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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Methylprednisolone Inhibits Tumor Growth and Peritoneal Seeding Induced by Surgical Stress and Postoperative Complications

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ABSTRACT

Background. Surgery often introduce inflammatory response, which may promote tumor growth and metastasis of residual cancer cells. We investigated the impacts of methylprednisolone on the tumor growth and peritoneal seedings in mice treated with lipopolysaccharide (LPS), which mimics systemic inflammation induced by surgical stress and postoperative complications.

Methods. The serum interleukin-6 (IL-6) levels, tumor volume, tumor weight, and the number of peritoneal nodules were investigated in tumor growth model and peritoneal seeding model using BALB/c mice and murine CT26 cancer cell lines in vivo. We conducted functional analyses of IL-6 in Western blotting and proliferation assays in vitro. We also investigated whether preoperative administration of methylprednisolone decreased postoperative serum IL-6 levels in cancer patients in a randomized clinical study.

Results. In the in vivo study, methylprednisolone inhibited the LPS-induced increase of serum IL-6 levels (mean, 33,756 pg/ml vs. 5917 pg/ml; $P < 0.001$), tumor volume (mean, 397 mm^3 vs. 274 mm^3; $P = 0.019$), tumor weight (mean, 0.38 g vs. 0.15 g; $P = 0.020$), and the number of peritoneal nodules (mean, 112 vs. 47; $P = 0.002$). In the in vitro study, IL-6 enhanced JAK/STAT signaling and increased the cell proliferation, and IL-6R-neutralizing antibody attenuated these effects. In the clinical study, serum IL-6 levels were significantly decreased by methylprednisolone (median, 97.5 pg/ml vs. 18.0 pg/ml; $P = 0.030$).

Conclusions. Surgical stress and postoperative complications may enhance tumor growth due to the increase of IL-6. However, methylprednisolone can decrease serum IL-6 levels, thus inhibiting tumor growth and peritoneal seeding.

Surgical excision is often necessary to cure solid tumors. However, postoperative recurrence is still a major problem. Several previous studies reported that surgical stress and the occurrence of postoperative complications negatively impacted the long-term outcomes of cancer patients.[1–3] One possible mechanism is the inflammatory response induced by surgical stress or postoperative complications, which may promote tumor growth and the metastasis of residual cancer cells. Interleukin-6 (IL-6), an inflammatory cytokine secreted by macrophages and T cells, is well known to enhance the proliferation of carcinoma cells.[4,5] Recent studies showed that IL-6 enhanced cell migration and invasion in several types of cancer.[6–8] Despite these findings, few studies have demonstrated the benefit of controlling inflammation induced by surgical stress and postoperative complications.[9] We hypothesized that elevation of serum IL-6 levels by surgical stress and postoperative complications influences the proliferation and metastatic spread of residual cancer cells.

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Methylprednisolone is a well-known corticosteroid used to suppress the immune system and inhibit inflammatory reactions. Several studies showed the efficacy of preoperative methylprednisolone in reducing complications following esophagectomy.[10,11] However, no studies have demonstrated the antitumor effect of methylprednisolone or its impact on long-term prognosis. In this study, we administered lipopolysaccharide (LPS) intraperitoneally to BALB/c mice to investigate whether methylprednisolone decreased the proliferation and metastatic spread of cancer cells. We also examined whether methylprednisolone decreased serum IL-6 levels in patients in a randomized clinical study.

MATERIALS AND METHODS

Animal and Cell Lines

Six-week-old male BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). All animal experiments were performed in accordance with the guidelines approved by Osaka University.

Tumorigenic CT26 mouse colon adenocarcinoma cells (CT26.WT; ADCC CRL-2638) were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (Life Technologies).

In Vivo Tumor Experiments

In the subcutaneous implantation experiments, 2×10^6 CT26 cells in 100 μl of PBS were inoculated subcutaneously into the back of mice. When the tumor volume reached 150 mm^3, mice were treated with methylprednisolone or saline intraperitoneally to decrease any subsequent inflammation, and LPS or saline was injected intraperitoneally. Mice were classified into four groups: the control group was administered 100 μl of saline two times; the methylprednisolone-only group was administered 100 μl of methylprednisolone (100 μg/g of body weight) followed by 100 μl of saline; the LPS without methylprednisolone group was administered 100 μl of saline followed by 100 μl of LPS (0.5 μg/g of body weight); and the LPS with methylprednisolone group was administered 100 μl of methylprednisolone (100 μg/g of body weight) followed by 100 μl of LPS (0.5 μg/g of body weight). Serum was collected from the caudal vein 2 h after administration of LPS or saline, and serum IL-6 levels were measured using an ELISA kit specific for mice (R&D Systems, Minneapolis, MN). Tumor volumes were calculated on day 5 using the equation V (mm^3) = $A \times B^2/2$, where A is the largest diameter and B is the smallest diameter. Mice were sacrificed on day 7, and tumor weights were evaluated.

In the peritoneal seeding experiments, 100 μl of methylprednisolone (100 μg/g of body weight) or 100 μl of saline was injected intraperitoneally followed by 100 μl of LPS (0.5 μg/g of body weight) or 100 μl of saline. Next, 1×10^6 CT26 cells in 500 μl of PBS were injected intraperitoneally. Laparotomy was performed on day 10, and the number of tumor nodules and the tumor weight were evaluated.

Immunohistochemistry

Tumor-bearing mice were sacrificed on the day after intraperitoneal injection of LPS or saline. The tumors were fixed in formalin and embedded in paraffin. Slides were incubated with Ki67 antibody (diluted 1:300; Novus Biologicals, Littleton, CO). Ki67 staining was recorded as the ratio of positively stained cells to all tumor cells in five fields from each section at 200 × magnification.

Western Blotting

CT26 cells were stimulated with recombinant mouse IL-6 (50 ng/ml) or recombinant mouse IL-6 and anti-mouse IL-6R-neutralizing antibody (100 μg/ml). Total protein (10 μg) was extracted from cultured cells 20 min after cells were stimulated and transferred to polyvinylidene difluoride membranes. The membranes were blocked and incubated with following antibodies: anti-phosphorylated signal transducer and activator of transcription 3 (pSTAT3) (1:1000 dilution, Cell Signaling Technology, Beverly, MA), anti-STAT3 (1:1000 dilution, Cell Signaling Technology), and anti-β-actin (1:1000 dilution, Sigma-Aldrich, St. Louis, MO). After incubation with secondary antibodies, the protein bands were detected.

Proliferation Assay

CT26 cells were seeded at a density of 2×10^3 cells per well in 96-well plates and cultured in RPMI 1640 medium supplemented with a low concentration of fetal bovine serum (0.1% FBS) for 24 h. Cells were stimulated with recombinant mouse IL-6 (50 ng/ml, R&D Systems, Minneapolis, MN) or recombinant mouse IL-6 and anti-mouse IL-6R-neutralizing antibody (100 μg/ml, Chugai Pharmaceutical, Tokyo, Japan). Cell proliferation was assessed on days 0, 2, and 5 after cell stimulation and compared between the three groups using a Cell Counting Kit-8 (Dojindo, Tokyo, Japan).

Wound Scratch Assay

Cells were seeded at a density of 1×10^6 cells per well in 6-well plates and cultured for 24 h to permit cell adhesion and the formation of a confluent monolayer. A scratch across the dish surface was created with a 200-µl pipette tip. The cells were cultured in RPMI 1640 medium with 0.1% FBS with recombinant mouse IL-6 (50 ng/ml, R&D Systems) or recombinant mouse IL-6 and anti-mouse IL-6R-neutralizing antibody (100 µg/ml, Chugai Pharmaceutical). Wound closure was monitored by collecting digitized images at 0 and 24 h after the scratch was created, and cell migration was evaluated by measuring the ratio of the cell coverage area to the initial cell-free zone in five random areas using Image J software.

Human Subjects

A randomized, clinical study was begun in December 2016 by the Clinical Study Group of Osaka University to evaluate the safety and efficacy of preoperative methylprednisolone administration in patients with resectable gastric cancer. This study was registered in UMIN-CTR, number UMIN000024465. Patients who were randomly assigned to the methylprednisolone group received preoperative intravenous administration of 5 mg/kg of methylprednisolone once just before the skin incision. This study was phase II/III study, and the primary endpoints were the maximum of serum level of postoperative CRP (phase II), and the recurrence free survival (phase III). In a correlative study, we examined serum IL-6 levels preoperatively (within 2 weeks before surgery) and postoperatively (the day after surgery) in patients who enrolled at our institute between December 2016 and September 2017. A testing center (SRL, Tokyo, Japan) was commissioned to measure levels of serum IL-6.

Statistical Analysis

In human subjects, differences between groups were examined for statistical significance by the Mann–Whitney *U* test. In both in vitro and in vivo analysis, data are expressed as mean ± standard error (SE). Differences between groups were examined for statistical significance by Student's *t* test. Two-sided *P* values were calculated, and a value of $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed with the SPSS statistics software package, version 22.

RESULTS

Impact of Methylprednisolone on Tumor Growth in Vivo

To investigate whether LPS accelerated tumor growth and whether this acceleration was inhibited by methylprednisolone in the tumor-bearing mouse model, we evaluated the volume and weight of the tumors in each of the four groups (Fig. 1a). LPS significantly increased serum IL-6 levels relative to saline control (mean, 33,756 pg/ml vs. 1352 pg/ml; $P < 0.001$), and this increase was prevented by methylprednisolone (mean, 5917 pg/ml; $P < 0.001$; Fig. 1b). Tumors volumes on day 5 in the LPS(+)/methylprednisolone(−) group (mean, 397 mm^3) showed a significant increase relative to both the LPS(−)/methylprednisolone(−) group (mean, 261 mm^3; $P = 0.013$) and LPS(−)/methylprednisolone(+) groups (mean, 236 mm^3; $P = 0.024$; Fig. 1c). Methylprednisolone administration suppressed the increase of tumor volumes even with LPS (mean, 274 mm^3; $P = 0.019$). Similarly, the increase of total weight of tumors on day 7 in the LPS(+)/methylprednisolone(−) group (mean, 0.38 g) was suppressed by methylprednisolone administration (mean, 0.15 g; $P = 0.020$; Fig. 1d). The tumor proliferation promoted by LPS (mean, 69%) also was inhibited by methylprednisolone administration in Ki67 immunohistochemistry (mean, 47%; $P = 0.032$; Fig. 1e). Resected tumor and tumor cells stained with Ki67 antibody, are shown in Supplementary Figure 1.

Impact of Methylprednisolone on Tumor Peritoneal Seeding in Vivo

To investigate whether LPS accelerated tumor growth and methylprednisolone inhibited peritoneal seeding of tumor cells in the mouse model, we evaluated the total number and weight of peritoneal tumors 10 days after inoculation with CT26 cells (Fig. 2a). The number of tumor nodules on day 10 in the LPS(+)/methylprednisolone(−) group (mean, 112) was significantly higher than in the LPS(−)/methylprednisolone(−) (mean, 39; $P = 0.001$) and LPS(−)/methylprednisolone(+) groups (mean, 50; $P = 0.013$; Fig, 2b). Methylprednisolone administration suppressed the increase in number of tumor nodules, even with LPS (mean, 47; $P = 0.002$). Similarly, the total weight of tumors on day 10 in the LPS(+)/methylprednisolone(−) group (mean, 1.11 g) was significantly higher than that in the LPS(−)/methylprednisolone(−) (mean, 0.35 g; $P = 0.011$) and LPS(−)/methylprednisolone(+) groups (mean, 0.49 g; $P = 0.049$), but the increase was suppressed by methylprednisolone administration (mean, 0.39 g; $P = 0.018$;

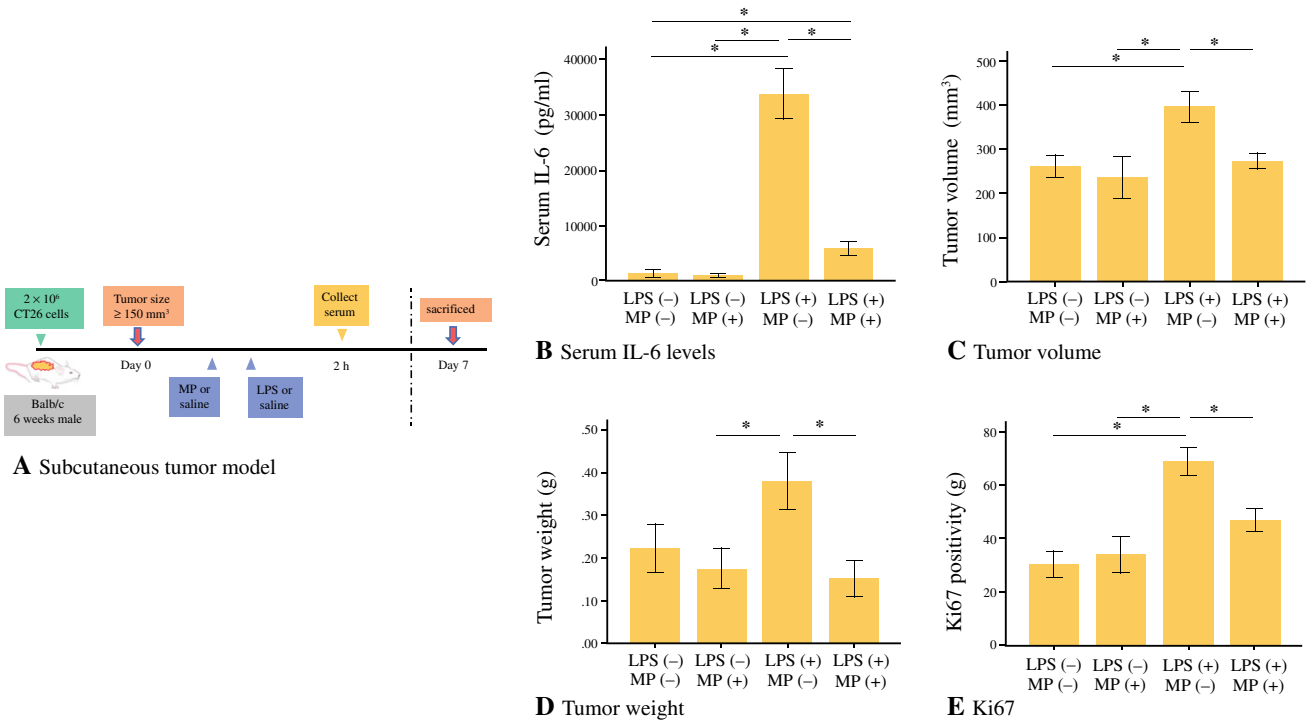

FIG. 1 Impact of methylprednisolone on tumor growth in vivo. **a** Schema of the subcutaneous tumor model. **b** Serum IL-6 levels at 2 h after LPS or saline administration. **c** Tumor volumes on day 5. **d** Tumor weight on day 7. **e** Proportion of cells staining positively for Ki67 antibody in five fields from each section at × 200 magnification. Bars represent mean ± standard error of five independent experiments. $*P < 0.05$. *LPS* lipopolysaccharide; *MP* methylprednisolone

FIG. 2 Impact of methylprednisolone on tumor peritoneal seeding in vivo. **a** Schema of peritoneal seeding model. **b** Number of tumor nodules on day 10. **c** Total weight of tumor nodules on day 10. Bars represent mean ± standard error of three independent experiments. $*P < 0.05$. *LPS* lipopolysaccharide; *MP* methylprednisolone

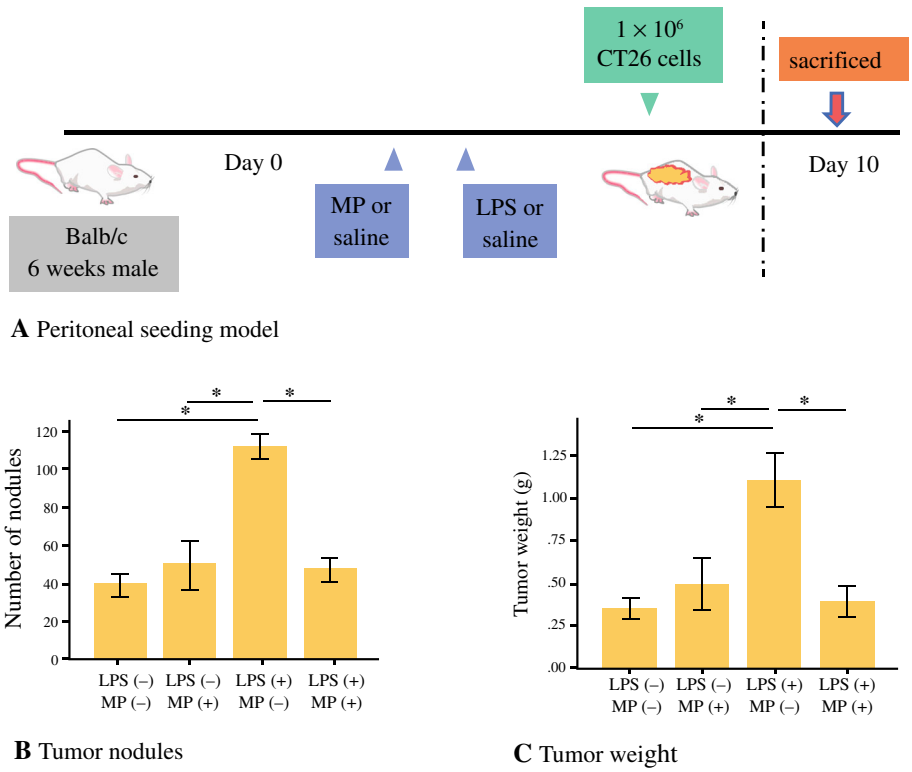

Fig. 2c). Resected tumor are shown in Supplementary Figure 2.

Proliferation and Migration Activity Regulated by IL-6

To investigate the role of the JAK/STAT pathway in our model, we conducted a proliferation assay and wound scratch assay. Western blotting showed that recombinant mouse IL-6 promoted the expression of pSTAT3, and anti-mouse IL-6R-neutralizing antibody attenuated the expression of pSTAT3 (Fig. 3a). Recombinant mouse IL-6 significantly increased the proliferation of CT26 cells ($P < 0.001$), but this was significantly mitigated by anti-mouse IL-6R-neutralizing antibody ($P < 0.001$; Fig. 3b). Tumor migration also was significantly promoted by recombinant mouse IL-6 (mean, 66% vs. 49%; $P = 0.026$), and it was relatively inhibited by anti-mouse IL-6R-neutralizing antibody, although it was not statistically significant (mean, 54%; $P = 0.206$; Fig. 3c). Representative images are shown in Fig. 3d.

Impact of Methylprednisolone on Postoperative Serum IL-6 Levels Induced by Surgical Stress in Human Subjects

In a correlative study of the clinical trial, we examined the pre- and postoperative serum IL-6 levels in six patients in the surgery-only group and six patients in the surgery plus methylprednisolone group. Preoperative serum IL-6 levels were similar in the two groups (median, 3.2 pg/ml in the surgery-only group and 5.4 pg/ml in the surgery plus methylprednisolone group; $P = 0.394$; Fig. 4a). However, postoperative levels were significantly higher in the surgery-only group than in the surgery plus methylprednisolone group (median, 97.5 pg/ml in the surgery-only group and 18.0 pg/ml in the methylprednisolone group; $P = 0.030$; Fig. 4b).

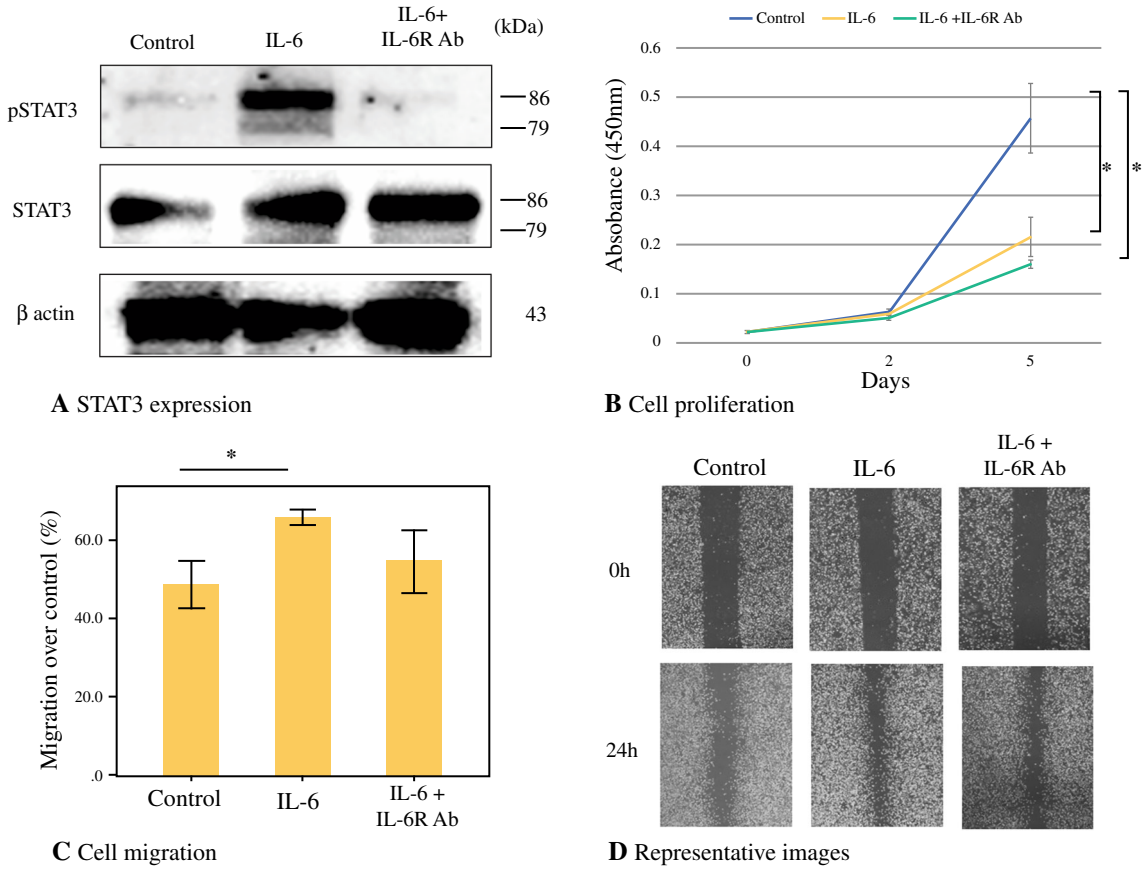

FIG. 3 Proliferation and migration activity regulated by IL-6. **a** Protein expression levels of pSTAT3 detected by Western blotting. **b** Proliferation assay. Absorbance was measured on days 0, 2, and 5 in triplicate. **c** Wound scratch assay. Cell migration was expressed as the ratio of the cell coverage area to the initial cell-free zone at 0 and 24 h. **d** Representative images in each group at 0 and 24 h. Bars represent mean ± standard error of three independent experiments. *$P < 0.05$

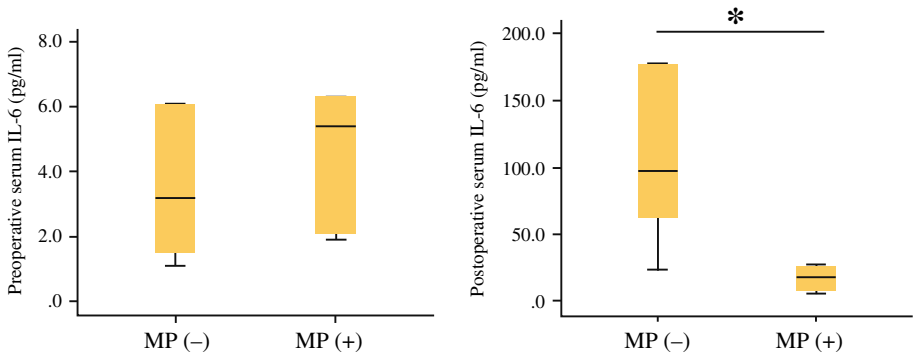

FIG. 4 Impact of methylprednisolone on postoperative serum IL-6 levels induced by surgical stress in human subjects. **a** Preoperative serum IL-6 levels in the two groups ($n = 12$). **b** Postoperative serum IL-6 levels in the two groups ($n = 11$). Box plot indicates the position of the first, second (median), and third quartiles. Bars show the lowest data points still within 1.5 times the interquartile range (IQR) from the lower quartile boundary, and the highest data points still within 1.5 times the IQR from the upper quartile boundary. $*P < 0.05$. *MP* methylprednisolone

DISCUSSION

In the in vivo study, LPS increased serum IL-6 levels in mice and promoted tumor growth and the adhesion of cancer cells to the peritoneum. However, methylprednisolone mitigated the increase of serum IL-6 levels, which led to significant inhibition of the tumor growth and peritoneal seeding induced by LPS. In Ki67 immunohistochemical analysis, the promotion of tumor proliferation by LPS also was inhibited by methylprednisolone administration. In the in vitro study, IL-6 enhanced both JAK/STAT signaling and the cell proliferation and migration of CT26 cells, and anti-mouse IL-6R-neutralizing antibody attenuated the proliferation and migration effects. We also confirmed that preoperative administration of methylprednisolone led to a significant decrease in serum IL-6 levels in a randomized clinical study.

IL-6 is mainly secreted by macrophages and T cells and is an acute-phase protein, along with interleukin-1 beta, tumor necrosis factor-alpha, and interferon gamma. IL-6 has been reported to be correlated with surgical stress and to be a reliable marker for detecting postoperative complications.[12,13] Moreover, IL-6 has been reported to play various roles in the pathogenesis and activity of human cancers.[14,15] The IL-6 receptor consists of the common receptor unit gp130 and the IL-6-specific subunit gp80, and activation of this receptor complex results in downstream activation of the JAK/STAT, PI3K/Akt, and MAPK signaling pathways.[16,17] Activation of the JAK/STAT pathway results in nuclear translocation of phosphorylated STAT3 and transcriptional upregulation of target genes and plays an important role in tumor proliferation, invasion, and metastasis.[5,18,19] A previous study reported that serum and abdominal fluid in patients with postoperative peritoneal infection enhanced tumor cell migration and invasion in vitro.[20] These fluids may contain proinflammatory cytokines and growth factors, which are primarily synthesized after trauma and initially released locally by leukocytes, macrophages, and endothelial cells, and then released systemically.[21,22] In this study, CT26 cells cultured with anti-mouse IL-6R-neutralizing antibody proliferated more slowly than control cells. Previous studies reported that a variety of malignant tumors contain or synthesize IL-6, and autocrine growth stimulation has been suggested as a possible mechanism for the action of IL-6.[23,24] These findings suggest that IL-6R-neutralizing antibody might suppress not only recombinant IL-6 but also IL-6 secreted by cancer cells, which is necessary for stimulating proliferation. Ashizawa et al.[25] reported that the preoperative serum IL-6 level was a prognostic factor in patients with gastric cancer. In contrast, postoperative serum IL-6 level is mainly reflected by postoperative inflammation. We hypothesize that suppressing postoperative inflammation will provide a positive impact on the prognosis of patients who have undergone surgery. Therefore, we performed in vivo experiments to investigate whether methylprednisolone, a strong anti-inflammatory drug, has an antitumor effect resulting from its suppression of the systemic inflammatory response.

We also demonstrated the influence of LPS-induced inflammation and methylprednisolone on peritoneal seeding. However, it is not clear how inflammation promotes peritoneal seeding of cancer cells. Surgical removal of primary tumors causes spillage of cancer cells and promotes peritoneal seeding.[26,27] Recently, Xiao et al.[28] reported that IL-6 promoted epithelial-to-mesenchymal transition (EMT) of peritoneal mesothelial cells, possibly through the JAK/STAT3 signaling pathway. EMT of these cells is an important process for peritoneal metastasis,

because cancer cells liberated from the primary tumor invade through the peritoneal membrane, which is covered with peritoneal mesothelial cells.[29] These facts suggest that IL-6 elevation by LPS-induced inflammation may promote peritoneal seeding by inducing adhesion of cancer cells to the peritoneal membrane.

To the best of our knowledge, no studies have demonstrated the prognostic benefit of preoperative methylprednisolone administration in cancer surgery. Although several previous studies have reported that preoperative methylprednisolone administration suppressed the postoperative increase in serum levels of IL-6 in esophageal cancer, the prognostic value of postoperative IL-6 was not evaluated.[11,30] In our clinical study, the serum IL-6 level was lower than that in our in vivo experiment, because serum was collected the day after surgery to simplify the study. Serum IL-6 is drastically increased by surgery and decreases rapidly thereafter. We think that preoperative methylprednisolone may play an important role in the acute phase after surgery in preventing tumor growth and peritoneal seeding induced by surgery. In the future, we will analyze the usefulness of preoperative methylprednisolone administration in improving outcomes of gastric cancer patients in our ongoing, randomized, controlled trial.

The main limitation of our study was that we evaluated only IL-6 and no other proinflammatory cytokines. Methylprednisolone is a strong anti-inflammatory drug and suppresses other acute-phase proteins, such as tumor necrosis factor-alpha and nuclear factor-kappa B. Therefore, there may be other cytokines besides IL-6 that influence tumor growth and seeding in our mouse model of inflammation.

In conclusion, surgical stress and postoperative complications may enhance tumor growth due to the increase of IL-6. However, preoperative methylprednisolone can reduce the elevation of serum IL-6 levels, thus inhibiting tumor growth and peritoneal seeding.

DISCLOSURE There are no conflicts of interest.

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