The Neutrophil Elastase Inhibitor Sivelestat Suppresses Accelerated Gastrointestinal Tumor Growth *via* Peritonitis After Cecal Ligation and Puncture

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Abstract. *Background: We examined whether tumor growth is enhanced by cecal ligation and puncture (CLP) and suppressed by a neutrophil elastase inhibitor, sivelestat. Materials and Methods: C57BL/6 mice were divided in CLP/sivelestat, CLP alone, and control (simple laparotomy) groups. Murine CT26 colon carcinoma cells were injected subcutaneously into the back of each mouse and tumor growth and serum cytokine levels were assessed. Results: Mice subjected to CLP alone exhibited enhanced tumor growth compared to controls with subcutaneously injected CT26 cells (0.64±0.24 g vs. 0.021±0.027 g, p<0.001), while treatment with CLP/sivelestat produced smaller tumors than CLP alone (0.28±0.23 g vs. 0.64±0.24 g, p=0.006). Cytokine assays showed suppressed production of interleukin (IL)-6 and IL-10 in the CLP/sivelestat group, and increased IL-6 and IL-10 in the CLP-alone group. Conclusion: Intra-abdominal inflammation induced by CLP enhances the growth of subcutaneously implanted tumors, while perioperative administration of sivelestat suppresses tumor growth by affecting systemic inflammation.*

It has been suggested that postoperative complications might increase the risk of tumor recurrence after various kinds of cancer surgery, and previous studies in gastrointestinal surgery have focused on the relationship between anastomotic leakage and tumor recurrence. For instance, Docherty *et al*. (1) identified the presence of an anastomotic leak as being a highly significant and independent prognostic factor for both recurrence and cancer-specific mortality after colorectal cancer surgery, while Fujita *et al*. (2) reported that anastomotic leakage after colorectal cancer surgery increased the incidence of local tumor recurrence and thus worsened prognosis. In addition, Siezega *et al*. (3) identified the occurrence of anastomotic leakage as being a major independent prognostic factor for long-term survival after total gastrectomy for gastric cancer. However, these factors were not confirmed in other clinical studies (4, 5).

Cecal ligation and puncture (CLP) is a commonly used experimental animal model of sepsis (6, 7) that closely resembles acute peritonitis occurring with bowel perforation, followed by tissue necrosis leading to microbial infection. CLP is a particularly useful model, as the severity of sepsis is easily modulated by varying the puncture size (8). We previously established this model in rodents and reported several results from experiments on inflammation (9-11).

Sivelestat sodium hydrate, which chemically is sodium N-{2-[4-(2,2-dimethylpropionyloxy) phenylsulfonylamino] benzoyl} aminoacetate tetrahydrate [Elaspol, ONO-5046-Na ($C_{20}H_{21}N_2NaO_7S$-Na.$4H_2O$, molecular weight of 528.51); Ono Pharmaceutical Co., Osaka, Japan], is a synthetic, specific, low-molecular-weight inhibitor of neutrophil elastase. The efficacy of sivelestat on acute lung injury (ALI) has been demonstrated in several investigations and the use of this drug in humans has been approved for ALI associated with systemic inflammatory response syndrome (SIRS). Furthermore, some authors have reported that sivelestat has anticancer properties (12-14).

We hypothesized that proliferation of cancer cells can be stimulated by systemic inflammation and controlled by suppressing inflammation. Accordingly, this study sought to

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determine whether systemic inflammation caused by CLP enhances tumor growth of CT26 colon cancer cells implanted in mice subcutaneously, and whether sivelestat (Elaspol) suppresses this acceleration of tumor growth.

Materials and Methods

Animals. Eight- to ten-week-old female C57BL/6 mice were purchased from Japan SLC, (Shizuoka, Japan). All animal procedures were conducted in accordance with institutional and national guidelines (Research approval number: 081032).

Cecal ligation and puncture. To establish live intra-abdominal infection, we subjected the mice to a CLP procedure as previously described (6, 7, 9-11). The animals were anesthetized by inhalation of diethyl ether in an induction chamber and maintained by delivery through a face mask. After shaving and cleaning the ventral abdominal wall with alcohol, a 20-mm transverse incision was made to expose the cecum, which was then mobilized and ligated with 3-0 silk at 10-mm from the tip. A 26-gauge needle was used to perforate the center of the ligated portion of the cecum once in a through-and-through manner. The cecum was then replaced in its original position and the wound was closed with 4-0 vicryl interrupted sutures. All animals received 0.6 ml of saline solution (0.9% subcutaneously) resuscitation immediately after the surgery.

Cell lines. *N*-Nitroso-*N*-methylurethane induced undifferentiated murine colon carcinoma cell line, CT26 cells, was purchased from the American Tissue Culture Collection (Manassas, VA, USA). Cells were maintained and cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum. Cells were collected and prepared as single-cell suspensions in phosphate-buffered saline (PBS).

Tumor growth assay. Initially, mice were selected randomly to undergo CLP (n=5) or simple laparotomy (n=5) in order to evaluate the influence of CLP on tumor growth. CT26 cells (1×10^4) suspended in 0.1 ml PBS were injected subcutaneously into the dorsum of mice four hours after CLP or simple laparotomy. The length (*a*) and perpendicular diameter (*b*) of each tumor was measured 7, 14, 21, and 28 days after implantation. The tumor volume (V) was calculated as follows: $V=(a\times b^2)/2$. Mice were sacrificed 28 days after implantation and the tumors were weighed (Figure 1 A).

In the second experiment, mice were randomly divided into three groups: CLP (n=8), CLP/sivelestat (n=8) and simple laparotomy (n=8). In the group of CLP with sivelestat, sivelestat (30 mg/kg/day) was injected subcutaneously for 14 consecutive days from the day before CLP to evaluate the inhibitory effect of sivelestat on tumor growth. CT26 cells (1×10^4) suspended in 0.1 ml PBS were injected subcutaneously into the dorsum of mice 24 hours after CLP. Mice were sacrificed 28 days after injection and the tumors were weighed (Figure 1 B).

Cytokine and growth factor levels. Blood was harvested from surviving mice following CLP (n=10, 9, 7 and 8 mice), CLP with sivelestat (n=10, 9, 7 and 9 mice), or simple laparotomy (n=10, 10, 10, and 10 mice) at 4, 8, 24, and 96 h after surgery, respectively. To examine the cytokines and growth factors

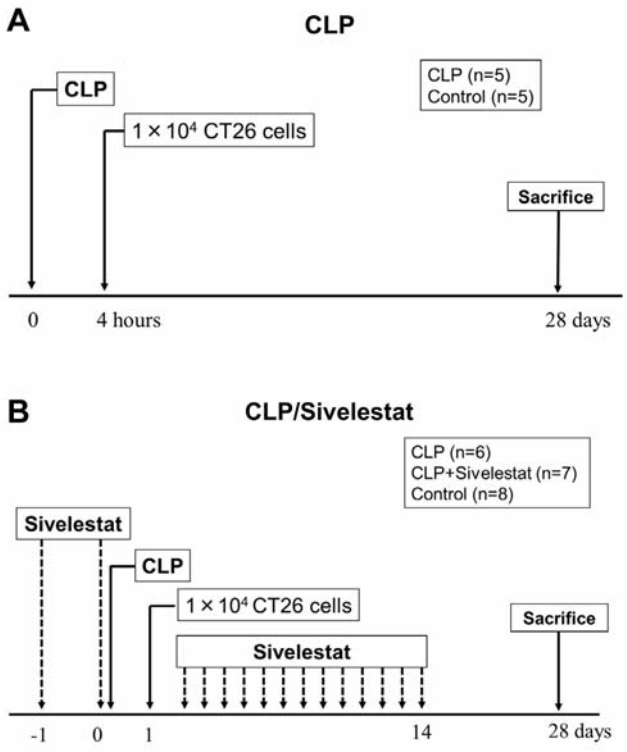

Figure 1. *A: Protocol for the assay to evaluate the influence on tumor growth of cecal ligation and puncture (CLP). CT26 cells (1×10^4) suspended in 0.1 ml phosphate buffered saline (PBS) were injected subcutaneously into the dorsum of mice four hours after CLP or simple laparotomy. The tumor volume was measured 7, 14, 21, and 28 days after implantation, and the tumors were weighed 28 days after implantation. B: Protocol for the assay to evaluate the influence on tumor growth of CLP and sivelestat. In the group treated with CLP with sivelestat, sivelestat (30 mg/kg/day) was injected subcutaneously for 14 consecutive days from the day before CLP to evaluate the inhibitory effect of sivelestat on tumor growth. CT26 cells (1×10^4) suspended in 0.1 ml PBS were injected subcutaneously into the dorsum of mice 24 hours after CLP or simple laparotomy. The tumor volume was measured 7, 14, 21, and 28 days after implantation, and the tumors were weighed 28 days after implantation.*

associated with tumor growth, nucleated cells were isolated from the peripheral blood by gradient centrifugation. Plasma was also collected and stored at –80˚C until use. The concentrations of interleukin (IL)-1α, IL-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α, basic fibroblast growth factor (bFGF) , and vascular endothelial growth factor (VEGF) in serum were simultaneously measured using a multiplex suspension bead array immunoassay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instruction (15).

Statistical analysis. All data are presented as the mean±standard deviation. A general linear model was used to analyze repeated measurements in the experiments of tumor growth over time. Student's *t*-test was used for comparison of tumor weights of two

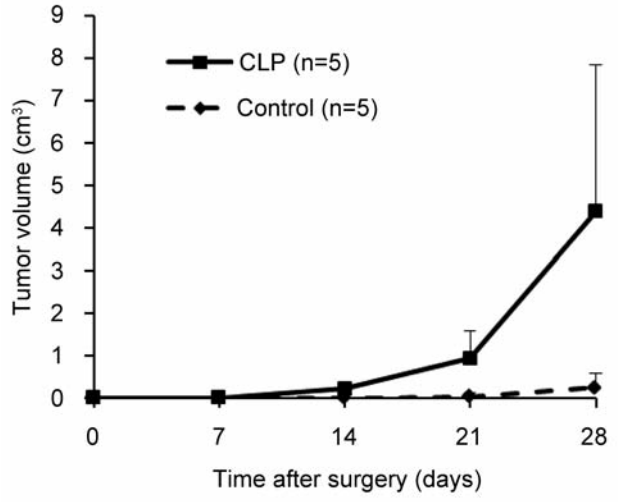

Figure 2. *Time course of the implanted tumor volume in the cecal ligation and puncture (CLP) group and simple laparotomy group. Growth of the subcutaneously injected cancer cells in mice was significantly faster after CLP compared with simple laparotomy (p=0.021). Data are the mean±SD.*

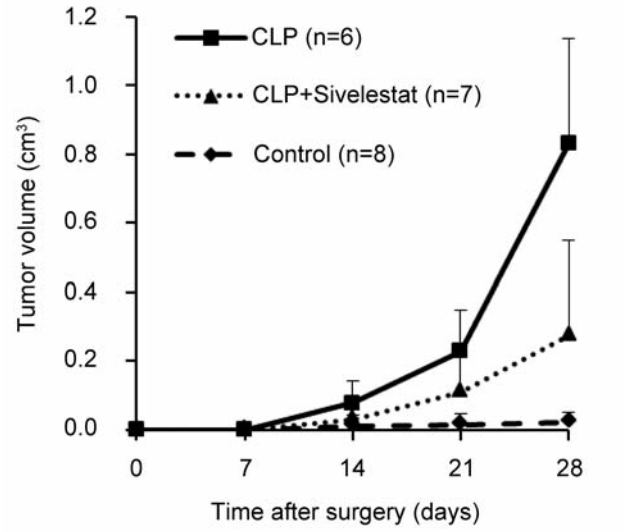

Figure 3. *Time course of the implanted tumor volume in mice in groups treated with cecal ligation and puncture (CLP) with sivelestat, CLP without sivelestat, and simple laparotomy. Growth of the subcutaneously injected cancer cells was significantly faster in the mice after CLP compared with those treated with simple laparotomy (p=0.001). Tumor volume in the mice treated with CLP and sivelestat was also significantly lower than that in the mice treated with CLP and daily saline injection (p=0.001). Data are the mean±SD.*

groups; analysis of variance (ANOVA) was used to compare the three groups in experiments for tumor weights and cytokine levels. The Bonferroni correction factor was applied to adjust for multiple comparisons. The value of $p<0.05$ was considered statistically significant. Statistical analyses were performed using SPSS version 11.0 (SPSS, Chicago, IL, USA).

Results

Acceleration of tumor growth by CLP. Each CLP was completed within 10 min. Growth of the subcutaneously injected cancer cells was significantly faster in mice subjected to CLP alone compared with control mice that underwent simple laparotomy (Figure 2). The tumor weight was more than 8 g at 28 days after implantation in two out of five mice, with mean tumor weight approximately 17-times greater in the CLP-treated group than in controls (5.3±3.4 g *vs*. 0.32±0.43 g, $p=0.029$).

Suppression of accelerated tumor growth by sivelestat. Animals that died of adverse events during surgery (for example, deep anesthesia) were excluded from the study (two in the CLP group and one in the CLP/sivelestat group). It was confirmed that tumor growth was accelerated by CLP (Figure 3). In addition, mice that received CLP as well as daily sivelestat injections (CLP/sivelestat group) had significantly smaller tumors than mice that received CLP and a daily saline injection (CLP alone) (Figure 3). Mean tumor weights 28 days after implantation were approximately 30-times greater by CLP than from simple laparotomy (0.64±0.24 g *vs*. 0.021±0.027 g, $p<0.001$), with significantly lower mean weights compared to those in the previous experiment. Mean tumor masses in mice treated with CLP and sivelestat were less than half those in the group treated with CLP alone at 28 days after implantation (0.28±0.23 g *vs*. 0.64±0.24 g, $p=0.006$).

Profiles of pro-inflammatory and anti-inflammatory cytokines and growth factors in each group. Blood was harvested from mice and sera were assayed for selected cytokines and growth factors. Pro-inflammatory mediators (IL-1α, IL-1β, IL-6 and TNF-α) and growth factors (bFGF and VEGF) were measured, as well as the anti-inflammatory cytokine IL-10. While levels of most cytokines followed similar time course trends across the groups, IL-6 and IL-10 exhibited completely different concentration profiles (Figure 4). In mice treated with CLP alone, IL-6 reached a maximum level four hours after CLP and decreased thereafter, whereas there was no increase in concentration of IL-6 in mice that underwent a simple laparotomy, with a significant difference ($p<0.001$) by multiple comparison analysis (Figure 4D). Furthermore, the mice of the CLP/sivelestat group displayed statistically significantly lower IL-6 concentrations compared to the group treated with CLP alone (Figure 4 D, $p=0.042$). In contrast, mice in the latter group exhibited a peak of IL-10

concentration at eight hours after CLP with a decrease thereafter, compared to no increase in mice which underwent simple laparotomy, with a significant difference (p=0.006) by multiple comparison analysis (Figure 4 G). The trend for decreasing IL-10 concentration was also observed in the CLP/sivelestat group compared to mice treated with CLP alone (Figure 4 G, p=0.634). Interestingly, no increase in IL-1α, IL-1β, TNFα, bFGF, or VEGF was observed in mice subjected to CLP, regardless of sivelestat treatment (Figure 4 A-C, E, F).

Discussion

The present study yielded two major findings. Firstly, systemic inflammation induced by CLP stimulated the growth of tumors from CT26 cells implanted subcutaneously, and secondly, sivelestat suppressed tumor growth induced by CLP *in vivo*.

The association between systemic inflammation caused by postoperative complications and cancer recurrence has yet to be clarified in gastrointestinal cancer surgery. Indeed, most of the relevant clinical studies have focused on the relationship between anastomotic leakage and local recurrence, and did not address the association between inflammation and recurrence in distant organs. Such a relationship has been more clearly escribed in breast cancer surgery. Murthy *et al*. (16) found that patients with wound complications at primary surgery had increased rates of systemic recurrence of breast cancer. Likewise, Yan *et al*. (17) reported postoperative fever as being an independent prognostic factor for relapse-free survival in patients with breast cancer as well as hormone receptor-related subgroups.

CLP is a model that appropriately represents complications after gastrointestinal surgery, such as anastomotic leakage or intra-abdominal abscess. In this study, CLP significantly enhanced CT26 tumor growth compared to the control laparotomy procedure, suggesting that the systemic inflammation induced by CLP enhanced tumor proliferation in a distant organ. This finding is of significance for this model in relation to the association between systemic inflammation and distant metastasis after cancer surgery. Notably, there was a significant difference in the tumor weights and volume between the two experiments (Figures 2 and 3), most probably due to differences in the respective protocols. In the first experiment (Figures 1A and 2), we implanted the CT26 cells four hours after CLP, while this was done 24 h after CLP in the second experiment (Figures 1B and 3) because of the technical difficulty in preparing CT26 cells within four hours of CLP. In addition, the high level of IL-6 and/or IL-10 present at four hours after CLP might have further accelerated tumor growth (Figure 4D and G).

The significant difference shown in the concentrations of IL-6 between mice subjected to simple laparotomy and those subjected to CLP without sivelestat, and between mice treated by CLP with and without sivelestat could reflect the tumor-promoting actions of IL-6. Thus, an IL-6-neutralizing antibody could potentially inhibit these actions, as previously used in pre-clinical and clinical studies, especially in ovarian cancer (18).

There are three possible mechanisms to explain the tumor-suppressive effect observed in the present study with respect to the kinetics of the IL-6 concentration. The first is an inhibition of neutrophil elastase, which is the original site of action of sivelestat, with studies reporting both a pro-cancer, proliferative effect of neutrophil elastase and a reciprocal anticancer effect of sivelestat (12-14, 19-23). In addition, Houghton *et al*. reported that neutrophil elastase directly induced tumor cell proliferation in both human and mouse lung adenocarcinoma cells, specifically by degrading insulin receptor substrate-1, a key regulator of phosphatidylinositol 3-kinase (23). Wada *et al*. also reported that sivelestat suppresses the growth of gastric cancer cells by inhibiting the release of transforming growth factor-α, which is stimulated by neutrophil elastase (12). It is also possible that sivelestat suppressed tumor proliferation *via* modulation of IL-6 levels. The second suggested mechanism is based on the modulatory effect of sivelestat on the immune response. It has been suggested that the immune response in sepsis represents the interplay of two contrasting phenomena, with early SIRS characterized by excessive production of pro-inflammatory mediators (hyper-inflammatory status) progressively suppressed by the developing compensatory anti-inflammatory response (hypo-inflammatory status) syndrome (CARS) (24-27). In this study, IL-6 reached its peak in mice subjected to CLP and normal saline injection four hours after CLP, while IL-10 reached its peak eight hours after CLP, which may represent SIRS and subsequent CARS, respectively. CARS has been implicated in accelerated tumor growth through the modulation of both cellular and humoral immunity (28-32). In this study, CLP with sivelestat treatment induced a significant decrease in IL-6 concentration and a downward trend in IL-10 concentration following CLP compared to CLP alone, possibly reflecting an immunomodulatory effect of sivelestat that ameliorated both SIRS and subsequent CARS caused by CLP. The third mechanistic possibility is a direct cytotoxic effect of sivelestat. In our preliminary study, sivelestat was cytotoxic towards CT26 cells at 1 mg/ml *in vitro* (data not shown), and although sivelestat was injected subcutaneously at least 5 cm away from the tumor site in the present study, the possibility of sivelestat directly exerting such an effect *in vivo* cannot be completely excluded.

Several studies have suggested that sivelestat may suppress the proliferation or metastasis of cancer by various mechanisms independently of direct neutrophil elastase inhibition (13, 19-22, 33). During surgical treatment of

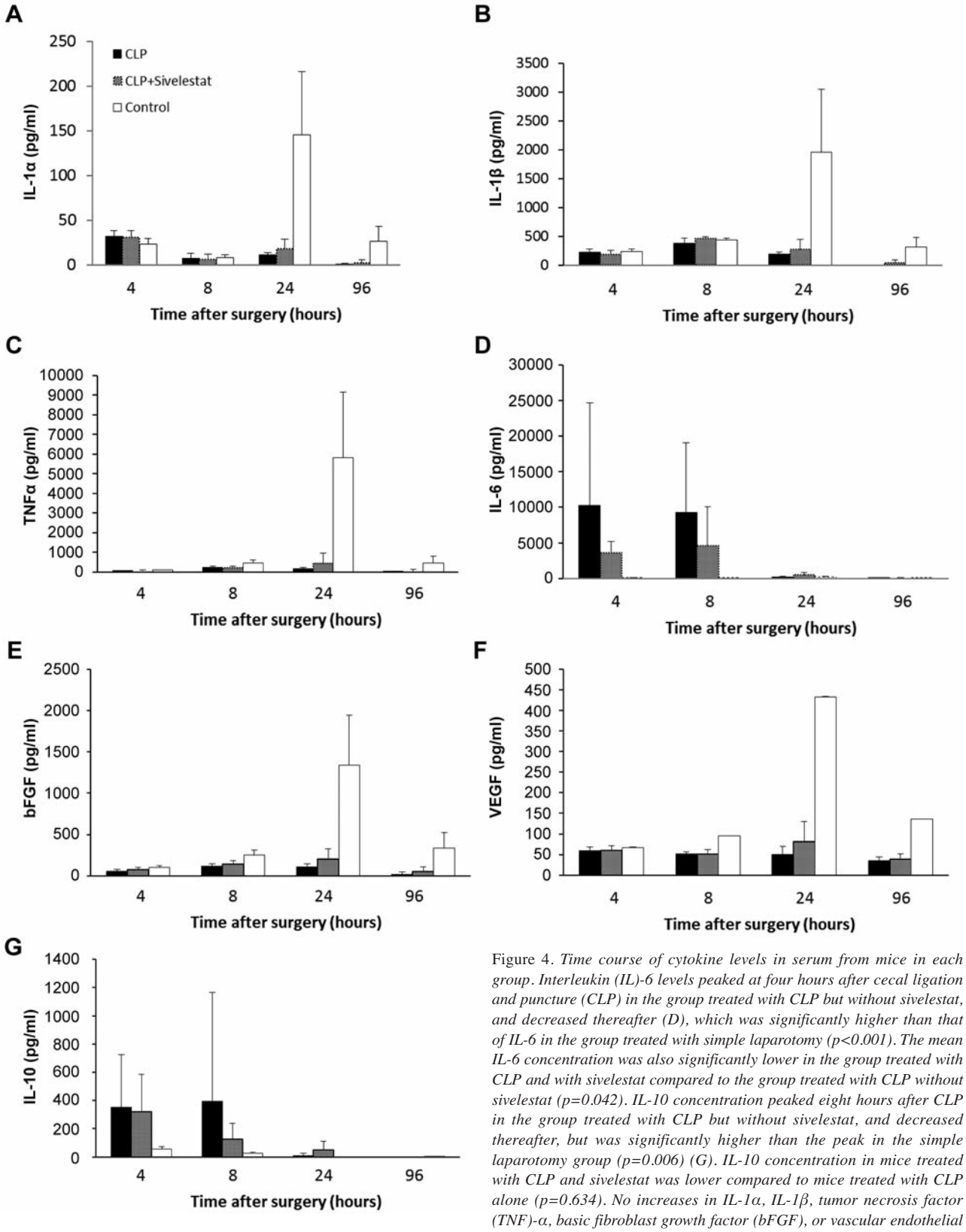

Figure 4. *Time course of cytokine levels in serum from mice in each group. Interleukin (IL)-6 levels peaked at four hours after cecal ligation and puncture (CLP) in the group treated with CLP but without sivelestat, and decreased thereafter (D), which was significantly higher than that of IL-6 in the group treated with simple laparotomy ($p<0.001$). The mean IL-6 concentration was also significantly lower in the group treated with CLP and with sivelestat compared to the group treated with CLP without sivelestat ($p=0.042$). IL-10 concentration peaked eight hours after CLP in the group treated with CLP but without sivelestat, and decreased thereafter, but was significantly higher than the peak in the simple laparotomy group ($p=0.006$) (G). IL-10 concentration in mice treated with CLP and sivelestat was lower compared to mice treated with CLP alone ($p=0.634$). No increases in IL-1α, IL-1β, tumor necrosis factor (TNF)-α, basic fibroblast growth factor (bFGF), or vascular endothelial growth factor (VEGF) were observed in mice subjected to CLP, regardless of sivelestat treatment (A-C, E, F). Data are the mean±SD.*

cancer, invasiveness is inevitable and postoperative complications accompanied by an inflammatory response can occur. These inflammatory responses can promote tumor proliferation and thus worsen the patient's prognosis. Further investigations may elucidate the mechanism by which sivelestat suppresses tumor growth and thus potential applications for cancer patients in clinical practice.

The results from this study have confirmed that intra-abdominal inflammation caused by CLP enhances the growth of subcutaneously-implanted tumor cells. Furthermore, the perioperative administration of sivelestat is a possible intervention to suppress tumor growth by affecting the systemic inflammatory state.

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