shown). Because both phenomena were reported to the initial signaling of typical cellular apoptosis, we speculated that the inhibitory effect of IL-6/sIL-6R on the growth of MIPS-2 was, at least in part, because of the apoptotic events.^{25,31} MRA antibodies completely blocked both apoptotic responses induced by IL-6/sIL-6R in MIPS-2 cells as expected (Figure 5, C and D; right lane and panel).

Discussion

The present results indicated that a novel human pleomorphic MFH cell line, MIPS-2, produced inflammatory cytokines as well as the majority of MFH cells so far described, ie, expression of IL-6, suggesting that inflammation and/or immune response may be closely associated with its pathogenesis.^{32,33} MIPS-2 cells remain stable in culture for a long time and immortal. As often observed in previous reports,^{34–36} these cells were also

associated with a significant aneuploidy and karyotypic study revealed the abnormalities in chromosome number and structure. MIPS-2 cells were tumorigenic in nude mice with marked cachexia, which is also frequently observed in the patient suffering from malignant neoplasms at advanced stage. Thus the present cell line MIPS-2 is a quite good model corresponding to human MFH.

Serological study showed that IL-6 has a positive correlation with the progression of various soft tissue sarcomas including MFH.³⁷ In our recent study, CRP, which directly reflects IL-6, proved to be significantly associated with the malignancy grade, tumor size, and histological subtypes of MFH.⁶ These findings indicate that IL-6 may be responsible for its biological behavior; tumor progression, proliferation, and morphology. It is noteworthy that IL-6 may be elevated as a metabolic abnormality in advanced malignant neoplasms. The sources of IL-6 could be different; it could arise from malignant cells, immunocompetent cells, or infiltrating inflammatory cells. Increased evidence confirmed that MFH cell itself produces IL-6 *in vitro* and *in vivo*.^{38–40} Melhem and colleagues³² immunohistochemically and serologically studied various cytokines in inflammatory type MFH. They proposed that different cytokines including IL-6 might be responsible for the expansion of the tumor cell population, fibroblast proliferation, and secretion of extracellular collagen. Liesveld and colleagues³³ established and characterized a giant cell-type MFH cell line that expressed several proinflammatory cytokines including IL-6. Black and colleagues⁴¹ showed that increased IL-6 concentrations may contribute to systemic inflammatory reaction in tumor-bearing nude mice. Our novel cell line MIPS-2 showed the consistent features with their reports, producing IL-6 which can be one of the molecules inducing PNS in MFH patients. Considering together, increased serum/tissue levels of several cytokines including IL-6, in patients with MFH, reflect a generalized inflammatory state. The inflammatory cells and cytokines found in tumors could make a contribution toward tumor growth, progression, and immunosuppression.^{42,43} Thus the links between MFH and inflammation may provide clues to unknown pathogenesis and make it possible to have implications for prevention and treatment of this devastating tumor.

IL-6 is a multifunctional proinflammatory cytokine produced by different cell types, and is involved in the response to different diseases.^{12,13} IL-6 was characterized as a growth regulator for different cell types, being able to either inhibit or stimulate the cell proliferation in various malignant neoplasms.^{11–13} Nevertheless, the potential roles of IL-6 on MFH cells have been poorly understood. As evidenced by numerous reports, the heterogeneous histological features of MFH may be explained by the ability of MFH cells to elaborate a variety of cytokines. Furthermore MFH tumors are typically surrounded by an inflammatory infiltrate or monocytes and macrophages probably migrating into the tumor because of elaboration of various chemokines such as IL-8, CSF-1, and MCP-1. Indeed, MIPS-2 cells were also found to overproduce hematopoietic growth factor IL-8 (data not shown). Yoshida and colleagues⁴⁴ considered chemoat-

tractants and/or differentiation factors such as CSF-1 produced by MFH to stimulate proliferation and infiltration of the host monocyte/macrophage lineage. Takeya and colleagues⁴⁵ suggested that histiocyte-like cells comprising MFH tumors are the infiltrated macrophages that originate from blood monocytes attracted by tumor-derived MCP-1. Taken together, MFH cells produce several cytokines and chemokines and consequently are susceptible to modulation of inflammatory mediators, which may cause tissue damage and a more aggressive behavior.

The activation of cells that do not express the membrane bound IL-6R α by IL-6 and the soluble IL-6 receptor (sIL-6R) is termed transsignalling. We firstly reported that two IL-6 receptor subunits, IL-6R α and glycoprotein 130 (gp130) were expressed in human MFH cells. The physiological roles of sIL-6R are not fully understood in normal and pathological states. Although studies showed that IL-6 is expressed in several mesenchymal tumors, little data are available concerning the association of IL-6 and its receptor proteins. Erices and colleagues⁴⁶ showed that IL-6R deficiency may represent for bone marrow-derived mesenchymal stem cells to escape the osteoblastic differentiation by the cell itself as well as other stromal cells. Human osteoblasts express cell surface IL-6R, which is unable to transmit IL-6-induced signals until it is shed into its soluble form.²⁹ These evidences suggest that the cellular responsiveness to IL-6 may be primarily determined and regulated by the expression of the appropriate receptor proteins. As an agonist, sIL-6R enhances the biological activity of IL-6, which depends on target cells. In this study IL-6/sIL-6R presented an anti-proliferative effect for the MIPS-2 cell. Furthermore this effect was partly because of apoptosis. After 7 days of incubation, the cell count decreased to 70% of control. The inhibitory effect was completely abolished by the treatment with MRA, however, MRA had no influence on tumor growth *in vitro* (data not shown). The inhibition of IL-6 signaling by MRA might improve the signs and symptoms of PNS of MFH patients and normalize the acute-phase reactants.

Our data could not reconcile the fact that IL-6 is a known growth factor for stromal and endothelial cells but appeared to inhibit the MIPS-2 cell line. Because the membrane IL-6R is only present on a relatively small number of cells, ie, hepatocytes, neutrophils, and monocytes, the pathophysiological effects may primarily depend on the soluble form receptor. It should also be required to investigate whether the physiological divergence may be related to the affinity of both receptors, or the density of gp130 and IL-6R on the cell surface. IL-6/sIL-6R complex induced striking changes in morphology, characterized by a spindle shape, in contrast to the polyclonal feature compared to control MIPS-2. Moreover, the complex dramatically attenuated invasion activity. IL-6 used the JAK/STAT cascades and in part the MAPK signaling pathway, as demonstrated by rapid phosphorylation of STAT3 and p44/42 MAPK. Our model would contribute to the better understanding of the biological and intracellular events that occur in MFH cells after simultaneous exposure to both the growth factors and proinflammatory cytokines that are invariably expressed

in the microenvironment of tumors. The precise mechanism should be elucidated in the future how IL-6/sIL-6R showed morphological alterations in combination with a growth inhibition and attenuation of invasion *in vitro*.

Collectively, all these data suggest that the IL-6/sIL-6R signaling pathway may play a pivotal role, via an auto-crine and/or paracrine manner, which may shed a light on the understanding of the pathogenesis and might be a novel therapeutic target for the treatment of MFH.

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