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Treatments for the Activating Macrophages that Reduces Surgical Stress and Postoperative Mortalities from Bacterial Infections and Tumor Metastases

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Abstract. *Background: Some of the mortalities caused by infectious diseases and/or distant metastases following surgery are thought to be due to immunological suppression. For this reason, techniques that reduce immunological suppression following surgery may reduce mortalities and/or incidences of micrometastases in distant organs. Materials and Methods: Mice were anesthetized and their peritoneal cavities were opened for 30 min. Immunological suppression was estimated by the presence of tumor necrosis factor-α (TNF) after injection with OK-432 (dead bacterial bodies). The mice were administered with either Staphylococcus aureus or cancer cells of Meth A fibrosarcoma. Survival times and lung metastastic foci were then observed at 3 weeks. Results were compared for mice with or without treatment by OK432 or TNF prior to surgery. Results: While significant suppression of TNF production was observed after laparotomy, administration of a macrophage-activating agent (TNF or OK-432) 3 h prior to laparotomy prevented immune suppression after the laparotomy. Laparotomy increased mortalities from bacterial infections and promoted the number of lung metastases. By contrast, administration of TNF or OK-432 3 h prior to the laparotomy decreased mortalities and metastases after the laparotomy. Conclusion: These results suggest that appropriate activation of macrophages prior to surgery is a method to reduce some of the detrimental effects caused by surgical operations.*

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Stress occuring after trauma and surgery is well known to induce profound immunosuppression (1-5). This immunologically suppressed state can affect the progression of infectious diseases and the development of tumor metastases (6-8). Some antibiotics and chemotherapeutic agents have been used after surgical operations to inhibit the progression of infectious diseases and metastases, but the efficacy of these treatments has been limited. Therefore, it appears that it would be beneficial to develop immunological methods for the prevention of immunosuppression after surgery to inhibit these detrimental side-effects. It is important to understand how macrophages behave under stressful conditions, since macrophages are known to play a pivotal role in the maintenance of homeostasis, by providing protection from invasive pathogens and by suppressing metastases (9-11).

Macrophages initially respond to external environmental stimuli and transmit this information throughout the body by paracrine, autocrine and juxtacrine systems through production of cytokines (9). This information-transfer system was named the "macrophage-network". This is a new concept that describes a mechanism for how animals control the homeostatic system (12). When under stress, macrophage activity in response to external stimulation may be suppressed. To investigate the macrophage function, we focused on the production of tumor necrosis factor-α (TNF) after bacteria or bacterial components administration. TNF is one of the major proteins produced from activated macrophages, and it induces antitumor and anti-bacterial activities *in vivo* and *in vitro* (13).

It has been reported that sequential-activated stages are necessary to produce mature TNF from resident (non-activated) macrophages. The first stage is called "primed stage" during which membrane-bound TNF (26 kDa) is expressed on the macrophages after appropriate stimulation with priming agents, such as interferon-γ (IFN-γ) (14). A subsequent stage is referred to as the "triggered stage" in which there is cleaving membrane-bound TNF of a metalloprotease family, TNF-α converting enzyme (TACE) (15), and mature TNF (17 kDa) is released after treatment with triggering agents such as lipopolysaccharide (LPS). The primed stage can also be induced by other biological response modifiers (16-18). Macrophages primed by stimulation with TPA could phagocytize significant amounts of particles, including pathogens, and reduce reactive oxygen species (19). Moreover, the primed stage is beneficial because it can induce the production of membrane-bound TNF which protects against mycobacterial infection. (20). Based on these facts, it was hypothesized that avoidance of the suppressed condition for TNF production could protect against the detrimental effects of surgical stress, loss of resistance to pathogen infections or promotion of metastases.

In this paper, the effect of prior administration of priming agents on TNF production after laparotomy was investigated.

Materials and Methods

Animals. BALB/c male mice were purchased from the Shizuoka Experimental Animal Farm (Shizuoka, Japan). They were 6-8 weeks of age and weighed 18-24 g at the start of the experiments. Mice were given a standard laboratory diet and water *ad libitum*. Prior to performing the experiments, an application to undertake experimentation was approved by the Animal Ethics Committee of the Biotechnology Research Center, Teikyo University.

Reagents. A human TNF mutein, TNF-SAM2 (purity: >99.5%, specific activities: $4.3x10^6$ units/ml protein, LPS content: 177 pg/mg protein) was obtained as recombinant proteins that used JM109 as the host and was purified in our laboratory (21). OK-432 (dead bacterial bodies of *Streptococcus pyrogenes*, 1 KE: 0.1 mg dry body weight) was donated by Chugai Pharmaceutical Co., Tokyo, Japan.

Surgical operation (laparotomy). BALB/c mice were anesthetized with intraperitoneally administered nembtal. Using aseptic techniques, a 2-cm longitudinal abdominal incision was made. The intestine was exposed and covered with sterile wet gauze. The intestine was put back into the abdominal cavity after a 30 min exposure period, and the incision was closed with a 6-0 suture.

TNF production. Detailed procedures for obtaining TNF-production are described elsewhere (17). Briefly, mice were intravenously administered with1 KE of OK-432 as a triggering agent at 0, 2, 4, and 6 h after a surgical operation. Two h after OK-432 administration, blood, liver and spleen were taken. Serum was collected from blood, and the supernatant of the homogenized liver and spleen was prepared by centrifuge (7000 g x 10 min.) and kept at –80˚C until used. TNF was assayed by a method described elsewhere (17). For the prevent effect of priming agents, three h before the laparotomy, mice were administered either TNF (1000 units, 0.23 μg) or OK-432 (0.1 KE). These values had previously been reported to be the optimal dosage and timing to induce the primed stage (22, 23). TNF production was the induced by intravenously administering 1 KE of OK-432, and TNF activity was measured.

Bacterial infection. Staphylococcus aureus (ATCC 25923) was cultured on a heart-infusion agar plate. Mice were intravenously inoculated with *Staphylococcus aureus* ($1x10^8$ cfu, colony forming unit) and then allowed to recover. BALB/c mice (n=18) were intravenously administered TNF (1000 units/mouse, 0.23 μg/mouse) or 0.1 KE of OK-432. 0 or 3 h later, the peritoneal cavities of the mice were opened for 30 min. Immediately after the operations, mice were intravenously injected with 10^8 cfu of *Staphylococcus aureus*. BALB/c mice (n=18) were intravenously administered OK-432 (0.1 KE/mouse) and 0 or 3 h later, the peritoneal cavities of the mice were opened for 30 min. Three hours after the operations, mice were intravenously injected with 10^8 cfu of *Staphylococcus aureus*.

Tumor metastasis. Meth A fibrosarcoma was passaged once a week as ascites in BALB/c mice. To establish a reproducible model of lung metastases of Meth A fibrosarcoma, tumor cells which showed highly metastatic activity in lung tissue were established through repeated passage of the cells obtained from the metastatic region. BALB/c mice (n=7-8) were intravenously administered with either TNF (1000 units/mouse) or OK-432 (0.1 KE); 0 or 3 h later, the peritoneal cavities of the mice were opened for 30 min. Meth A ($5x10^5$ cells) was administered intravenously to laparotomy-operated BALB/c mice; 21 days after inoculation lungs were removed, fixed in 10% formalin solution, and the number of lung metastases was counted.

Statistical analysis. Statistical evaluations of differences between groups were made by Student's *t*-test. The probability of survival after bacterial inoculation was compared using Kaplan-Meier analyses and log-rank statistics.

Results

Influence of surgical stress on TNF production induced by OK-432 and mortality from Streptococcus infection. We previously reported that TNF production after administration of LPS or OK-432 (dead bacterial bodies) was a useful parameter for estimating immunological status in terms of the innate

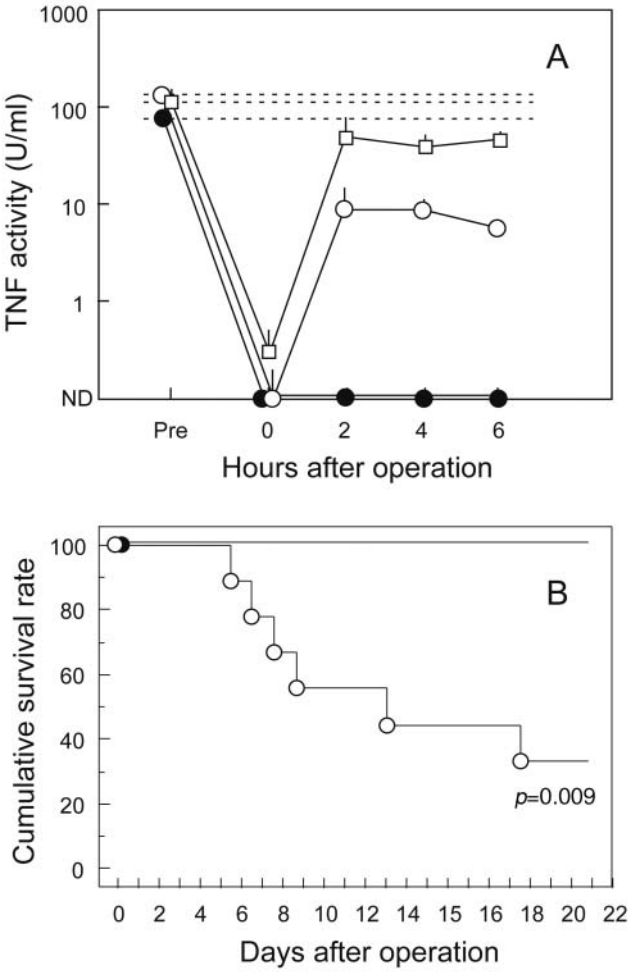

Figure 1. *Time course of endogenous TNF production induced by OK-432 after laparotomy in mice and survival rate after Staphylococcus aureus infection. A) At specified times after the operation, mice were treated intravenously with OK-432 (1 KE), and 2 h later, their livers (○), serum (●), and spleens (□) were obtained and tested for TNF activity. Symbols and bars indicate averages and standard deviations. B) Immediately after the operations, mice were intravenously injected with 10^8 cfu of Staphylococcus aureus. Survival rates were recorded for the group with (○) and without the operation (●). Survival rates were analyzed using the Kaplan-Meyer method, and the statistical significance between the control and operation group was calculated with the log-rank test.*

immune function (14, 18). TNF production after surgical stress has been described in only a few reports (3, 24). These showed significant suppression of TNF production when LPS was administered immediately after a laparotomy operation, but the kinetics of immune suppression following surgery has not yet been examined. For this reason, TNF production with respect to time after a surgical operation was investigated first. As shown in Figure 1A, TNF activity immediately after an operation was significantly reduced when compared to the level measured before the operation. TNF activity gradually returned to its normal level after 24 h (data not shown). This result showed that the stress of laparotomy by itself suppressed TNF production acutely but only transiently.

In order to investigate the detrimental effect of surgical stress, the mice were injected with *Staphylococcus aureus* ($1x10^8$ cell/mouse) after the laparotomy. No mice died in the two control groups (mice that were anesthetized and injected with *Staphylococcus aureus* but did not receive a laparotomy, and laparotomy mice that were not injected with *Staphylococcus aureus*). By comparison only 33% of the mice survived in the test group (laparotomy plus injection) (Figure 1B). This experiment confirmed that surgical stress aggravated pathogen infection.

Recovery of TNF production and inhibition of mortality from Staphylococcus aureus infection when priming agents were administered before laparotomy. Based on the above results, we hypothesized that if macrophages could maintain their functions even after a surgical operation, the mice would also retain their resistance to invasive bacteria. In this experiment we used a priming agent to determine whether the immunological status of innate immune functions (as measured by TNF production) would be maintained even after a surgical operation. The results showed that there was almost the same level of TNF production even in the mice receiving surgery as long as they were administered priming agents with the optimal dose and timing (Figure 2A, 2B). By contrast, the mice that received the priming agent just after the operation did not show such an effect (Figure 2C, 2D). These data indicate that maintenance of the primed stage is possible even following an operation and that the suppression of macrophage activity by laparotomy can be avoided.

Impairment of macrophage functioning caused by surgery could be responsible for the reduced ability to eliminate detrimental substances including pathogens and cancer. It is a well known fact that one of the detrimental side effects of cancer surgery is the promotion of morbidity and/or mortality by infectious diseases and metastasis. From the data in Figure 2A, 2B, we believe that proper administration of priming agents could provide protection from morbidity and/or mortality of infectious diseases after surgery. For this reason we investigated whether the administration of priming agents prior to surgery really protects against mortality from infectious disease by using *Staphylococcus aureus*. As shown in Figure 3A and 3B, the survival rate of the control was 33%, but the survival rate of the group that received 1000 units of TNF 3 h before the operation was 89% (log-rank test: $p=0.037$), and the group with 0.1 KE of OK-432 was 89% (log-rank test: $p=0.034$). Conversely, the groups that received the priming agent immediately after the operation were not statistically different from the control group. Therefore, the timing of

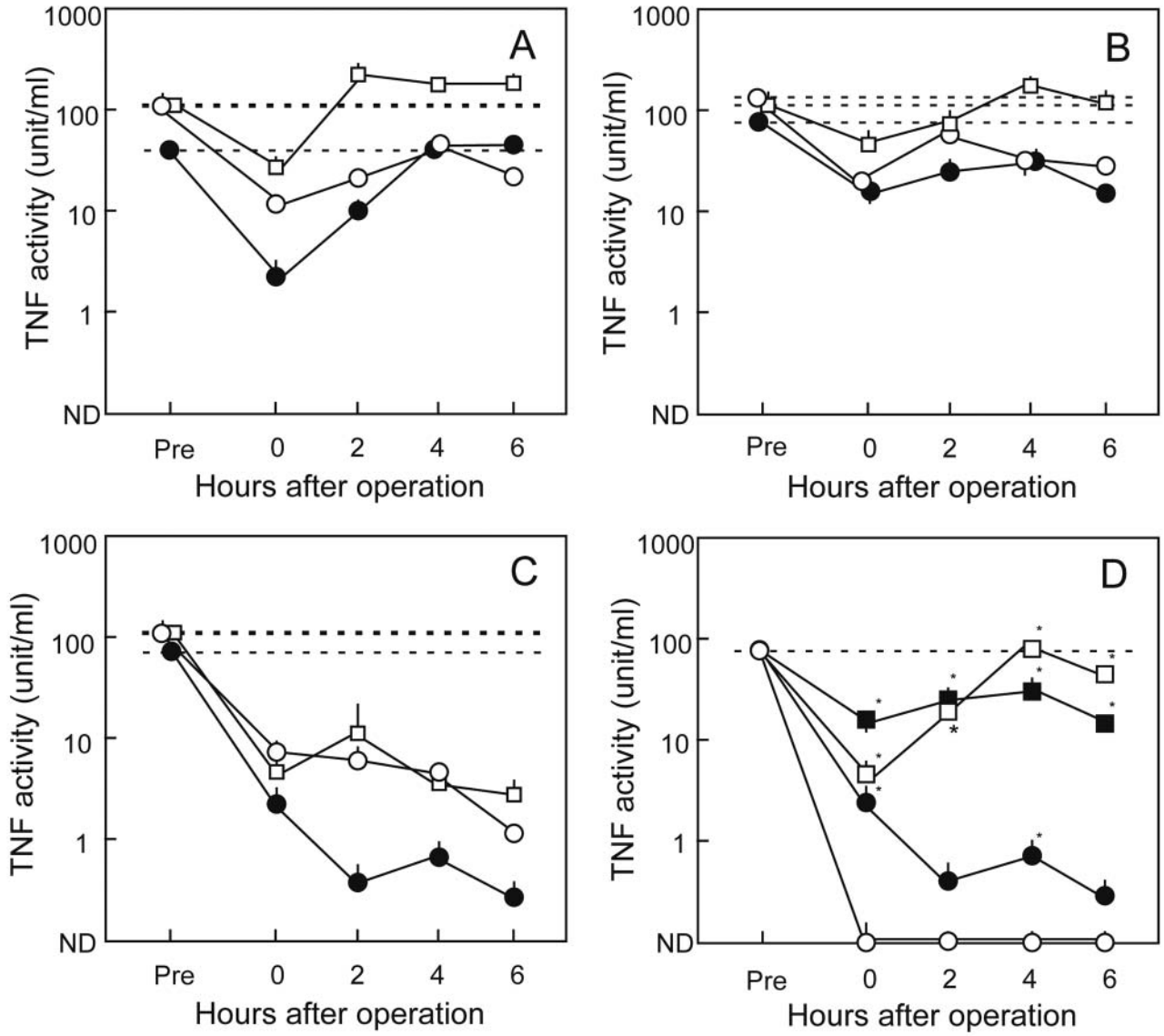

Figure 2. *Time course for endogenous TNF production induced by OK-432 after laparotomy in mice. A) The effect of pre-administration of TNF 3 h before the operations: endogenous TNF production in livers (○), serum (●), and spleens (□) were shown. B) The effect of pre-administration of OK-432 (0.1 KE) 3 h before the operations: endogenous TNF activity in the livers (○), serum (●), and spleens (□), were shown. C: The effect of just-administration of TNF after the operation: endogenous TNF production in livers (○), serum (●), and spleens (□). D: All serum data from A, B and C are assembled for statistical analysis. Untreated (non-primea) control (○), just-administered of TNF (●), pre-administration of TNF (□), pre-administration of OK-432 (■). Symbols and bars indicate averages and standard deviations. Significant differences (*$p<0.01$) between the average of the non-primed control and the primed samples for each time period.*

induction of the primed stage is critical for reducing the mortality rate from *Staphylococcus aureus* infections following surgical operations.

Inhibition of tumor metastases in the lung after surgery by administering priming agents. To investigate the protective effect of priming agents for reducing metastases after surgery, test mice were administered priming agents before surgery and were then injected with tumor cells. As shown in Table I, the number of lung foci in the mouse group with TNF (1000 units/mouse) or OK-432 (0.1 KE) administration 3 h before the laparotomy was significantly decreased. By contrast, the mouse group in which TNF was administered just before the laparotomy tended to have an increase in metastatic foci ($p=0.058$). These data demonstrate that the creation of a primed stage could be obtained by the appropriate administration of priming reagents and that this provided protection from metastases after surgery.

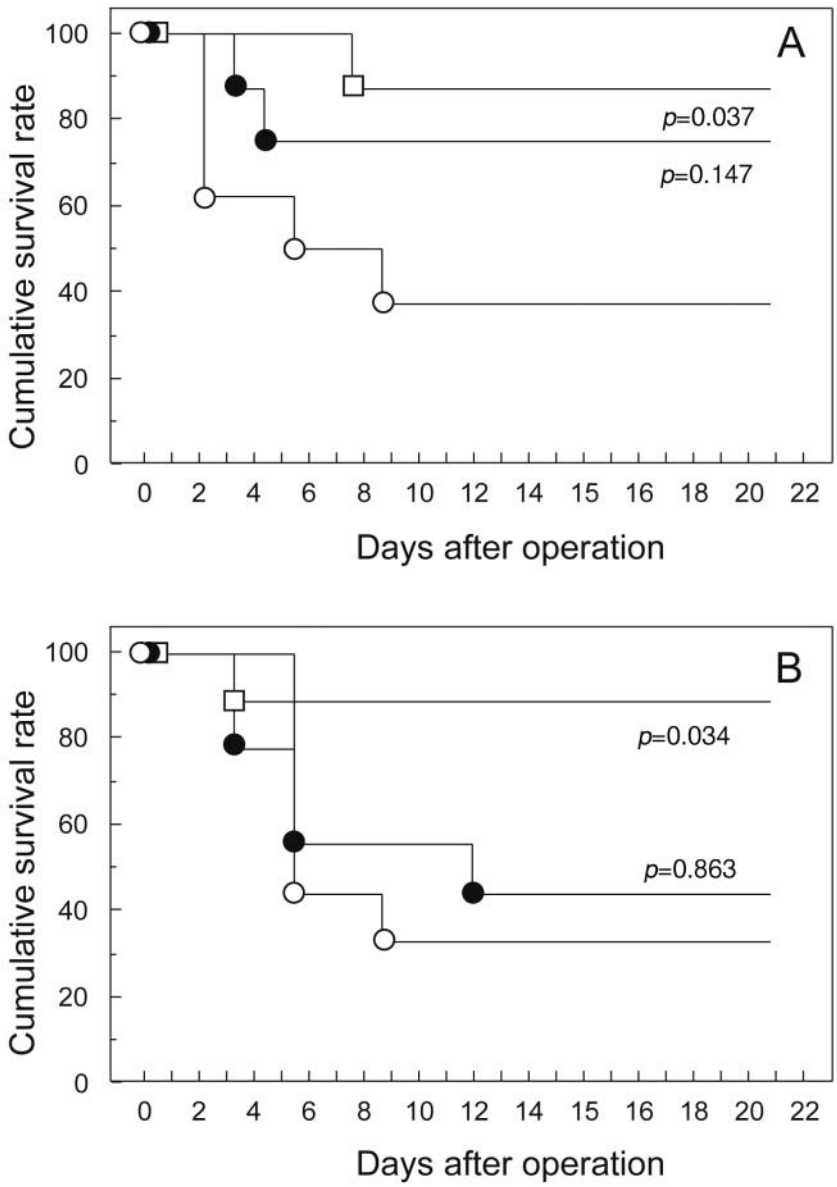

Figure 3. *Survival rate after Staphylococcus aureus infection. A) Survival rates were observed for the three groups: the control (○), 0 h TNF (●), and 3 h TNF (□). B) Survival rates were observed for the group three with the control (○), 0 h OK-432 (●), and 3 h OK-432 (□). Survival was analyzed with the Kaplan-Meyer method, and statistical significance between control and test groups was calculated by the log-rank test.*

Discussion

Some surgical operations and/or severe trauma can cause additional damage. Surgery to resect tumors may disperse tumor cells into the blood stream, which is probably one reason why distant metastases sometimes happen after surgery. Because of the impairment of immune function caused by surgery, tumor cells might easily establish micro metastases in distal tissues during the post-operative period when they could evade the host defense system. Several reports have shown a relation between surgical stress and immunosuppression (1, 5, 8, 24), but there are few reports that have demonstrated success with any treatment to avoid immune suppression from the surgical stress. As a result, extreme caution is required to protect against infection or tumor progression in compromised hosts after a surgical operation. In this paper we demonstrated the efficacy of a method that prevented the detrimental side-effect of surgical stress. This method relied on the administration of priming agents prior to surgery.

Table I. *Anti-metastatic effect of priming agents after Meth A tumor cells were injected into laparotomized mice.*

Laparotomy*	Treatment	Number of metastatic foci Average±SD	*p*-value**
+	Saline	54.3±25.6	
–	Saline	37.4±20.0	0.189
+	TNF-SAM2 (0 h, 1000 units)	83.5±51.4	0.058
+	TNF-SAM2 (3 h, 1000 units)	10.1±10.7	0.0082
+	OK-432 (3 h, 0.1 KE)	0±0	0.0004

The number of metastatic foci on the lungs were counted after 21 days of Meth A fibrosarcoma inoculation. *– indicates no laparotomy operation, + indicates laparotomy operation. **Statistical analysis between laparotomy control and other treatment groups were performed using Student's *t*-test.

The primed stage was used as the parameter of macrophage condition in surgically operated animals. The present investigation demonstrated that laparotomy significantly suppressed TNF production, and that appropriate administration of priming agents prior to surgery prevented the suppression of macrophage activity as measured by TNF production. The dose and timing of priming agents to overcome the suppression caused by surgery is quite similar to that used for achieving optimal TNF production following the administration of OK-432 (22, 23). For example, the optimal dose and timing of recombinant TNF is 1000 units/head 3 h before OK-432 administration (22). Therefore, determining the conditions for optimal administration of the priming agents is very important for obtaining the maximum effect for avoiding immune suppression caused by surgery. In clinical settings, we demonstrated that TNF or PPD administered 3 h before administration of OK-432 could induce endogenous TNF in tumor patients (25, 26).

One of the mechanisms of immunological suppression caused by surgical operations has been attributed to neuro-endocrine regulation by the pituitary gland. This is because serum cortisol, which is well known as a suppressive factor to macrophage functions, increased during a 6-h period after the surgical operations (27, 28). Macrophage suppression in our study also occurred immediately and continued for 6 h after the operation (Figures 1 and 2). This suggests that one of the suppressive factors of macrophage function may include cortisol.

There are two macrophage-activated stages involved in TNF expression, *i.e.* the primed and the triggered stages. In the triggered stage, mature TNF is secreted by macrophages after triggering agents such as LPS have been administered. By contrast, the primed-stage macrophage has membrane-bound TNF (pro-TNF), but does not release mature TNF (29, 30). Interferon-γ, TNF, LPS, and OK-432 can induce the primed stage transiently (16, 17, 22, 23). In this study, 0.1 KE of OK-432 had a significant protective effect against *Staphylococcus* infection (Figure 3-B). This dose of OK-432 (0.1 KE) was too small to produce mature (soluble) TNF, but was adequate to induce a primed stage in mice (22, 23). These data suggest that when macrophages are in the primed stage, they play a key role in protection against death from bacterial infection.

It has been reported that murine tumor metastases were significantly promoted when more than 10 μg of human TNF was administered in conjunction with an injection of tumor cells in the time period from 5 h before injection to 1 h afterwards. Promotion did not occur when less than 2 μg of TNF was administered (31). Our results showed a significant suppression of tumor metastasis when 1000 units (0.23 μg) of human TNF had been injected 3 h before surgery (Table I), but not for injections that were administered immediately before the operation. Optimal priming doses of TNF have been reported as 100 to 1000 units (0.023-0.23 μg) per mouse. These data suggest that higher amounts of TNF should be avoided for use as priming agents in surgery, especially for tumor resection, because higher amounts of TNF were not optimum for producing the primed condition and could increase the risk for enhanced tumor metastases.

We found that primed macrophages expressed the precursor TNF, which formed 26 kDa membrane-bound proteins on cell surfaces. Membrane-bound TNF can act as a ligand and cause intracellular signals in TNF-receptor-II expressed cells (32). Moreover, membrane-bound TNF is known to act as the receptor that transmits signals after ligation with TNF receptors expressed on the surface of macrophages (it is called as reverse signal) (14, 29, 30, 33, 34). Thereafter, they transmit this primary information to neighboring cells by membrane-bound TNF by cell adhesion. Thus, it is possible for macrophages, which are distributed in every organ and tissue, to act as sentinels by cross talking with each other, thus assuring cellular integrity of the body from internal and/or external changes. We have hypothesized that there is a network composed of tissue macrophages, which we have named a "macrophage network" (12).

When in the appropriate physiological condition, tissue macrophages have the potential to produce TNF after treatment with triggering reagents such as LPS or OK-432. This means that the physiological status of macrophages in the body would be somehow primed with respect to TNF production. Thus, it appears that preserving the primed stage

is important for maintaining homeostasis. The present study provides suggestive evidence that "the macrophage network" acts as a regulatory system that protects against disorders of the body due to invasive disruptions such as surgery. However, additional research is required to fully understand the biological significance of the macrophage network and the primed stage of macrophages. For example, each type of tissue macrophage behaves differently.

The phenotype of intestinal macrophages is very different from other macrophages. Intestinal macrophages have phagocytotic activity, but no CD14, TLR-4, or Fc-receptor expression. Moreover, they are hyporesponsive to LPS and other macrophage-activating agents (35, 36). Cellular and/or molecular analyses of tissue macrophages will be necessary to clarify the biological significance of macrophage networks and the primed stage of macrophages.

The "macrophage network" is still hypothetical. Nevertheless, the concept can still be used to develop novel approaches for the treatment of various intractable diseases: a particularly attractive research target are tumors with tumor-associated macrophages (TAMs), it has been reported that there is a better prognosis when the tumor contained higher amounts of TAM (37-39).

Conclusion

Using the surgical-stress model it was demonstrated that maintenance of a primed macrophage stage is essential for maintaining integrity of the body. The existence of a primed stage provides indirect evidence of how the macrophage network behaves. This concept may lay the groundwork for a new era for therapeutic and/or protective approaches for intractable diseases. A greater understanding of this phenomenon can be obtained by targeting further studies on both the signal-transduction mechanism of membrane-bound TNF and the biological characteristics of tissue macrophages.

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