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Wound Healing of Intestinal Anastomosis after Digestive Surgery under Septic Conditions: Participation of Local Interleukin-6 Expression

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Abstract. This study aimed to evaluate the integrity of anastomotic wound healing after digestive surgery under septic conditions and to observe local interleukin-6 (IL-6) expression around the anastomotic segment. Experimental animals were separated into lipopolysaccharide (LPS) and control groups. Each was injected with LPS or normal saline solution into the peritoneal cavity 24 hours before transection and anastomosis of the colon. The anastomotic bursting pressure (ABP) and tissue hydroxyproline concentration (HP) were measured as indicators of wound healing. Immunohistochemical staining for IL-6 was performed on tissue samples obtained from the anastomotic segment, lung, liver, and kidney. The reactive cells were counted by light microscopy. The ABP and HP were significantly lower in the LPS group than the control group 7 days after the surgery. In the LPS group, IL-6 expression around the anastomotic segment was enhanced 1 and 6 hours after surgery but suppressed 24 hours afterward. In contrast, IL-6 expression in lung, liver, and kidney was enhanced in the LPS group 24 hours after surgery but not in the control group. It is suggested that anastomotic wound healing is impaired after digestive tract surgery under septic conditions, and local IL-6 expression participates in wound healing.

Leakage of an intestinal anastomosis is a major complication after surgery, with a reported incidence of up to 50% associated with significantly increased mortality and morbidity [1, 2]. Many factors contribute to the healing and integrity of an anastomosis, such as blood supply, tension of the anastomosis, bowel preparation, the patient's condition, and inflammation [2–5]. In addition, several chemical mediators, including growth factors and cytokines, are reported to contribute to wound healing during the early phase after surgery [6–10].

Interleukin-6 (IL-6) is a well studied cytokine that performs as a local regulator and a systemic signal of inflammation. IL-6 has been reported to be produced by various types of cells, including polymorphonuclear leukocytes (PMNs), macrophages, fibroblasts, lymphocytes, epithelial cells, and endothelial cells [11]. Some studies showed that this molecule might play a role in wound healing [8, 12, 13].

Most surgeons understand that wound healing is impaired in patients who have been hypercytokinemic, induced by surgical trauma and endotoxemia. Recent work has demonstrated that IL-6 concentration in peripheral blood and in fluid drained from the peritoneal cavity is increased in these patients [13–16].

We reported that IL-6 in portal blood is increased soon after the commencement of digestive surgery [17]. Local expression of IL-6 in the intestinal anastomotic segment, and the comparison between the expression of IL-6 and the wound healing are not fully understood. The purpose of this study is to analyze the influences of surgical trauma under septic conditions on wound healing of an intestinal anastomosis. In particular, we describe the local expression of IL-6 in the intestinal anastomosis and in important organs such as the lung, liver, and kidney.

Materials and Methods

Animals and Treatments

Adult male Sprague-Dawley (SD) rats, weighing 300 to 350 g, or male ICR mice, weighing 40 to 50 g, were employed for these experiments. The SD rats were used for measurement of anastomotic bursting pressure, the hydroxyproline assay, and histopathologic studies; and the ICR mice were used for immunohistochemical studies. The animals were separated into two groups: (1) Lipopolysaccharide (LPS) (*Escherichia coli* 0127: B8; Difco Laboratories, Detroit, MI, USA), 0.5 mg/kg body weight, was injected into the peritoneal cavity 24 hours before the digestive surgery as follows: Transection and anastomosis by one-layer, end-to-end and interrupted sutures with 7/0 polypropylene string were carried out in the colon via a 3 cm sterile midline incision under inhalant anesthesia with isoflurane (Abbott Laboratories, North Chicago, IL, USA) (*LPS group*). (2) The same volume of normal saline solution was injected instead of LPS (*control group*).

Animals were fasted from solid food overnight before surgery but were maintained on routine laboratory chow and water ad libitum after surgery. They were sacrificed at 1, 6, 24 hours and at 3 and 7 days after surgery under full inhalant anesthesia with isoflurane. Then samples were obtained from the anastomotic segment, lung, liver, kidney, and peripheral blood immediately. Total blood leukocyte counts in peripheral blood were measured at 1 and 24 hours and 3 and 7 days after the operation.

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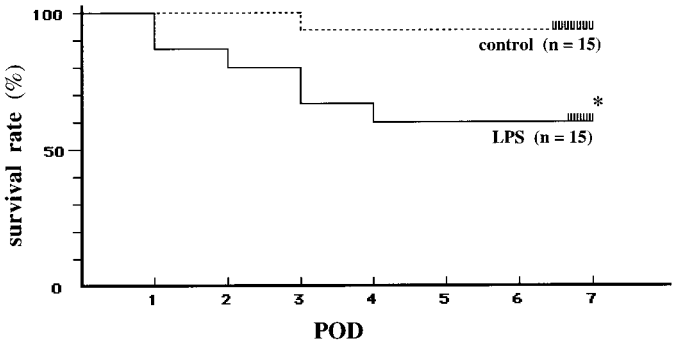

Fig. 1. Seven-day survival curves for the two groups by the Kaplan-Meier method. The 7-day survival rate of the LPS group was significantly lower than that of the control group. POD: postoperative days. $^*p < 0.05$ versus the control group.

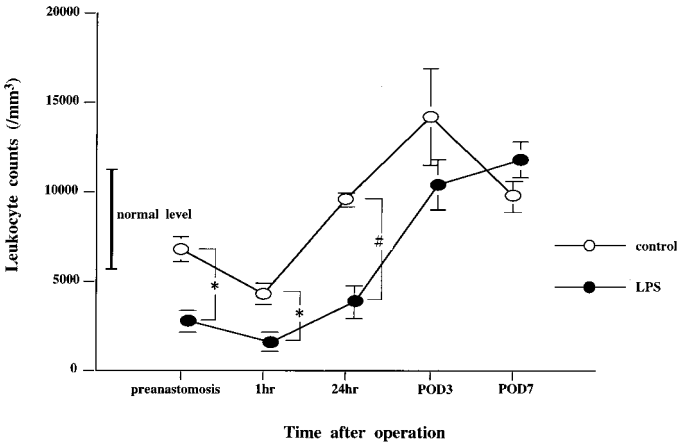

Fig. 2. Changes in the peripheral leukocyte counts after surgery. The peripheral leukocyte counts at 1 and 24 hours after surgery in the LPS group (filled circles) were significantly lower than in the control group (open circles). Values are expressed as the mean ± SEM of six rats. $^*p < 0.05$, $\#p < 0.001$ versus the control group.

Anastomotic Bursting Pressure

The anastomotic bursting pressure (ABP) was measured on postoperative day (POD) 7 using the method reported by Van der Ham et al. in 1993 [18]. A tube was inserted at 3 cm anal side of the anastomosis to the oral side; and two ligations were made at 1 cm oral and anal sides of the anastomosis. This tube was connected to an infusion pump and a pressure transducer linked to a recorder (Polygraph, Nippon Kouden, Tokyo, Japan). The pressure was measured with infusion of normal saline solution through this tube at a constant rate of 1 ml/min. The pressure recorded immediately before the abrupt loss was the ABP.

Hydroxyproline Assay

Hydroxyproline concentration in the anastomotic segment on POD 7 was measured as an indicator of collagen accumulation [19, 20]. This assay was performed on tissue samples frozen in liquid nitrogen and kept at −80°C in a deep freezer. After measuring the wet tissue weight, the samples were hydrolyzed by adding 6 N HCl at 110°C for 24 hours in sealed test tubes.

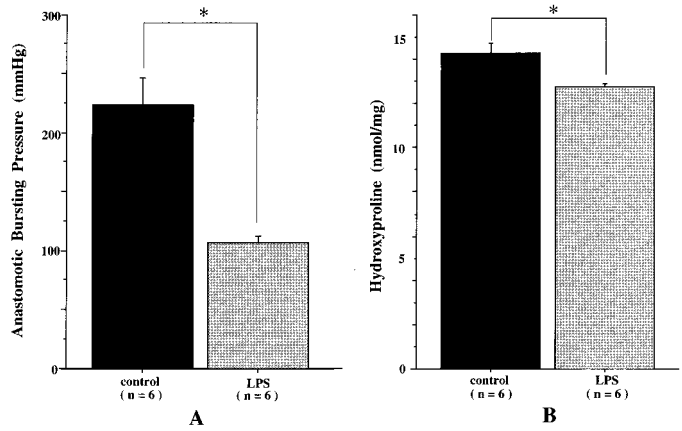

Fig. 3. A. Anastomotic bursting pressure 7 days after surgery. The anastomotic bursting pressure in the LPS group was significantly lower than that in the control group. **B.** Hydroxyproline concentration of the anastomotic segment 7 days after surgery. Hydroxyproline concentration in the LPS group was significantly lower than in the control group. Values are expressed as means ± SEM. $^*p < 0.01$ versus the control group.

Hydrolysates were transferred to flasks and dried in a desiccator overnight to remove the hydrochloric acid by evaporation. After dissolving in 3 ml of 0.02 N HCl, 0.1 ml of the solution was extracted and added to 0.9 ml of 0.02 N HCl. Final solutions were applied in 50-μl aliquots to the amino acid analyzer (Amino Acid Analyzer Model 835, Hitachi, Tokyo, Japan) to determine the hydroxyproline concentration.

Histopathologic Study

The samples obtained from the anastomotic segment, lung, liver, and kidney on PODs 1, 3, and 7 were fixed in 10% formalin and enbedded in paraffin. Sections were obtained at 3-μm intervals and stained with hematoxylin and eosin (H&E) or AZAN for histopathologic examination by light microscopy.

Immunohistochemical Staining

Immunohistochemical staining for IL-6 was performed on tissue samples obtained from the anastomotic segment, lung, liver, and kidney of ICR mice 1, 6, and 24 hours after surgery. We also investigated the IL-6 in mice 48 hours after LPS administration without surgery to evaluate the effect of LPS on IL-6 expression. These samples were fixed in 4% paraformaldehyde, processed in Tissue-Tek (Miles, Elkhart, IN, USA), cut into 5-μm thick frozen sections, and dried at room temperature (RT). Absolute methanol containing 3% hydrogen peroxide was added to block endogenous peroxidase. After washing three times with phosphate buffer solution (PBS) for 5 minutes each, these sections were treated with 1% polyoxyethylene-10-octylphenyl ether in PBS for 20 minutes at RT. After washing in the same way, they were reacted with 100 μl of biotinylated rat antimouse IL-6 monoclonal antibody (PHARMINGEN, San Diego, CA, USA), diluted in 9 ml of PBS and 1 ml of whole goat serum at RT in a moist chamber

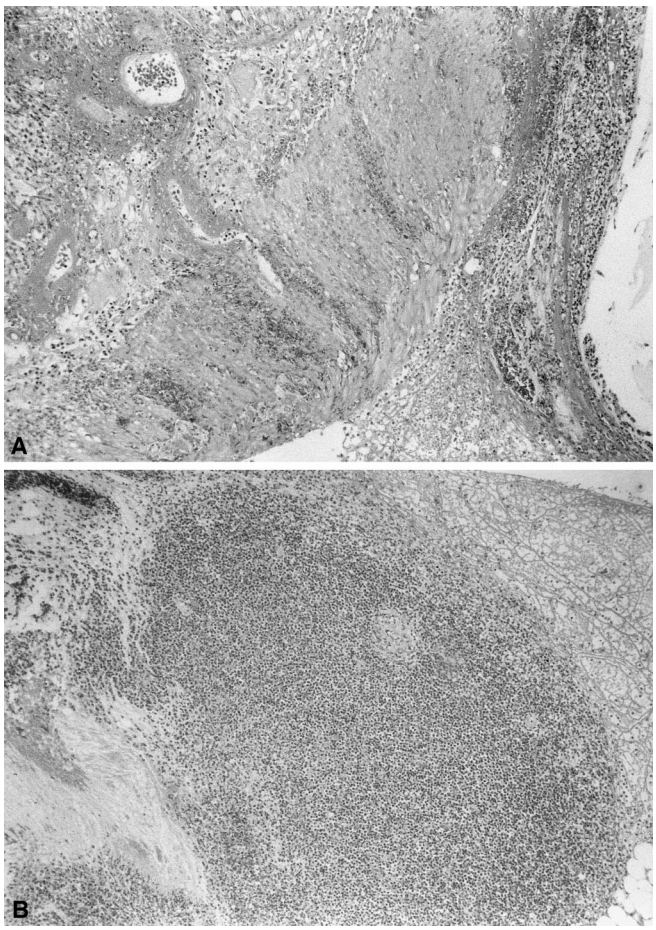

Fig. 4. Rat colonic anastomosis on postoperative day 1. The inflammatory cells including polymorphonuclear leukocytes infiltrated markedly around the anastomosis in the control group **(B),** whereas infiltration of the inflammatory cells was scattered in the LPS group **(A).** (H&E, ×100)

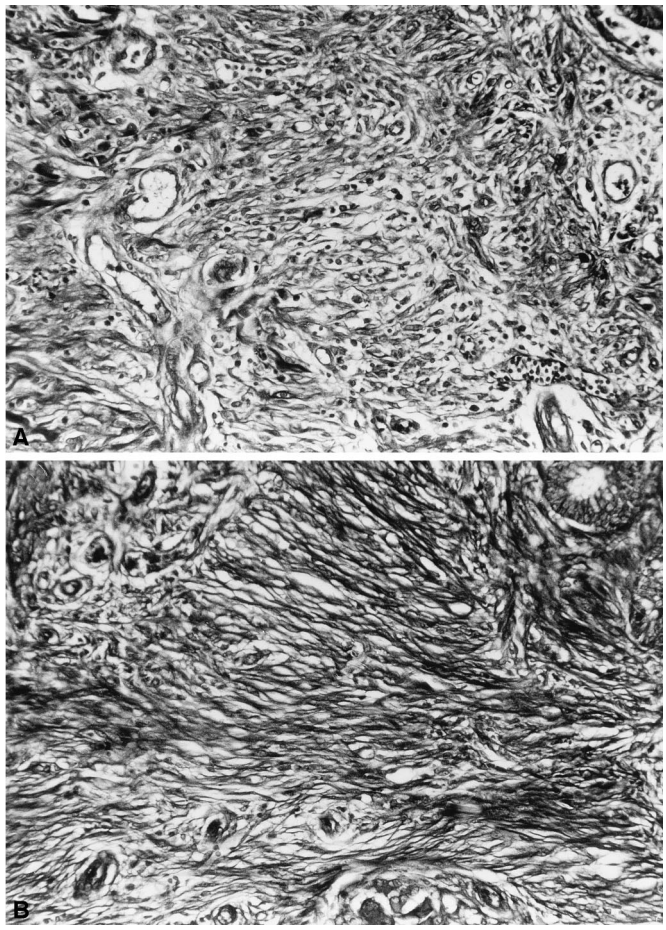

Fig. 5. Histologic findings of collagen fibers in the anastomotic segment of the colon on postoperative day 7. There were degeneration and irregular formation of collagen fibers in the LPS group **(A)** compared with those in the control group **(B).** [Heidenhain's azocarmine stain (AZAN), ×200]

for 2 hours. After washing, the preparations were incubated with two drops each of avidin solution and biotinylated horseradish peroxidase solution included in the kit (ABC KIT, VECTOR, Burlingame, CA, USA) in 4.5 ml of PBS and 0.5 ml of 5% skim milk for 2 hours at 37°C. After PBS rinsing, diaminobenzidine and nickel were applied for 8 minutes at RT to achieve a permanent color change.

Six views were selected randomly for each section and observed under light microscopy (×100). The mean numbers of reactive cells in six views were regarded as the data for each sample. We observed six samples each time.

Statistical Analysis

The data are presented as the mean ± standard error of the mean (SEM). Survival rate was calculated by the Kaplan-Meier product-limit method and analyzed statistically with the generalized Wilcoxon test. Other results were analyzed with the unpaired *t*-test. Statistical significance was assumed at $p < 0.05$.

Results

Survival Rate

The survival curves for the two groups are shown in Fig. 1. The 7-day survival rate for the LPS group (60.0%) was significantly lower than that of the control group (93.3%) ($p < 0.05$).

Peripheral Leukocyte Count

The peripheral leukocyte counts were smaller in the LPS group than the control group during the first 3 days after surgery. In particular, the leukocyte counts at 1 and 24 hours after surgery in the LPS group (1 hour 1533.3 ± 176.3/mm^3; 24 hours, 3825.0 ± 480.2/mm^3) were significantly lower than those in the control group (1 hour 4233.3 ± 611.9/mm^3; 24 hours 9600.0 ± 152.7/mm^3) ($p < 0.05$). In addition, the number of leukocytes at 1 and 24 hours after surgery in the LPS group were lower than normal (6,000–12,000/mm^3 in the rat) (Fig. 2).

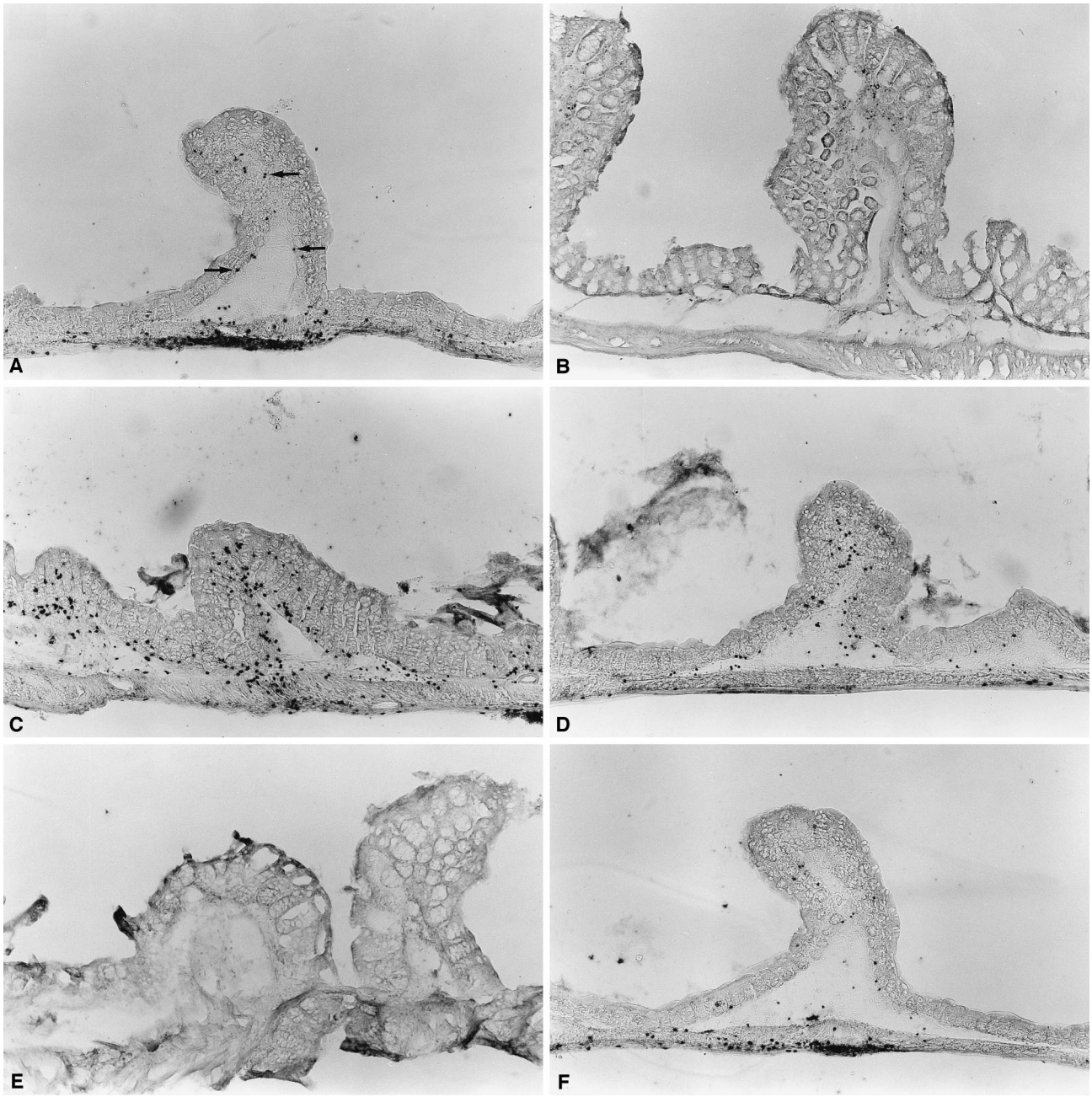

Fig. 6. Immunohistochemical staining using biotinylated rat antimouse IL-6 monoclonal antibody around the colonic anastomosis in the LPS group **(A, C, E)** and the control group **(B, D, F).** A, B. One hour after surgery. C, D. Six hours after surgery. E, F. At 24 hours after surgery. The expression of IL-6 produced by polymorphonuclear leukocytes and macrophages was observed mainly in the submucosal region (arrows) (A). The reactive cells for IL-6 were enhanced markedly in the LPS group (A, C) compared with those in the control group (B, D) until 6 hours after surgery. IL-6 expression was scarcely observed in the LPS group 24 hours after surgery (E) compared with that in the control group (F). (×100)

Anastomotic Bursting Pressure

The ABP was significantly lower in the LPS group (112.7 ± 2.8 mmHg) than in the control group (205.0 ± 33.0 mmHg) 7 days after surgery ($p < 0.01$) (Fig. 3A).

Hydroxyproline Assay

Tissue hydroxyproline concentration around the anastomotic segments 7 days after surgery were 14.26 ± 0.47 nmol/mg wet tissue in the control group and 12.75 ± 0.10 nmol/mg in the LPS group (Fig. 3B). There was a significant difference between the two groups ($p < 0.01$).

Histopathologic Study

Histopathologic observations of sections stained with H&E showed that infiltration of inflammatory cells such as PMNs or macrophages into the anastomosis was more inhibited in the LPS

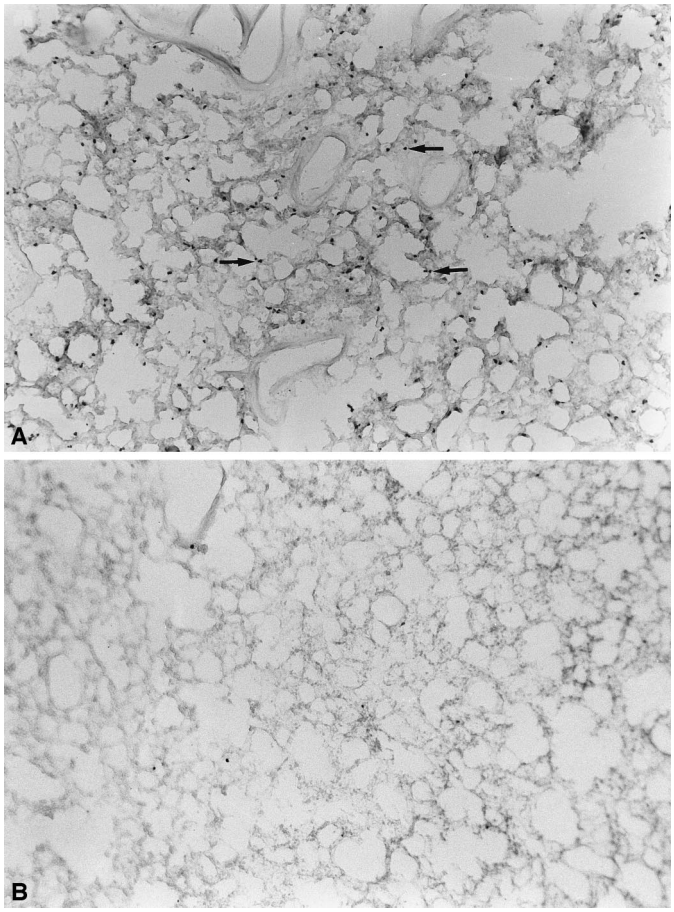

Fig. 7. Immunohistochemical staining of IL-6 in the lung 24 hours after surgery in the LPS group **(A)** and the control group **(B).** Many reactive cells for IL-6 could be seen in the LPS group (arrows) (A), whereas they were not observed in the control group. (×100)

group (Fig. 4A) than the control group (Fig. 4B) 1 day after surgery. Minimal morphologic changes were detected in lung of the control group. In contrast, moderate infiltration of PMNs and macrophages into alveolar septa and alveoli with focal fibrinous exudate, as well as congestion of the interstitium were observed in lung of the LPS group on POD 3. Liver and kidney did not show obvious histologic differences between the LPS and control groups at any time point. After AZAN staining of the anastomosis, degeneration and irregular formation of collagen fiber were observed in the LPS group (Fig. 5A), compared to that in the control group (Fig. 5B) on POD 7.

Immunohistochemical Staining

The reactive cells for IL-6 in the intestinal anastomosis were observed mainly in the submucosal region. IL-6 expression around the anastomosis was significantly more enhanced in the LPS group than the control group 1 and 6 hours after surgery (Fig. 6A–D). It was markedly suppressed in the LPS group (Fig. 6E) compared to that in the control group (Fig. 6F) 24 hours after surgery. In contrast, IL-6 expression in the lung, liver, and kidney was enhanced in the LPS group 6 and 24 hours after the operation. In particular, it was detected prominently in the lung 24 hours after the operation (48 hours after LPS administration) (Fig. 7A). It was not observed in the control group (Fig. 7B) (Table 1). The expression of IL-6 was not enhanced in the mice 48 hours after administration of LPS without surgery (the numbers of reactive cells in the anastomosis were 23.33 ± 3.85, lung 12.33 ± 2.09, liver 9.00 ± 0.89, and kidney 2.33 ± 0.67).

Discussion

The influence of surgical trauma under septic conditions on wound healing of an intestinal anastomosis were studied in an attempt to define the local expression of IL-6. Cytokine messenger RNA expression of intestinal mononuclear cells has been evaluated for a certain disease using the in situ hybridization method [21]. To the best of our knowledge, however, the present study is the first to examine local expression of IL-6 in an intestinal anastomosis under the septic condition. Our results showed that there is a significant decrease in the expression of IL-6, followed by significantly increased production, in the anastomotic segment 24 hours after the operation in the LPS group. The accumulation of PMNs in the anastomosis was suppressed markedly on POD 1 in the LPS group, where anastomotic bursting pressure and concentration of hydroxyproline were also significantly lower than in the control group. These results indicate that an inflammatory reaction, which is necessary for successful wound healing, was suppressed locally under septic conditions.

The effect of IL-6 on wounds has been shown to have both positive and negative effects during the healing [12, 22–24]. Depending on the target cell, IL-6 can stimulate or inhibit cell proliferation, and it appears to be an integral part of the cytokine network regulating immune responses [11, 25]. In the present study, it remains to be seen if IL-6 has any direct effect on wound healing. Our results showed that wound healing was impaired in the LPS group as evaluated by the ABP assay, hydroxyproline assay, and histopathologic studies. It is believed that the bankruptcy of the local cytokine network that induces abnormal production of IL-6 might participate in inhibition of the healing process.

Sakamoto et al. have reported that IL-6 in fluid drained from the operative field was still readily detectable at high levels 24 hours after surgery [14]. In our study, IL-6 expression was scarcely observed in the anastomosis 24 hours after the operation in the LPS group. The difference between the two studies can be explained by the fact that they measured the IL-6 released from leukocytes and infiltrated into the thorax or abdominal exudate.

Interestingly, we observed that IL-6 expression in the lung, liver, and kidney was increased in the LPS group 6 and 24 hours after the operation but not in the control group. This outcome was contrary to the result observed in the anastomosis.

Lipopolysaccharide induces the production and release of cytokines by activating several transcription factors, particularly nuclear factor-κB [26–28]. In our study the administered LPS might have induced production of IL-6, although IL-6 was not expressed in these organs in mice treated with LPS without surgery. The serum IL-6 level was not measured in the present study, whereas other studies have demonstrated that the serum IL-6 level was increased, with a peak at 6 to 12 hours after the operation [14, 16, 17]. Increased IL-6 in peripheral blood is believed to induce the expression of IL-6 in lung, liver, and kidney. The time lag for IL-6 expression in colonic anastomosis and other

Table 1. Reactive cells for IL-6 in each view using light microscopy (×100).

Time after surgery	Anastomosis	Lung	Liver	Kidney
Control group				
1 Hour	56.00 ± 9.48	1.00 ± 0.52	1.33 ± 0.49	0.67 ± 0.33
6 Hours	137.50 ± 8.50	2.33 ± 0.62	3.50 ± 1.48	3.00 ± 0.97
24 Hours	72.00 ± 10.08	6.83 ± 1.01	7.16 ± 2.31	2.33 ± 0.84
LPS group				
1 Hour	127.00 ± 8.77***	3.67 ± 0.76*	7.00 ± 1.27**	5.33 ± 1.23**
6 Hours	224.16 ± 14.58***	49.00 ± 5.34****	31.33 ± 4.96***	6.17 ± 1.19
24 Hours	3.33 ± 0.84****	230.83 ± 14.40****	60.67 ± 6.10****	8.00 ± 1.24**

Values are expressed as mean ± SEM ($n = 6 \times 6$).
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, versus the control group.

important organs may be due to this mechanism. IL-6 expressed in lung might bring on the infiltration of inflammatory cells on POD 3, via its own effect or by activating other chemical mediators. Histopathologic findings observed in the lung of the LPS group by H&E staining indicated that organ dysfunction was followed by IL-6 expression. The animals in the LPS group were considered to be in worse condition than the control group based on the survival rates and peripheral leukocyte counts. The results of Holman et al. support this contention [29]. These results suggest that an inflammatory reaction, which may induce multiple organ dysfunction, occurred in important organs (e.g., lung, liver, kidney) under severe stress (i.e., infection and surgery).

The limitations of the present study were the small number of animals (six per group) and the 24-hour observation period, which may have been too short for proper evaluation of cytokine production. We believe that these limitations did not significantly influence the outcome of the study because the difference between the groups was too marked to have resulted from chance, and cytokine including IL-6 acts during the early phase of the wound healing process.

The repressive process of IL-6 expression remains to be established. PMNs and macrophages have been reported to be important sources of IL-6 during inflammatory and septic states [27]. The suppression of inflammatory cell margination, which may produce IL-6, is believed to be a factor in this repression process. In the histologic study, we observed some marginated cells (e.g., PMNs and macrophages) around the colonic anastomosis on POD 1 in the LPS group, whereas there were fewer reactive cells for IL-6 relative to the number of infiltrated inflammatory cells in the same animal. Therefore it is believed that there are other factors in this process. Recent work [11] has demonstrated that IL-6 itself controls its own expression via activation of nuclear factor IL-6, which suggests that one of the factors of the repression process may be down-regulation, which is followed by a significant increase in IL-6 expression.

Conclusions

Expression of IL-6 in the anastomotic segment was suppressed after the operation in mice treated with LPS. It is suggested that wound healing was impaired by local suppression of the inflammatory reaction following surgical trauma under the septic condition. It is also suggested that IL-6, in particular, may play an important role in wound healing. Systemic or local administration of cytokine-specific antibody may be able to prevent impairment of wound healing and inhibit the multiple organ failure induced by surgical trauma.

Résumé

Cette étude expérimentale, animale, vise à évaluer la cicatrisation de l'anastomose intestinale dans des conditions septiques et de déterminer l'expression de l'interleukine-6 (IL-6) dans les tissus autour de l'anastomose. Les animaux ont été divisés en deux groupes, l'un recevant une injection de lipopolysaccharide (groupe LPS), l'autre, du sérum physiologique dans la cavité abdominale (groupe de contrôle), 24 heures avant la section intestinale et l'anastomose du côlon. On a mesuré la pression de rupture anastomotique (ABP) et la concentration en hydroxyproline (HP), utilisées comme indicatrices de la qualité de la cicatrisation. Une coloration immunohistochimique a été utilisée pour l'expression de l'IL-6 dans les échantillons provenant des tissus anastomotiques, du poumon, du foie et du rein. Les cellules réactives ont été comptées sous microscopie optique. Les valeurs d'ABP et de HP étaient significativement plus basses dans le groupe LPS par rapport au groupe de contrôle, 7 jours après chirurgie. L'expression d'IL-6 dans les tissus périanastomotiques était retrouvée 1 et 6 heures après chirurgie mais elle était déprimée 24 heures plus tard. En revanche, l'expression d'IL-6 dans le poumon, le foie et le rein était retrouvée dans le groupe LPS 24 heures après chirurgie mais pas dans le groupe de contrôle. On suggère que la cicatrisation des anastomoses est défectueuse dans les conditions septiques et que l'IL-6 participe à la cicatrisation.

Resumen

El presente trabajo fue orientado a evaluar la integridad de la cicatrización anastomótica en cirugía del tracto digestivo realizada en condiciones sépticas y a determinar la expresión local de interleucina-6 (IL-6) alrededor del segmento anastomótico. Los animales experimentales fueron separados entre un grupo LPS y un grupo control, y cada uno fue inyectado con liposacárido (LPS) o con solución salina normal, en la cavidad peritoneal, 24 horas antes de la transección y anastomosis del colon. Se efectuó la medición de la presión de estallido de la anastomosis (PEA) y de la concentración de hidroxiprolina (HP) como indicadores del proceso de cicatrización. Se practicó la coloración inmunohistoquímica para IL-6 en muestras tisulares obtenidas a partir del segmento anastomótico del pulmón, hígado y riñón y se hizo el

conteo de células reactivas mediante microscopía de la luz. Tanto la PEA como la concentración de HP aparecieron significativamente más bajas en el grupo LPS que en el grupo control a los 7 días luego de la cirugía. En el grupo LPS, la expresión de IL-6, alrededor del segmento anastomótico resultó incrementada 1 y 6 horas luego de la cirugía, pero suprimida a las 24 horas. Por el contrario, la expresión de IL-6 en el pulmón, hígado y riñón resultó incrementada en el grupo LPS a las 24 horas, pero no así en el grupo control. Tales datos parecen sugerir que el proceso de cicatrización de la anastomosis resulta alterado luego de cirugía del tracto digestivo practicada en condiciones sépticas, y que la expresión local de IL-6 participa en el proceso.

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Invited Commentary

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The study of Ishimura and colleagues has examined whether colonic anastomoses heal normally in septic animals and if the wound healing process in septic animals is associated with a normal pattern of tissue proinflammatory cytokine production. Using both rats and mice, the authors observed that animals subjected to a prior septic insult (endotoxemia) had reduced colonic wound healing and survival following surgical anastomosis. Local interleukin-6 (IL-6) production was increased early (within 6 hours) at the anastomotic site but then decreased.

As in all good scientific endeavors, the present study raises more questions than it has answered. The findings confirm a presumption by most surgeons that wound healing and recovery from intestinal surgery are often delayed in the infected or septic patient. Although the present study raises the possibility that IL-6 is involved in the healing process, the authors acknowledge that "it remains to be seen if IL-6 has any direct effect on wound healing."

These essentially descriptive studies do not address whether a causal relation exists between IL-6 expression and colonic wound healing. The problem is that the humoral response to sepsis or endotoxinemia is complex, and a variety of proinflammatory cytokines such as TNFα, IL-1, IL-6, the C-C, C-X-C families of chemokines, LIF, IL-12, and interferons are released concordantly. The role that an individual cytokine such as IL-6 plays in wound healing is still undefined.

As observed here and by others, increased IL-6 expression is often part of the early inflammatory response during wound healing [1]. Only recently have interventional efforts been made to examine the contribution of individual cytokines to the healing response. In a study of peritoneal wound healing and adhesion formation, Saba and colleagues noted that blocking an endogenous IL-6 response with antibodies had no effect on skin wound healing but reduced adhesion formation after colonic serosal injury, suggesting that IL-6 plays a role in fibroblast proliferation [2]. In the present report, a similar approach could have specifically addressed whether the differences in wound healing and survival were causally related to local or systemic IL-6 production. We noted as early as 1993 that passive immunization of mice with a monoclonal antibody to IL-6 could prevent much of the innate inflammatory and hepatic acute phase responses to inflammation [3]. Another approach would have been to use IL-6 null mice, as was performed by Kopf and colleagues [4].

The studies of Ishimura and his group remind us of the words of Joseph Priestly: "In completing one discovery we never fail to get an imperfect knowledge of others of which we could have no idea before, so that we cannot solve one doubt without creating several new ones." Ishimura and his colleagues must be encouraged to continue their investigations and use more interventional approaches to better identify the specific role played by IL-6 in wound healing and the septic responses.

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