

Effect of Preoperative Administration of Methylprednisolone and Ulinastatin on Tumor Cell Metastasis after Surgical Stress

KAZUYA MOMOSAKI, NOBUYA ISHIBASHI, SHOGO YOSHIDA, TATSUYA MURAOKA,
KATSUYUKI TANAKA, NOBUTAKA IWAKUMA, YOHSUKE OKA,
ATSUSHI KAIBARA, YOSHITO AKAGI AND KAZUO SHIROUZU

Department of Surgery, Kurume University School of Medicine, Kurume, 830-0011, Japan

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Summary: Using a rat laparotomy stress model, we conducted a comparative analysis of postoperative organ metastasis after administration of ulinastatin (UTI) or methylprednisolone (MP), which have an inhibitory effect on cytokine production. The subjects were classified into 4 groups: 1) minimal laparotomy group (C group), 2) major laparotomy group (L group), 3) preoperative MP intravenous administration + major laparotomy group (MP group), and 4) preoperative UTI intravenous administration + major laparotomy group (UTI group). Either MP or UTI was administered intravenously before surgery, and RI-labeled cells were injected into the portal vein immediately after laparotomy to collect tissue specimens in order to measure radiation dosage. Then, the concentrations of serum IL-2 and IL-6, liver interleukin 1 beta (IL-1 β) and interleukin 10 (IL-10), and liver E-selectin were measured. In addition natural killer cell, (NK cell) activation and neoplastic nodules on the liver surface at 3 weeks after surgery were also measured. The adhesion rate of malignant cells to the liver was higher in the L group than in the C group, higher in the MP group than the L group, and lower overall in the UTI group. The concentration of IL-1 β and IL-6 were decreased in the MP and UTI groups compared to the L group. IL-2 was decreased significantly in the MP group compared with the C and L groups. E-selectin expression level decreased in the UTI group compared with the L group. NK cell activation decreased in the MP group compared with the C group and L group, but no differences were observed between the UTI and L groups. The number of tumor nodules on the surface of the liver increased in the MP group compared with the L group, and decreased in the UTI group compared with the L group. Postoperative alleviation of invasive reaction was suggested in both the MP and UTI groups. However, preoperative administration of MP increased metastasis while that of UTI inhibited metastasis. MP was considered to have decreased anti-tumor immunocompetence and promoted metastasis, while UTI was considered to have inhibited the expression of adhesive molecules and decreased metastasis.

Key words tumor cell metastasis, surgical stress, cytokine, ulinastatin, methylprednisolone

INTRODUCTION

When surgical stress is applied to living organisms, harmful invasive responses such as a decrease in immunocompetence and a deterioration in nutritional status caused by the loss of body protein are induced

along with reactions indispensable to recovery such as promotion of wound healing. Surgery for a malignant tumor has a harmful effect in which proliferation of malignant tumor cells and metastasis formation could be promoted by the invasive reaction [1,2]. Adhesion of cancer cells and endothelial cells in sec-

Corresponding author: Nobuya Ishibashi, Department of Surgery, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan. Tel: 81-942-35-3311 Fax: 81-942-34-0709 E-mail: nob@med.kurume-u.ac.jp

Abbreviations: IL-1 β , interleukin 1 beta; IL-10, interleukin 10; MP, methylprednisolone; NK cell, natural killer cell; TNF- α , tumor necrosis factor alpha; UTI, ulinastatin.

ondary organs is one of the important steps in hematogenous metastasis. Adhesion molecules produced in vascular endothelial cells after surgical invasion, migratory competence of tumor cells, and production of proteolytic enzyme that destroys extracellular matrix are all controlled by the local or systemic production of cytokine [3].

Preoperative administration of methylprednisolone in radical surgery for esophagus cancer has been recommended because preoperative administration of steroid hormone was considered to regulate the production of the cytokine that triggers this invasive reaction [4]. However, despite the fact that the postoperative invasion reaction could be reduced by the steroid hormone medication before radical surgery, no reduction in recurrence and no improvement in prognosis have been reported. Protease inhibitor is also reported to inhibit production of cytokine [5], which suggests a possibility that preoperative administration of protease inhibitor would also reduce the invasive reaction.

Therefore we administered a preoperative intravenous protease inhibitor (ulinastatin: UTI) that inhibits cytokine production, and conducted a comparative analysis against methylprednisolone (MP) using a rat laparotomy stress model in order to investigate the hematogenous metastasis of tumor cells to organs and the influence on various cytokine expressions, with an aim to search for a treatment approach that can not only regulate the postoperative invasive reaction but also reduce the postoperative recurrence rate in patients with malignant tumor.

MATERIALS AND METHODS

1. Experimental Animals

Male Donryu rats (n=82, BW: 210-230 g, 5 weeks of age) were purchased from the Shizuoka animal center and housed in the animal facility of Kurume University under 12 hours light and dark conditions. The animals were fed standard rat chow (Clea Inc., Tokyo) and water ad libitum for 7 days prior to the start of the experimental protocol. The experimental protocol was approved by the Kurume University Ethics Committee.

2. Cell line

The rat AH109A ascites hepatoma cells were cultured in RPMI1640 (Gibco Life Technologies Inc., Grand Island, NY) for two weeks, harvested from the culture dish and injected into the rat abdominal cavity for subculture. AH109A cells were withdrawn from the abdominal cavity and counted. AH109A cells were radiolabeled according to the method of Isaiah [6], as

follows. Briefly, AH109A cells were cultured with ¹²⁵I-iodo-deoxyuridine in CO2 incubator at 95% humidity and 37 degrees centigrade. Cells were harvested and divided into 2×10⁵ per 0.5 ml PBS, and then radioactivity was measured by gamma counter. Around 20,000 cpm per 0.5 ml of cell suspension was used for intra-superior mesenteric vein injection.

3. Medicines [Drugs]

Each dose of methylprednisolone was dissolved in 1.5 ml of distilled water. Rats were dosed at 5 mg/kg, 10 mg/kg, and 20 mg/kg to determine the dosage which showed the same pattern of cytokine suppression as observed in the postoperative reduction of invasive reaction in human esophageal cancer [esophageal carcinoma]. As a result, the dosage at 10 mg/kg was established. In addition, ulinastatin (trade name: MIRACLID, Mochida Pharmaceutical Co), which is a glycoprotein derived from human urine with molecular weight of 67000, was dissolved in 5% glucose solution at a final concentration of 50.000 units/kg/dose for rat, which is equivalent to 5,000 units/kg for human to suppress cytokine production [7].

4. Cell labeling with RI

AH-109A cells were cultured and labeled with ¹²⁵I-deoxyuridine for 3 hours, then cells were washed with PBS (phosphate buffer saline solution) 3 times, and 2×10⁵ cells were suspended in 0.5 ml PBS. After the radioactivity of each labeled cell suspension was measured using a gamma counter, cell suspensions with around 20,000 cpm/0.5 ml were chosen to fill 1 cc syringe for use.

5. Rat Invasive Laparotomy Model

The large incision group (L group) in which cruciform laparotomy (vertical incision: 6 cm, transverse incision: 4 cm) was performed was treated as a high invasive model, and the small incision group (C group) in which small midline laparotomy incision (vertical incision: 1 cm) was performed was considered as a control (low invasive model). In addition, an MP group treated with methylprednisolone and a UTI group treated with ulinastatin by intravenous injection prior to large incision, were investigated (four groups in total).

6. Drug Administration Method

After rats underwent general anesthesia with pentobarbital sodium (50 mg/kg, intraperitoneal injection), 1.5 ml of 5% glucose solution was given to rats in the C and L groups, and methylprednisolone at 10mg/kg

or ulinastatin at 50,000 units/kg in 1.5 ml of 5% glucose solution were administered to those in the MP and UTI groups, respectively, through vein of bulb of penis at the rate of 0.5 ml per 10 seconds. Ten minutes after drug administration, rats in each group underwent invasive stress by laparotomy for 30 minutes.

7. Investigation of Adhesion of Tumor Cells to Organs

Rats (n=36) were treated with drugs as mentioned above, and 10 minutes later, surgical stress [operation invasion] was given by laparotomy, after which RI-labeled tumor cells were immediately injected intraportally, then after a waiting period of 30 minutes abdominal closure was achieved by knotted suture (in one throw for C group, and a total of 9 throws for L group) with 4-0 Nylon (Ethicon Inc.). Rats were sacrificed at 5 hours after surgery, and blood, liver, lungs, and thyroid were harvested to measure radioactivity using a gamma counter. Also we weighed each removed organ to obtain cell adhesion rates on organs for further evaluation. The cell adhesion rates were calculated as follows: first the value was obtained by dividing radioactivity of each sample of organs by the level of radioactivity of intraportally injected cells, and then adjusted for the weight of each organ.

8. Measurement of Cytokine Levels in Blood and Liver

We injected AH-109A cells without RI-labeling intraportally to rats in each of the 4 groups. After waiting 30 minutes the surgical incision was closed, and the rats were then sacrificed at 3 hours after surgery to harvest whole blood and liver as mentioned above. Whole blood was centrifuged at 3000 rpm for 15 minutes, and supernatants were collected. About 3 g of liver sample were washed with D-PBS 2-3 times, and the samples in Falcon tubes were homogenized in 10 ml of 1× Sample Buffer (2.5 ml of 0.5 M Tris-HCl (pH 6.8), 2 ml of 10% SDS, 1 ml of 2-mercapto ethanol, 4 ml of glycerol, and 0.5 ml of H₂O) for 3 minutes with cooling. After centrifugation of homogenates at 3,000 rpm for 15 minutes at 4°C, supernatants were collected and amounts of proteins were quantified by Bradford Protein Assay with BECKMAN DU-600 spectrophotometer. Blood serum levels of IL-6 and IL-2, and interleukin 1 beta (IL-1β) concentration in liver homogenates were evaluated by ELISA (Immunoassay Kit; BioSource International, Camarillo, CA).

9. Measurement of E-selectin Levels in Whole Blood and Liver

Whole blood and liver were harvested at 3 hours after surgery to measure E-selectin concentration. Liver

samples were homogenized in sample buffer (2.5 ml of 0.5 M Tris-HCl (pH 6.8), 2 ml of 10% SDS, 1 ml of 2-mercapto ethanol, 4 ml of glycerol, and 0.5 ml of H₂O), and then supernatants were collected and protein contents of samples in each group were quantified by Bradford Protein Assay using a BECKMAN DU-600 spectrophotometer. Subsequently, samples were boiled for 5 minutes at 100°C, and resolved proteins by 7.5% SDS-PAGE were subjected to Western blot analysis.

Proteome Works System with 7.5% Ready Gel (BIORAD) was used. Twenty μg protein for each sample was loaded into each well of SDS-PAGE gel and electrophoresed in 1× Running Buffer (3.025 g Tris, 1.0 g SDS, and 14.410 g Glycine in 1 l distilled water) for 120 minutes at 80 V with MODEL 1000500 POWER SUPPLY (BIO-RAD). PVDF membrane (pore size: 0.2 μm) was pretreated in 100% methanol. The resolved proteins were transferred from gel to PVDF membrane [using gel-PDF membrane sandwich] with electrophoretic transfer apparatus at 60 V for 120 minutes. After blotting, the membrane was preincubated in blocking buffer (5% skim milk) to block non-specific binding, following overnight incubation with anti-E-selectin primary antibodies (1:500 dilution; M-20, sc-6939, Santa Cruz) at 4°C. The membrane was washed with PBS supplemented with 0.1% TWEEN20 for 3 times (for 40 minutes each). Then the membrane was incubated with donkey anti-goat IgG secondary antibodies (HRP conjugate) (sc-2020 Santa Cruz) at 1:3000 dilution in blocking solution, for 1 hour at room temperature, followed by washing 5 times with PBS supplemented with 0.1% TWEEN20 and further incubation for 5 hours.

Chemiluminescent signals were detected using ECL Plus Western Blotting Detection Reagent (RPN2132, Amersham Pharmacia) and autoradiography film. E-selectin signal intensity was normalized by the intensity of β-actin detected with anti-β-actin antibody (SIGMA) and HRP-conjugated anti-mouse secondary antibody as mentioned above. Analysis was performed using NIH Image 1.60.

10. Activation of NK cells

NK cell activity of spleen cells at 1 hour after surgery was measured using Yac-1 cells. Harvested spleen was cut on a sterilized ice-cold dish with 5 ml of PBS, passed through stainless steel mesh and then centrifuged with added Percoll (GE Health Care Bioscience, UK) at 2,200 rpm, room temperature for 15 minutes. The separated mononuclear cells layer was washed in ice-cold PBS and mononuclear cells were poured into

96 well U bottom microplates to become 4×10^4 in each well. We used T-cell lymphoma YAC-1 cells derived from A/st mouse as target cells. 1×10^6 Yac-1 cells were incubated with 100 micro Ci of $\text{Na}^{51}\text{CrO}_2$ (Japan Radioisotope Association, Tokyo, Japan) for 60 minutes at 37 degrees Celsius in 5% carbon dioxide atmosphere and then washed with culture medium. 1×10^4 Yac-1 cells were then suspended in 4×10^4 mononuclear cells in 96 well U bottom microplates at an E/T ratio of 40 to 1 for 5 hours at 37°C in 5% carbon dioxide atmosphere and then the microplate was centrifuged at 1,000 rpm for 5 minutes. Supernatants were collected and radioactivity was measured by gamma counter. Natural killer cell activity was calculated as follows:

Natural killer cell activity (% cytotoxicity) = (specimen dissociation(cpm) – nature dissociation(cpm)) / (maximum dissociation(cpm) – nature dissociation(cpm)) \times 100.

11. Evaluation of Number of Liver Metastases

In postoperative week-3, livers were harvested from rats injected with AH-109A cells without intraportal labeling in the four groups, and the numbers of neoplastic nodules on the liver surface were counted.

12. Histopathological Investigation

The harvested livers from rats in postoperative week-3 were fixed in 10% formalin for 7 days. Follow-

ing hematoxylin-eosin staining, microscopic examination and pathological studies of neoplastic nodules were performed.

13. Statistical Processing

In the experiments [study], statistical analysis and significance tests were performed using one way ANOVA and Fisher's PLSD test, respectively. $P < 0.05$ was considered to be statistically significant.

RESULTS

1. Investigation of Organ-specific Metastatic Tumor Cell Adhesion

The adhesion rate of malignant cells to liver was higher in the L group than in the C group ($5.77 \pm 0.5\%$ in C group vs. $7.42 \pm 0.3\%$ in L group, $p < 0.05$), and preoperative MP administration increased the adhesion rate of malignant cells to liver compared to that in the L group ($7.42 \pm 0.3\%$ in L group vs. $11.4 \pm 0.9\%$ in MP group, $p < 0.05$). The adhesion rate for liver after preoperative UTI administration was decreased compared to that in the L group ($7.42 \pm 0.3\%$ in L group vs. $4.8 \pm 0.3\%$ in UTI group) (Fig. 1). No difference in adhesion rate for lung, blood, and thyroid were observed among the 4 groups (Figs. 2, 3, and 4).

2. The Effects of Operation Invasion [Operative Stress], and Action of MP and UTI on Cytokine Production

IL-1 β concentration in liver at 1 hour after surgery was higher in the L group than in the MP and UTI groups (60.62 ± 1.6 pg/ml in L group vs. 46.3 ± 2.5 pg/ml in UTI group and 44.2 ± 2.2 pg/ml in MP group, $p < 0.05$) (Fig. 5). However, no significant difference

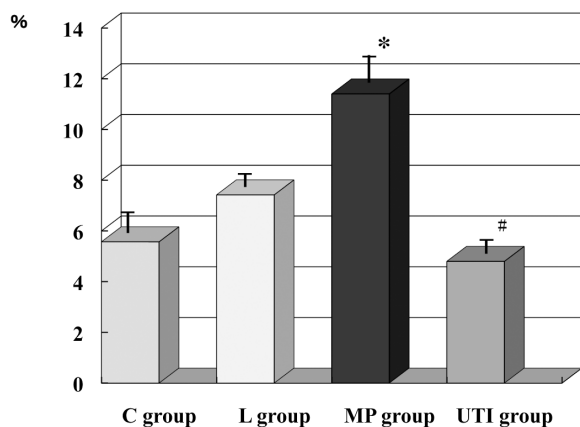


Fig. 1. RI activity in the liver at 5 hours after operation. Preoperative MP treatment increased radioactivity in the liver compared to the other groups. UTI pretreatment significantly reduced radioactivity in the liver compared to large incision group.

Data was presented as mean \pm SD. One way ANOVA followed by Fisher's PLSD test was used for statistical analysis. *, $p < 0.05$ MP group vs the other groups, #, $p < 0.05$ UTI group vs L group.

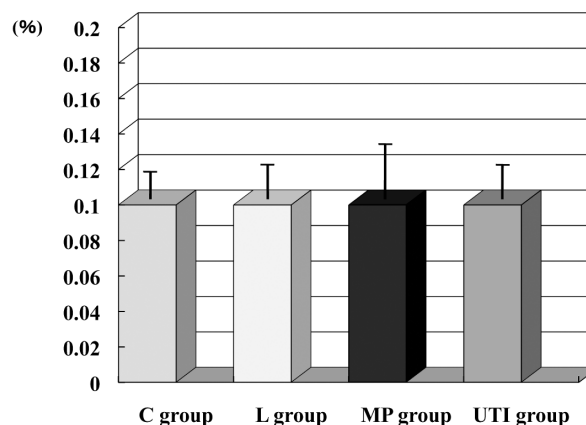


Fig. 2. RI activity in the lung at 5 hours after operation. There was no significant difference among the groups. Data was presented as mean \pm SD. n=9 rat/group.

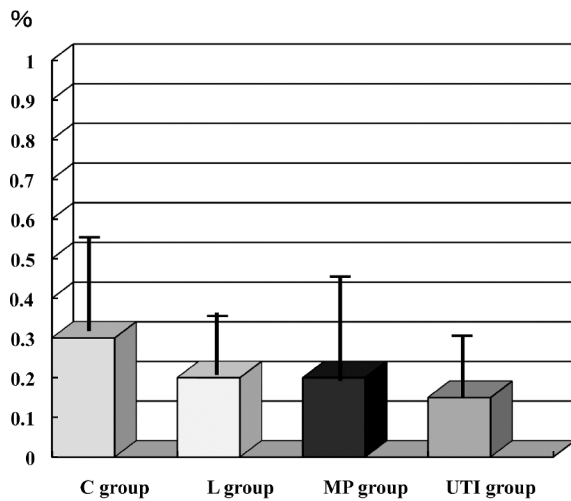


Fig. 3. RI activity in the blood at 5 hours after operation. There was no significant difference among the groups. Data was presented as mean \pm SD. n=9 rats/group.

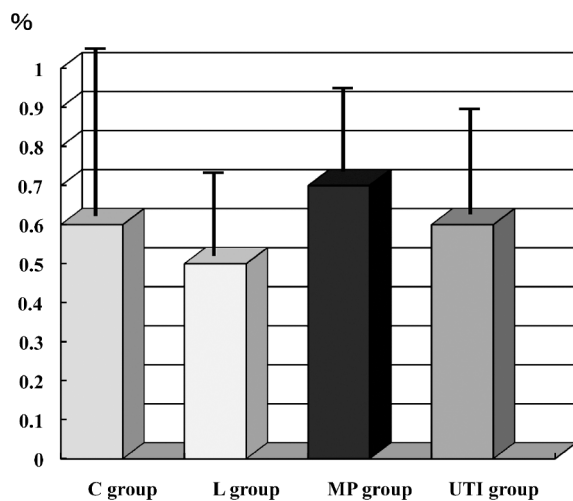


Fig. 4. RI activity in the thyroid at 5 hours after operation. There was no significant difference among the groups. Data was presented as mean \pm SD. n=9 rats/group.

was observed between the MP and UTI groups. Similar results were observed in serum.

Furthermore, serum IL-6 concentration at 3 hours after surgery was higher in the L group than in the MP or UTI groups (37.2 pg/ml in L group vs. 29.3 pg/ml in MP group and 26.6 pg/ml in UTI group, $p < 0.05$). However, no significant concentration difference in these cytokines was observed between the MP and UTI groups (Fig. 6). Serum IL-2 concentration at 1 hour after surgery was significantly decreased in the L and MP groups compared to that in C group (11.19 ± 0.19 pg/ml in C group vs. 8.55 ± 0.39 pg/ml in L group,

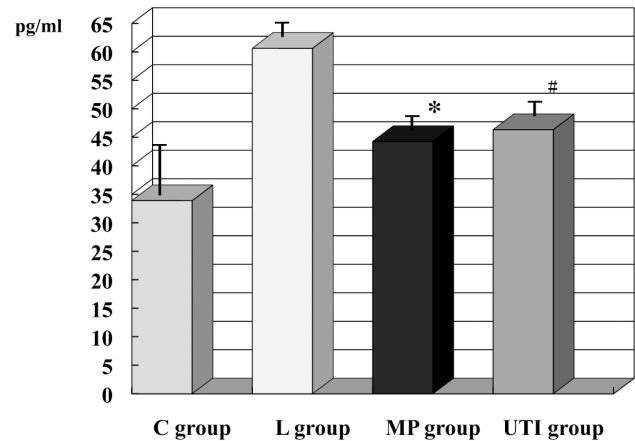


Fig. 5. IL-1 β levels in the liver at 1 hour after operation. Surgical stress increased IL-1 β levels in the liver. MP and UTI treatment reduced postoperative IL-1 β levels in the liver compared to the large incision group. Data was presented as mean \pm SD. One way ANOVA followed by Fisher's PLSD test was used for statistical analysis. *, $p < 0.05$ MP group vs L group, #; $p < 0.05$ UTI group vs L group. n=12 rats/group.

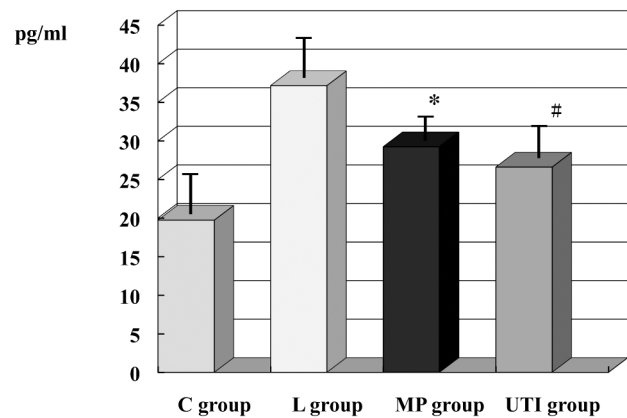


Fig. 6. IL-6 levels in the plasma at 3 hours after operation. Surgical stress increased plasma IL-6 levels. MP and UTI treatment reduced plasma IL-6 concentration compared to the large incision group.

Data was presented as mean \pm SD. One way ANOVA followed by Fisher's PLSD test was used for statistical analysis. *, $p < 0.05$ MP group vs L group, #; $p < 0.05$ UTI group vs L group. n=12 rats/group.

$p < 0.05$; 7.19 ± 0.41 pg/ml in MP group, $p < 0.05$) and significantly increased in the UTI group (10.56 ± 0.35 pg/ml in UTI group, $p < 0.05$) compared to that in L group (Fig. 7).

3. The Effects on Activation Mechanism of NK Cells and Action of MP and UTI

NK cell activity was significantly suppressed in the MP group compared to that in the large-incision only L group ($11.22 \pm 1.0\%$ in L group vs. $6.1 \pm 0.6\%$ in MP group, $p < 0.05$), and significantly activated in the UTI group compared to that in the L group ($11.22 \pm 1.0\%$ in L group vs. $14.1 \pm 0.8\%$ in UTI group,

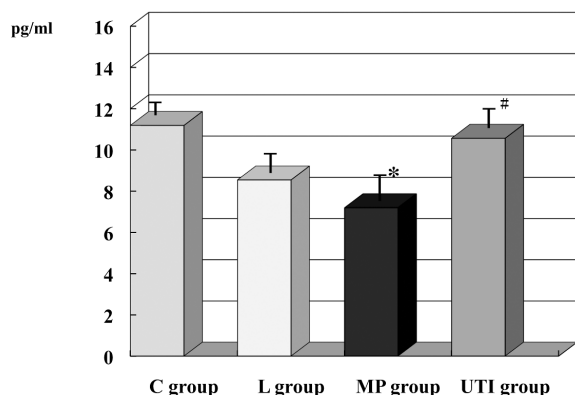


Fig. 7. IL-2 levels in the plasma at 3 hours after operation.

Surgical stress reduced IL-2 levels at 3 hours after surgery. IL-2 levels in the plasma were significantly suppressed by MP treatment compared to UTI. Data was presented as mean \pm SD. One way ANOVA followed by Fisher's PLSD test was used for statistical analysis. *; $p < 0.05$ MP group vs the other groups, #; $p < 0.05$ UTI group vs L group. n=12 rats/group.

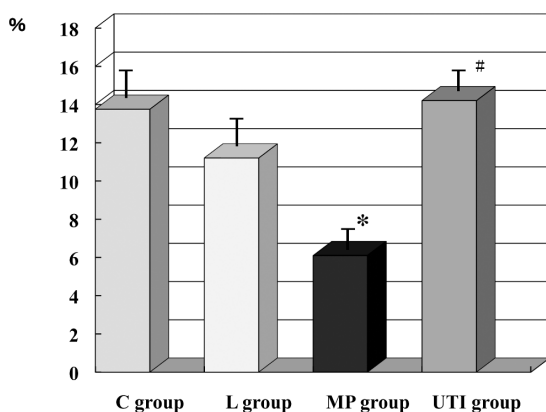


Fig. 8. NK cell activities at 1 hour after operation. NK cell activity was suppressed by MP treatment and preserved by UTI treatment. Data was presented as mean \pm SD. One way ANOVA followed by Fisher's PLSD test was used for statistical analysis. *; $p < 0.05$ MP group vs the other groups, #; $p < 0.05$ UTI group vs L group. n=10 rats/group.

$p < 0.05$) (Fig. 8).

4. The Effects of Operation Invasion [Operative Stress], and Action of MP and UTI on Adhesion Molecules

E-selectin concentration in liver at 3 hours after surgery assessed by Western blot analysis was higher in the L group than in the C group, and was significantly decreased in the UTI group compared to the L group (0.941 ± 0.089 in L group vs. 0.629 ± 0.072 in UTI group, $p < 0.05$). No significant difference in E-selectin level was observed between the MP and UTI groups (0.822 ± 0.083 in MP group vs. 0.629 ± 0.072 in UTI group, $p < 0.05$) (Fig. 9).

5. Effects of MP and UTI on the Number of Liver Metastases

The number of neoplastic nodules on liver surface in each group at 3 weeks after surgery was increased in the MP group compared to that in large-incision only L group (1.71 ± 0.28 nodules in L group vs. 5.28 ± 0.77 in MP group, $p < 0.05$), and decreased in UTI group compared to that in L group (Fig. 10).

6. Histopathological Investigation

The neoplastic nodules on the liver surface at 3 weeks after surgery were fixed in 10% formalin, and stained with hematoxylin-eosin. The histopathologi-

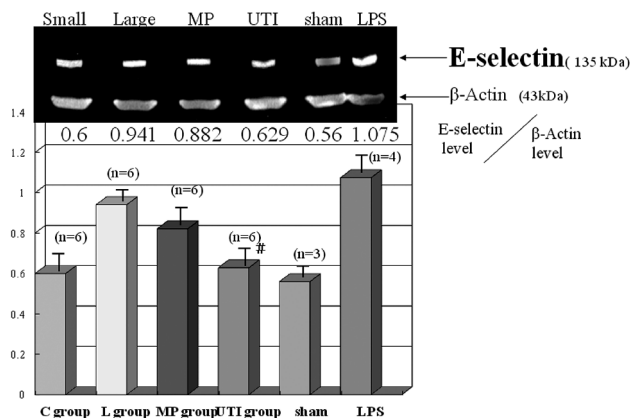


Fig. 9. E-selectin levels in the liver at 3 hours after surgery.

E-selectin levels in the liver were evaluated using western blotting. Data was normalized with β -actin levels in the liver. Preoperative UTI treatment suppressed E-selectin expression in the liver compared to L group. There were no significant difference between MP and UTI. Data was presented as mean \pm SD. One way ANOVA followed by Fisher's PLSD test was used for statistical analysis. #; $p < 0.05$ UTI group vs L group. n=4 rats/group.

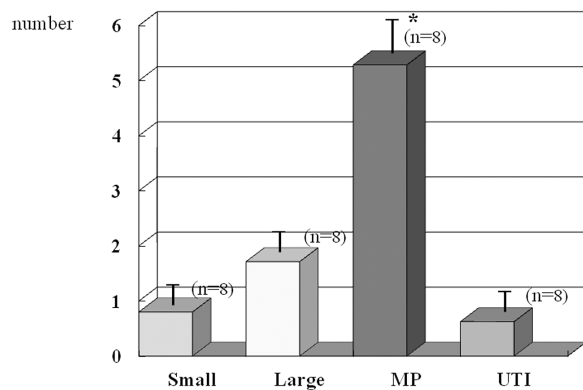


Fig. 10. The number of tumor nodules on the liver surface at 3 weeks after operation.

MP treatment significantly increased post operative tumor nodules on the liver surface compared to the other groups. Data was presented as mean \pm SD. One way ANOVA followed by Fisher's PLSD test was used for statistical analysis. *; $p < 0.05$ MP group vs. the other groups. $n = 8$ rats/group.

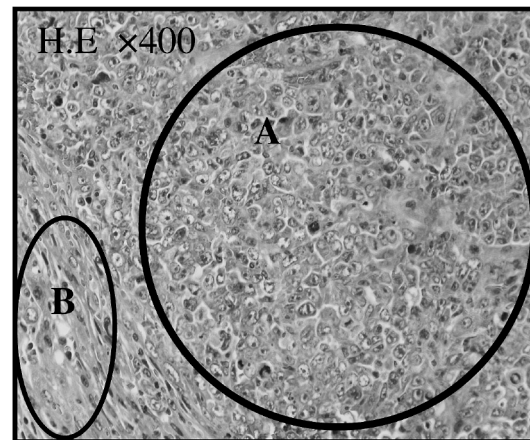


Fig. 12. Histopathological findings of metastatic tumor region ($\times 400$).

The Hematoxylin- Eosin stained metastatic tumor region (A: red circle) showed high NC ratio, irregular spherical nucleus with clear nucleolus, eosinophilic ~ clear cytoplasm, and high mitotic counts. Tumor showed medullary growth pattern, and boundary with surrounding hepatocyte tissue (B: blue circle) was clear.

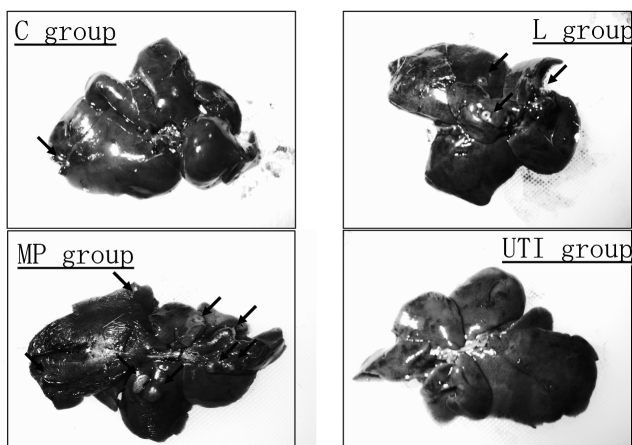


Fig. 11. Macroscopically represented metastatic tumor nodules on the liver surface.

Black arrow shows tumor nodules on the liver surface at 3 weeks after operation. MP group demonstrated a significant increase in tumor nodules on the liver surface compared to the other groups.

cal findings of tumors showed high NC ratio, irregular spherical nucleus with clear nucleolus, eosinophilic-clear cytoplasm, and high mitotic counts. Tumors showed medullary growth pattern, and clear boundary with surrounding hepatocyte tissue (Figs. 11 and 12).

DISCUSSION

IL-1 β and tumor necrosis factor (TNF- α) secreted

by activated macrophages at the initial phase of inflammatory response are known to be an endogenous mediator in a series of inflammatory responses [8]. Invasive reactions are induced mainly with these cytokines as a trigger. Postoperative inflammatory cytokine production showed a significantly higher level of postoperative IL-1 β and IL-6 the major laparotomy group (L group) than in the minimal laparotomy group (C group). In other words, the postoperative inflammatory cytokine production in this model was confirmed to correlate with the magnitude of the surgical invasion.

Hematogenous metastasis of cancer consists of a multistep process, but in this model we focused on the adhesion to the liver vascular endothelial cells and extravasation on account of the infusion of RI-labeled tumor cells into the blood vessel. This model is considered to be useful for investigating hematogenous metastasis, especially the adhesion to the vascular endothelial cells and extravasation, because there was no significant difference in radiation dose in the whole blood, lung, and thyroid gland after the infusion of labeled tumor cells among the 4 groups, and because the accumulation of RI was observed only in the metastasizing liver, and metastasis was observed only in the liver at 3 weeks after the infusion of tumor cells.

However, in this study we could not pathologically confirm adhered cancer cells or extravasated cancer cells. This is because, as Skolnik G. et al. [9] reported,

since tumor cells that were infused into the blood vessel disappeared from within the blood vessels within 5 hours and metastasized to organs such as the liver, by the time we could conduct an observation of the adhesion of tumor cells to organs under electronic microscopy. These processes are thought to be completed in a short period of time, and most cancer cells that had invaded into the blood vessels would break up in the bloodstream, with only 0.1% of them possibly surviving [10].

As an approach to regulate inflammatory cytokine, antibody (anti-TNF antibody, anti-IL-8 antibody), antagonist (recombinant IL-1 receptor antagonist) and intercellular signal transduction interception by antisense DNA are being considered [11]. Currently, corticosteroid and protease inhibitors are being suggested as drugs that are clinically applicable and could modify the reaction specifically at an early stage before the cytokine network is activated.

Steroid hormone is reported to inhibit not only the production of cytokine mRNA [12] but also the production of physiologically active substances such as leukotriene, protease, and prostaglandin [13]. For this reason, preoperative methylprednisolone administration has been recommended before radical esophagus cancer surgery as a means to reduce the invasive response, and a 10 mg/kg dose of this has been found to be clinically effective [4]. The dosage of methylprednisolone for an adult human is 1,000 mg/body, but it had been reported that NK activity, lymphocyte weakening reaction, and immunoglobulin production would be significantly inhibited when the dosage exceeds 10 mg/kg (blood level: 10 µg/ml) [14]. In a mice operation invasive model, the perioperative intraperitoneal dosage of methylprednisolone that inhibited serum IL-6 production significantly was 1 mg/mouse (an adult human: 250 mg-500 mg/body), which indicates that administration at 30 to 60 minutes before surgery would be effective [15]. In this study, preoperative intravenous dosages of 5 mg/kg, 10 mg/kg, 20 mg/kg were administered to rats, and it was found that the 10 mg/kg dosage group had the highest inhibitory effect on IL-6. The serum level in these rats with 10 mg/kg of administration was less than 0.1 µg/ml.

When we measured splenocyte NK cell activity as an index of immune response, we observed a decrease due to the methylprednisolone administration as compared to the major laparotomy-only group as well as a decrease in IL-2 productivity. Deguchi et al. [16] conducted an experiment on preoperative dexamethasone administration using rats regarding the postoperative metastasis of malignant tumor cells after the adminis-

tration of steroid hormone and reported that exogenously administered steroid hormone and endogenously secreted steroid hormone after surgery reduced the immune response in the host and promoted liver metastasis, and that administration of metyrapone, a glucocorticoid antagonist, inhibited metastatic colony formation. We also administered metyrapone using this invasive model and obtained similar results of inhibited metastasis (unlisted).

Recently, there has been a report of an *in vivo* experiment showing that glucocorticoid promoted metastasis under mild invasion and inhibited it under severe invasion [17]. Therefore, there is a possibility that this laparotomy-only model, which does not need thoracotomy as in radical esophagus cancer surgery, may not be highly invasive.

It is also reported that ulinastatin, one kind of protease inhibitor, inhibits not only granulocyte elastase and plasmin activity but also cytokine production at the post transcriptional level [18].

The dosage of ulinastatin was set at 50,000 unit/kg based on the experimental report that the dosage needed for a rat to inhibit the cytokine production corresponds to that of 5,000 unit/kg in a human [7]. According to Takeuchi et al. [19], ulinastatin administration was found to inhibit production of IL-6 and enhance the immunocompetence activating effect after hepatectomy. Moreover, Kobayashi et al. [20] reported regarding the influence of ulinastatin on the metastasis of malignant tumor cells that although the intravasation of tumor cells was inhibited by plasmin inhibitory effect, adhesion of tumor cells was not affected *in vitro*.

Since we obtained a result in this *in vitro* experiment that preoperative single administration of ulinastatin could inhibit the adhesion of tumor cells to the liver, we examined E-selectin, one of the adhesion molecules that has an important role in hematogenous metastasis to the liver.

E-selectin is a cell adhesion protein that transiently appears on the surface of vascular endothelial cells activated by inflammatory cytokine such as IL-18, and it is reported that the adhesion mechanism between this protein and carbohydrate antigen of cancer cells is involved especially in the hematogenous metastasis of colorectal cancer [10]. As for the serial Lea and serial Lex, E-selectin ligand sugar chain of cancer cells, a significant relationship is shown between the serum level and hematogenous metastasis [21,22].

Our results showed that preoperative administration of ulinastatin decreased the postoperative E-selectin level as compared to the major laparotomy group and inhibited hematogenous metastasis to the liver.

This result is considered due to both the adhesion molecule reducing effect of vascular endothelial cells caused by the inhibition of cytokine production and enhancement of anti-tumor immunocompetence by the above-mentioned immunocompetence activating effect. It is considered important that even cytokine production, which enhances immunocompetence by increasing IL-2, was inhibited by steroid. Despite the fact that no difference in the expression of IL-1 β and IL-6 between MP group and UTI group was observed, the E-selectin level in the MP group was significantly higher than that in the C group. There have been many reports regarding the E-selectin expression inhibitory effect of glucocorticoid, and the inhibition of activation of NF-kB is considered to be its major action. However, NF-ELAM-1 also exists as a gene promoter of E-selectin, and has an important role similar to NF-kB. In recent years, it had been reported that glucocorticoid could inhibit NF-kB but has no influence on activation of NF-ELAM-1 [23], which has led to the assumption that the activation of MAP Kinase, which transmits signals to ATC/c-Jun, was overexpressed in MP group by a certain mechanism.

On the other hand, regarding the mechanism responsible for increased adhesion of tumor cells to the liver and hematogenous metastasis despite the inhibition of cytokine production by preoperative administration of methylprednisolone, the decrease in immunocompetence due to steroid hormone could be one of the reasons for the increase in hematogenous metastasis to the liver, as Deguchi et al. [16] has reported.

From our findings, preoperative administration of ulinastatin can be considered a useful approach to inhibit the invasive reaction caused by excessive cytokine while inhibiting hematogenous metastasis.

In addition, regarding preoperative administration of methylprednisolone, the dosage should be set so as to not reduce the immunocompetence of the host.

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