The Effect of Surgical Wound on Ovarian Carcinoma Growth in an Animal Model

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Abstract. *Background/Aims: We examined the effects according to the extent of surgical wound mimicking laparoscopy or laparotomy on ovarian cancer growth in an orthotopic mouse model. Materials and Methods: To mimic surgery effects, we performed laparoscopy or laparotomy on athymic nude mice under isoflurane inhalation at four days after tumor cell injection. For the laparoscopy model, we performed pneumoperitoneum with CO_2, by inserting three cannulars. Results: Mice in the laparoscopy-mimicking group had significantly lower tumor weight compared to mice in the laparotomy group ($p<0.05$). In the immediate postoperative period, serum levels of vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP2) were significantly lower in the laparoscopy group. Conclusion: These results indicate that a minimal surgical wound such as that on laparoscopy, appears to induce little surgical stress on enhancing tumor growth compared to laparotomy in an ovarian cancer animal model, possibly because it minimally influences the secretion of VEGF and MMP2.*

Surgery is the mainstay of treatment for human cancer, including ovarian carcinoma. However, the stress of surgery itself is suggested to facilitate the post-surgery growth of pre-existing micrometastases, as well as small residual tumors (1-4). Laparoscopic surgery, accepted as a minimally invasive procedure, has recently been adapted to treat various types of cancers, and a few clinical studies have shown the oncologic feasibility of laparoscopic surgery (5-7). However, most animal studies have found that laparoscopic procedures are associated with significantly reduced increases in tumor growth and metastasis compared to open surgery (1-3, 8-11). Several animal and clinical studies have shown differences between laparoscopic and open surgery in the induction of adhesion molecules, immune suppression, inflammatory response, cytokines, and growth factors (2, 3, 7, 9-12). Although the mechanisms of surgical stress on promoting tumor growth have not been fully elucidated, potential mechanisms focusing on tumor cells because of physical manipulation by surgeons (13, 14), a drop in the level of antiangiogenic factors (15), local and systemic release of growth factors or cytokines (16), and suppression of cell-mediated immunity (17).

The standard treatment for epithelial ovarian cancer is maximal cytoreductive surgery of the primary and metastatic tumor and adjuvant taxane-carboplatin combination chemotherapy, which is the most effective treatment to date (18). The use of minimally invasive surgical techniques, such as laparoscopy, continues to expand because such methods offer reduced intraoperative and postoperative complications, less intraoperative blood loss, and a shorter postoperative recovery (19). Remaining concerns about the use of laparoscopy for surgical staging of ovarian cancer include the adequacy of abdominal exploration and staging compared to conventional laparotomy and the risks and implications of intraoperative tumor rupture and port-site metastases. Nevertheless, surgical staging using laparoscopy might be occasionally acceptable, especially in early-stage ovarian cancer (19, 20).

Recently, Lee *et al*. suggested that increased angiogenic processes mediated by surgical stress promoted ovarian cancer growth in an orthotopic mouse model. Increased tumor

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Key Words: Surgical stress, ovarian carcinoma, laparoscopy, mouse model.

growth was associated with increased angiogenesis and was completely stopped by β-adrenergic receptor blockade. Based on these findings, they suggested that perioperative use of a β-blocker could have preventive effects on surgical stress-induced tumor growth in patients with ovarian cancer (4). However, the influence on ovarian cancer growth of different levels of surgical stress from laparoscopy compared to laparotomy, and the underlying mechanisms are still unclear.

In this study, we examined the influence of the surgical wound by laparoscopy and laparotomy-mimicking procedures on ovarian cancer growth and determined underlying mechanisms responsible for the increased growth. Our results indicate that a minimal surgical wound such as the one created by laparoscopy had little effect in ovarian cancer animal models on enhancing tumor growth compared to laparotomy, and this might be because of minimal surgical stress effects on angiogenesis and induction of adhesion molecules.

Materials and Methods

Ovarian cancer cell lines and culture conditions. The human ovarian cancer cell lines HeyA8 and SKOV3ip1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 supplemented with 15% fetal bovine serum and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA, USA). For in vivo injections, cells were trypsinized and centrifuged at 233 ×g for 7 min at 4˚C, washed twice with PBS, and reconstituted in serum-free Hanks balanced saline solution (HBSS) (Gibco, Carlsbad, CA, USA). Only single-cell suspensions with >95% viability, as determined by trypan blue exclusion, were used for in vivo injections. All experiments used cells grown to 60% to 80% confluence, and all cell lines were routinely tested to confirm absence of Mycoplasma.

Animal care and orthotopic implantation of tumor cells. Female BALB/c nude mice and C57BL/6 mice were purchased from Orient Bio (Sungnam, Korea). This study was reviewed and approved by the Institutional Animal Care and Use Committee of the Samsung Biomedical Research Institute (SBRI) (IRB # 2009-09-014). SBRI is an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International, protocol No. H-A9-003)-accredited facility and abides by the Institute of Laboratory Animal Resources guide. To generate tumors, HeyA8 (2.5×10^5 cells/0.2 ml HBSS) or SKOV3ip1 (1.0×10^6 cells/0.2 ml HBSS) were injected intra-peritoneally into the peritoneal cavity of BALB/c nude mice (4, 21) that were eight to 12 weeks old. Mice (n=10 per group) were monitored daily for tumor development and postoperative complications and were sacrificed on days 25 to 30 (HeyA8), or days 35 to 40 (SKOV3ip1), or if they seemed moribund. Total body weight, tumor incidence and mass, and the number of tumor nodules were recorded. Tumors were fixed in formalin and embedded in paraffin or snap frozen in OCT compound (Sakura Finetek USA, Inc.) in liquid nitrogen.

Generation of an orthotopic in vivo model of laparoscopy and laparotomy for surgical stress. To mimic the effects of surgery, we performed a laparotomy or laparoscopy-mimicking procedures on mice, four days after tumor cell injection. For surgery, animals were exposed to experimental laparoscopy or laparotomy under isoflurane inhalation (Baxter, Deerfield, IL, USA) anesthesia. The surgical procedure for laparotomy was a 2-3 cm midline abdominal incision followed by externalization of intestines for four minutes as described previously (4, 22). During laparotomy, the small intestine was rubbed with two saline-soaked cotton Q-tips in four locations to simulate a surgical procedure. The intestine was then returned to the abdominal cavity and irrigated with saline, and the abdominal wall was closed with surgical clips (4, 22). For the laparoscopy-mimicking procedure, a 22-gauge cannular was inserted in the center of the abdomen and used as an insufflation needle for CO_2 pneumoperitoneum with 1-2 mmHg intra-abdominal pressure; an additional two cannulars were inserted in the lower quadrant of the abdomen (10). To mimic laparoscopic surgery, gentle stimulation of the bowel with cannulars under CO_2 pneumoperitoneum was performed for four minutes (Figure 1). To determine effects according to the extent of surgical stress in the immediate postoperative period, we performed a separate experiment using an SKOV3ip1 model. We harvested tumor tissues and serum from mice at six and 24 hours, and three and six days after surgery (n=5).

Immunohistochemistry for cluster of differentiation (CD)31 and proliferating cell nuclear antigen (PCNA). To quantify angiogenesis, microvessel density (MVD) was ascertained by counting CD31-positive vessels as described previously (4). In brief, 8-μm sections of fresh-frozen tumor samples were fixed and incubated with anti-mouse CD31 (1:800; PharMingen, BD Biosciences, San Diego, CA, USA) at 4˚C overnight. Immunohistochemical procedures for PCNA were as described previously (23).

Microscopic quantitative analyses of MVD and PCNA. To quantify MVD, 10 random fields at ×100 magnification per slide were examined for each tumor (one slide per mouse, five slides per treatment group) and the number of microvessels per field was counted by two investigators (J-W. L. and J-J. C.) blinded to the samples. A single microvessel was defined as a discrete cluster or single cell that stained positively for CD31 with the presence of a lumen (24). To quantify PCNA expression, the number of PCNA-positive cells and the total number of tumor cells were counted in five random fields at ×100 magnification to calculate the percentage of positive cells.

Assessment of tumor/serum vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)2 and MMP9 levels. The samples of mice blood were centrifuged for 10 min at 2000 ×*g*, and serum collected when mice were sacrificed. Tumor samples were placed in 200 μL of protein lysis buffer and homogenized. The lysate was centrifuged at 3350 ×*g* for 15 min at 4˚C. We quantified concentrations of VEGF, MMP2 (R&D Systems, Minneapolis, MN, USA) and MMP9 (Abnova, Taoyuan Country, Taiwan) in serum and tumor tissue homogenates by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols. Absorbance was measured on an (ELISA) reader at a test wavelength of 540 nm. Samples were measured in triplicates.

Statistical analysis. Continuous variables were compared with a Student's *t*-test or ANOVA if normally distributed, and the Mann-Whitney rank sum test, if distributions were non-parametric, using the GraphPad Prism 4 for Windows version 4.0 software (GraphPad Software, Inc., La Jolla, CA, USA). A *p*-value less than 0.05 was considered statistically significant.

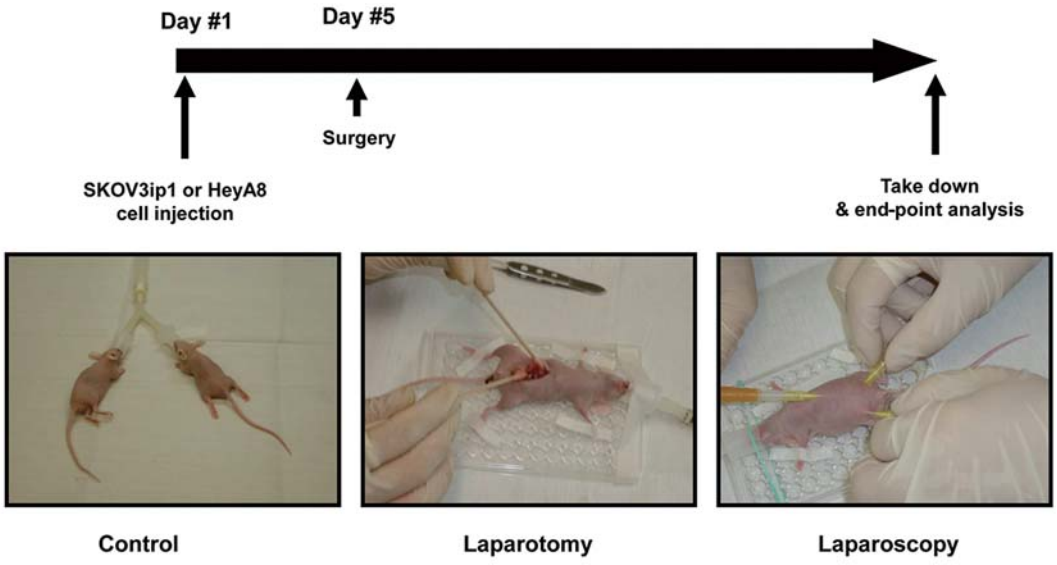

Figure 1. *Representative images for surgical stress including control (anesthesia alone), laparotomy, and laparoscopy-mimicking.*

Results

An increased surgical wound increased tumor growth of ovarian carcinoma. In the SKOV3ip1 and HeyA8 models, laparotomy resulted in significantly increased tumor weight compared to control and the laparoscopy-mimicking group ($p<0.05$ for both models; Figure 2). However, the laparoscopy-mimicking group did not show any difference in tumor weight compared to controls. Similarly, the number of tumor nodules was also significantly greater following a laparotomy than in the control and laparoscopy-mimicking groups ($p<0.05$ for both models; Figure 2) but no difference in the laparoscopy-mimicking and control groups of both models. No significant differences were seen between groups in animal body weight, suggesting that surgery did not adversely affect the overall well-being of the animals.

Effect of surgical wound on angiogenesis, adhesion and cell proliferation. Based on our prior findings on the effects of surgical stress on tumor growth and angiogenesis (4), we considered whether differences in tumor angiogenesis might appear after different types of surgery. The laparotomy group showed significantly increased VEGF levels in tumor tissues compared to the control and laparoscopy-mimicking groups for both models (Figure 3A). However, no difference was seen in VEGF expression between the laparoscopy-mimicking and control groups (Figure 3A). When we examined the MVD in harvested tumors, the laparotomy groups had significantly higher MVD counts ($p<0.05$) compared to the laparoscopy-mimicking and control groups in the SKOV3ip1 model (Figure 4A). However, these findings were not seen in the laparoscopy-mimicking group, when compared to the control group. The surgical effect on induction of adhesion molecules MMP2 and MMP9 was determined, and no significant difference was seen in the tissue levels between the three groups at the time when the mice in the laparotomy group became moribund (Figure 3B and C). We also examined the effects of surgery on tumor cell proliferation using PCNA staining. In the SKOV3ip1 models, positive PCNA staining was significantly increased in tumors following laparotomy compared to laparoscopy-mimicking and controls ($p<0.05$ for both) (Figure 4B). No significant difference was seen between the laparoscopy-mimicking and control groups.

Little change in serum VEGF and MMP2 immediately after surgery occurred in the laparoscopy-mimicking group. The perioperative period, especially the immediate postoperative period, is crucial for the long-term prognosis of surgical patients because of changes in systemic and local factors (25). We determined the change in the serum levels of VEGF and MMPs during the immediate postoperative period under different surgical stress in a SKOV3ip1 model. Serum VEGF expression was significantly higher in the laparotomy group compared with the laparoscopy-mimicking and control groups until six days after surgery (Figure 5A). However, the laparoscopy-mimicking group showed no significant difference from the control group, except at day 3 after surgery. The laparotomy group had significantly increased serum MMP2 levels compared to the laparoscopy-mimicking or control groups at postoperative day 3, and the level in the laparoscopy-mimicking group was modestly increased

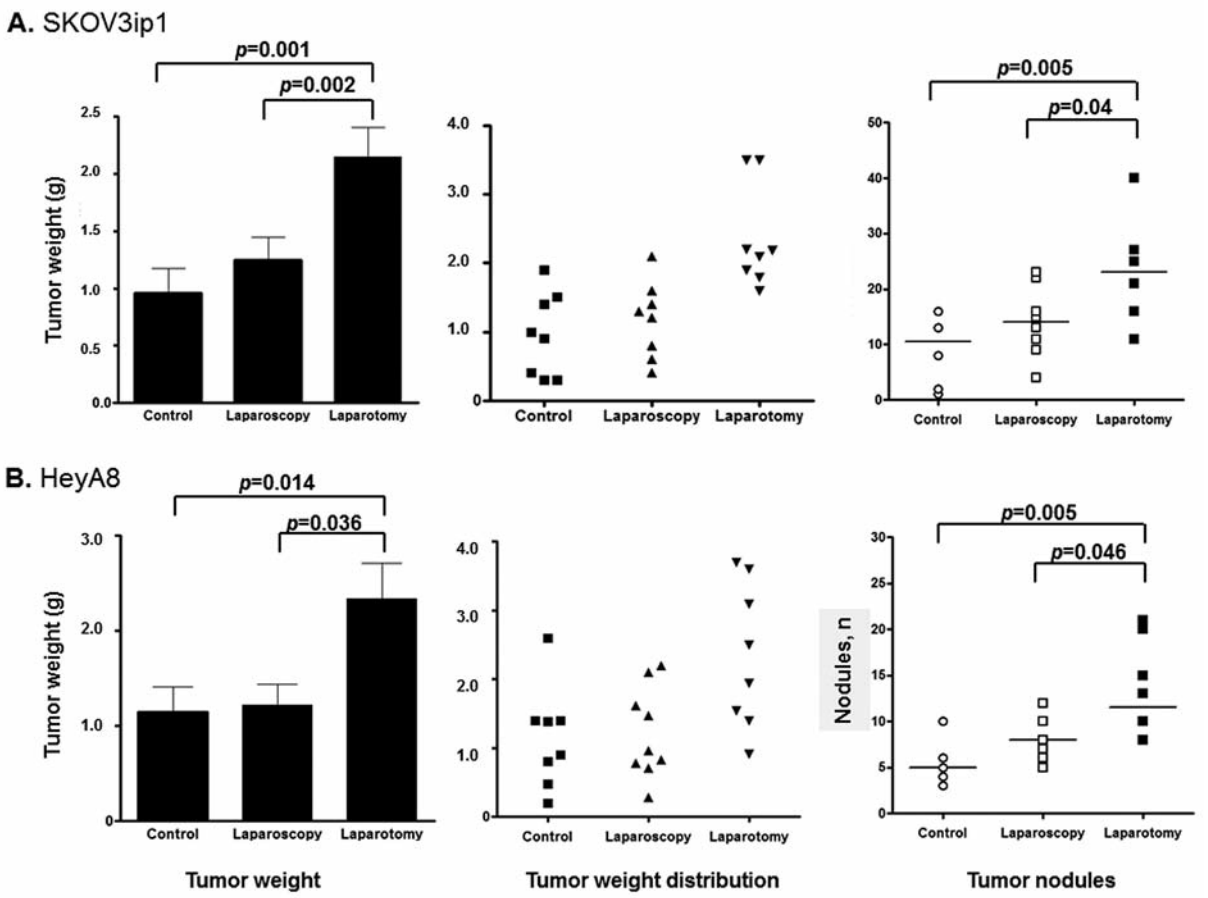

Figure 2. *Effect of surgical wound on in vivo ovarian cancer growth. Quantification of tumor weights and number of nodules in control (anesthesia alone) and surgically stressed mice (laparoscopy-mimicking or laparotomy) injected i.p. with SKOV3ip1 or HeyA8 ovarian cancer cells. Results are means±standard error (SE); n=10 mice per group.*

compared to that of the laparotomy group (Figure 5B). No difference in MMP9 expression was seen among the three groups (data not shown).

Discussion

We found that mice in the laparotomy group had significantly greater tumor weight and nodules compared to the laparoscopy-mimicking and control groups for both HeyA8 and SKOV3ip1 models. Laparoscopy, with minimal surgical wound, had little effect on tumor growth, which might be because of minimal influence on angiogenesis and induction of adhesion molecules.

Our results comparing surgical stress in laparoscopy-mimicking and laparotomy groups are consistent with previous studies (9-11). Southall *et al*. showed that both colon-26 adenocarcinoma and B-16 melanoma tumors injected intra-dermally in the dorsal skin of a murine model grew larger after laparotomy than after pneumoperitoneum (11). Allendorf *et al*. reported that increased tumor growth after laparotomy *versus* laparoscopy or pneumoperitoneum was observed in C3H/He female mice with an intradermal inoculation of tumor cells in the dorsal skin (9). In addition, Shiromizu *et al*., using a murine model with colon-26 cancer cells with injection into the tail vein, reported that laparotomy accelerated tumor metastasis to the lung and that laparoscopy did not increase the frequency or growth of metastases and a laparoscopic approach may suppress hematogenous metastasis to the lung because of reduced surgical stress and reduced cytokine response (10).

1. Surgical trauma was shown to enhance locoregional metastasis (2). Severity of trauma was furthermore shown to correlate with the tumor load, as laparoscopy induced less locoregional tumor load compared to laparotomy (26). Interestingly, the influence of surgery on tumor development is not confined to local peritoneal sites. Several reports described surgical trauma to cause systemic alterations that accelerate tumor development (27, 28). For example,

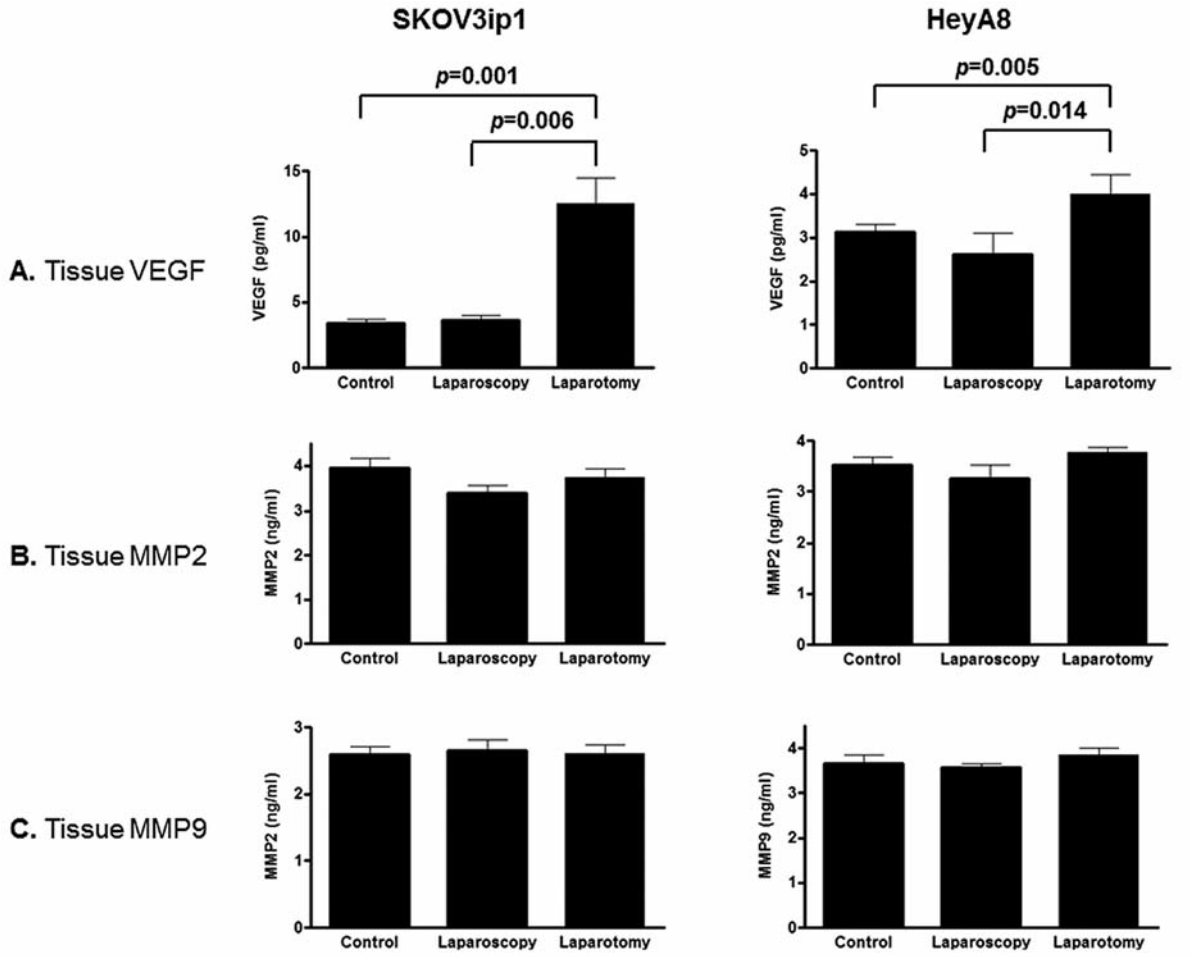

Figure 3. *Tissue levels of vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP) 2, and MMP 9 in tumors from animals exposed to surgical stress (laparoscopy-mimicking or laparotomy). Tissue level of VEGF increased in tumors of the laparotomy group compared to control and laparoscopy-mimicking groups at the time that mice of the laparotomy group became moribund. Results are means±SE.*

thoracotomy enhances tumor development in the peritoneal cavity (29). Thus, surgery induces both local and systemic changes that facilitate development of metastases. After surgery, the angiogenic balance of pro- and anti-angiogenic factors is shifted in favor of angiogenesis to facilitate wound healing, and, in particular, levels of VEGF are persistently elevated (3, 30), This may promote not only tumor recurrence and the formation of metastatic disease, but also result in the activation of dormant micrometastases (31). In this study, we found that VEGF levels were increased in tumor tissues when mice in the laparotomy group became moribund, and increased in the serum immediately after surgery until six days after surgery. Moreover, several studies reported compositional changes in blood plasma after open surgery, including to levels of MMP, interleukin, tumor necrosis factor-α, and insulin-like growth factor-binding protein, which have been shown to enhance *in vitro* tumor growth and which are not present after laparoscopic surgery (3, 32-35). In this study, we found that MMP2 and MMP9 were not changed in tumor tissues when mice of the laparotomy group became moribund, but MMP2 in serum was significantly increased in the laparotomy group at three days after surgery.

Considerable experimental and human data support that laparoscopic surgery results in reduced risk of tumor recurrence and formation of metastases compared with open surgery for colorectal cancer (CRC) (36-38). Although consensus has not yet been reached, laparoscopic surgery for CRC shows a potentially favorable patient outcome compared to open surgery. However, in epithelial ovarian cancer, a definitely disseminated disease, surgical stress might have more profound effects, with systemic and local influences, as opposed to localized cancer such as CRC and breast cancer, because maximal cytoreductive surgery of the primary and

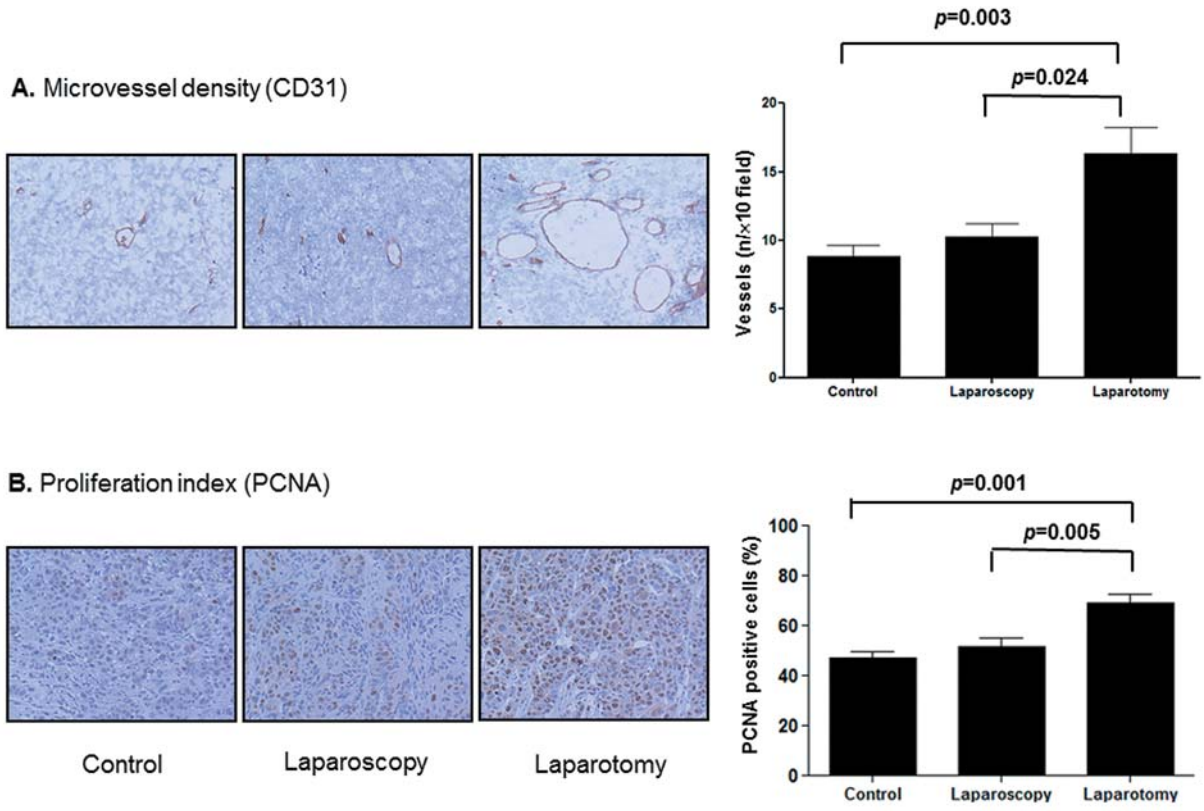

Figure 4. *Proliferation and angiogenesis in tumor tissues from animals exposed to surgical stress. SKOV3ip1 tumor samples from control (anesthesia alone) and surgically stressed (laparoscopy-mimicking or laparotomy) animals were stained for cluster of differentiation (CD) 31 and proliferating cell nuclear antigen (PCNA) by immunohistochemistry. All photographs were taken at original magnification ×100. Bars=SE.*

metastatic tumor is the most effective treatment for advanced ovarian carcinoma. This suggests that minimally invasive surgery such as laparoscopy rather than laparotomy, combined with better incisional and surgical techniques could reduce surgical stress with minimal effects on metastasis and re-growth of residual tumor in this type of cancer.

Limitations of this study include not fully evaluating the possible mechanisms for the surgical wound effect on tumor growth in ovarian cancer, for example the effect on growth factors, stress hormone, cytokines, natural killer cell activity, or other molecules associated with angiogenesis. Moreover, in addition to the extent of surgical wound, several mechanisms such as hypoxia and acidosis may be associated with increased tumor growth after surgery. Further experiments are necessary to clarify if a more detailed mechanism other than surgical stress regulates tumor growth and activates microscopic tumors after surgery.

In clinical the setting, the staging of cancer is the most important factor for treatment choices. In particular, staging laparoscopy is useful for the diagnosis of peritoneal dissemination in order to avoid unnecessary laparotomy (39). This study suggests that unnecessary exploration with laparotomy can cause activation of dormant cancer cells. In fact, laparoscopic surgical staging of ovarian cancer in early stages could be justified as a good option, considering the results of this study on surgical stress-induced tumor growth.

In conclusion, we found that a minimal surgical wound, such as the one created by laparoscopy, resulted in significantly reduced tumor weight and fewer nodules compared to laparotomy for an orthotopic ovarian cancer model, suggesting that this effect was due to minimal influence from surgical stress on angiogenesis and induction of adhesion molecules. Further clinical and translational research is warranted to evaluate more detailed mechanisms of surgical stress-induced tumor growth and ways to prevent it.

Conflicts of Interest

J-W Lee, Y-A Park, Y-J Cho, K-H Kang, J-J Choi, Y-Y Lee, T-J Kim, CH Choi, B-G Kim, D-S Bae have no conflicts of interest or financial ties to disclose.

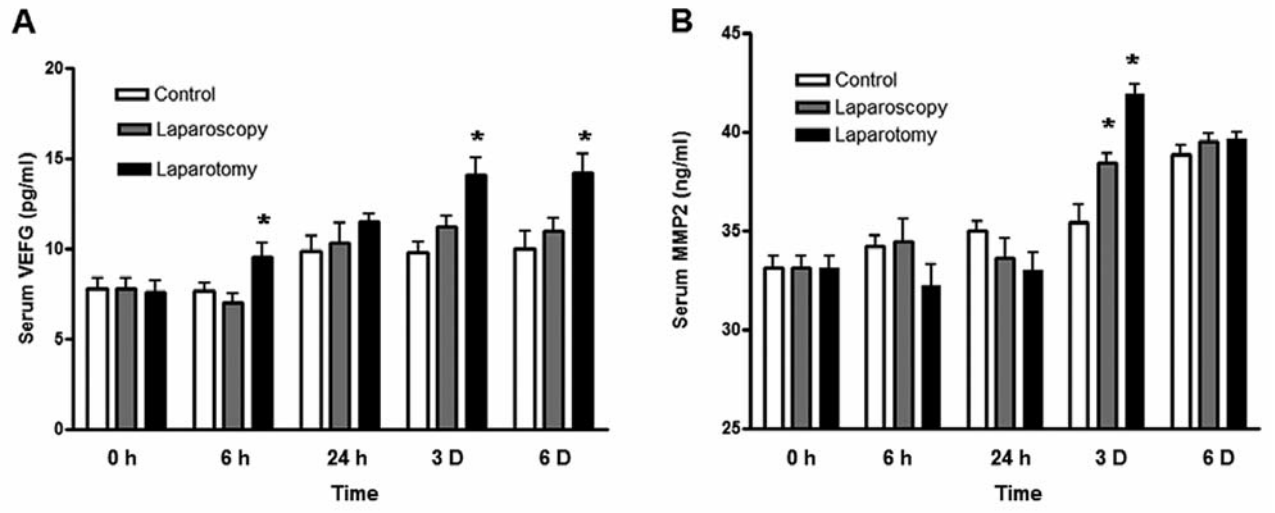

Figure 5. *Serum level of vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP)2 immediately after surgery in animals exposed to surgical stress (laparoscopy-mimicking or laparotomy). Serum VEGF levels were significantly increased in the laparotomy group during the postoperative period until six days after surgery compared to control and laparoscopy-mimicking. The MMP2 levels were significantly increased at three days for laparotomy compared to control and laparoscopy-mimicking groups, and mice of the laparoscopy-mimicking group showed significantly increased expression compared to the controls. Results represent the mean±SE. *p<0.001.*

Grant Support

This study was supported by a grant from the Korea Health 21 R&D Project of the Ministry of Health & Welfare, Republic of Korea (A090088) and the Samsung Biomedical Research Institute grant (#SBRI C-B0-229).

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Received April 28, 2013
Revised May 24, 2013
Accepted May 28, 2013