Responses of hepatic TNF-\(\alpha\) mRNA to repeated hemorrhage in the conscious rat

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Yamashita, Masatomo, and Mamoru Yamashita. Responses of hepatic TNF-\(\alpha\) mRNA to repeated hemorrhage in the conscious rat. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E27–E31, 1998.—Trauma victims may suffer from repeated hemorrhage, but responses of cytokines to it have not been described. To study this question, we first detected the time course of changes in serum tumor necrosis factor (TNF) activity and hepatic TNF-\(\alpha\) mRNA by cytotoxicity against L929 cells and by reverse transcription (RT) and polymerase chain reaction (PCR), respectively, after different sizes of hemorrhage (10–20 ml/kg) with chronically cannulated rats. Then we examined the changes in TNF-\(\alpha\) mRNA when two sequential 10 ml/kg hemorrhages were performed. TNF activity showed no significant increases after either size of hemorrhage. At mRNA level, both 15 ml/kg and 20 ml/kg hemorrhages induced significant increases after hemorrhage, whereas a 10 ml/kg hemorrhage did not. When the 10 ml/kg hemorrhage was repeated 24 h later, however, TNF-\(\alpha\) mRNA showed a significant increase. There were no significant differences in blood pressure and heart rate after single and repeated 10 ml/kg hemorrhage. This potentiation persisted for \(\geq 48\) h. These results show that responses of hepatic TNF-\(\alpha\) mRNA are augmented when moderate hemorrhage is repeated.

Cytokines are small polypeptides or glycoproteins secreted by a cell that affects growth and metabolism either of the same (autocrine) or of another (paracrine) cell. Since 1980, when interferon-\(\alpha\) became the first cytokine to be isolated, the number has grown to \(\geq 50\) distinct molecules (1). Cytokines show pleiotropy and redundancy in their actions and form a complex network by either inducing or suppressing the expression of other cytokines. In responding to external stimuli, the vertebrates set in motion molecular and cellular interactions designed to facilitate a return to physiological homeostasis and to aid tissue repair, and the cytokine network is deeply involved in these complex interactions. Tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) is one of the proinflammatory cytokines; it has a great influence on deciding temporal and spatial patterns of inflammatory responses. Its role in the development of organ failure after massive hemorrhage as well as sepsis is strongly suggested (3, 12, 26, 29).

In clinical settings, trauma victims often suffer simultaneously from different kinds of stimuli, including pain, tissue injury, and hemorrhage. They may also suffer from repeated stimuli, for example, rebleeding. Therefore, it is important to study the physiological responses to the combination of stimuli and to repeated stimuli, because the responses may differ from those observed after a single stimulus. It is well-known that the hypothalamic-pituitary-adenal (HPA) axis shows a potentiated response to repeated hemorrhage as well as to a combination of stimuli (5, 13, 17, 18). Because the cytokine network is subject to endocrine and neural control and exerts a reciprocal effect on neuroendocrine systems (19), responses of the cytokine network may be modified by a combination of stimuli and by repeated stimuli. It has already been reported that the cytokine network shows a potentiated response to the combination of trauma and hemorrhage (3). To our knowledge, however, responses of the cytokine network to repeated hemorrhage have not yet been described. To study this question, we first detected the time course of changes in serum TNF activity and hepatic TNF-\(\alpha\) mRNA after different sizes of hemorrhage (10–20 ml/kg) by using chronically cannulated conscious rats. Then we examined the changes in hepatic TNF-\(\alpha\) mRNA when rats were subjected to two sequential 10 ml/kg hemorrhages spaced 24–72 h apart.

MATERIALS AND METHODS

Animal preparation. Male Wistar rats (Charles River, Tsukuba, J apan), weighing 300–400 g, were anesthetized with pentobarbital sodium (50 mg/kg ip), and a femoral arterial cannula was placed using sterile procedures as previously described (11). After recovery, all rats were housed individually in a temperature-controlled environment with a 12:12-h light-dark cycle and were allowed to move freely. Access to food and water was given ad libitum. On the morning of the 4th day after cannulation, the following three experiments were begun. All procedures were performed by manipulating the cannulas outside the cage so as not to disturb the rats in any way. All the experiments were approved by the Animal Care Committee of the University of Tsukuba.

Experiment 1. Ninety chronically cannulated rats were subjected to either 10, 15, or 20 ml/kg hemorrhage over 3 min, and the blood shed into a sterile heparinized (25 U) syringe was reinfused 60 min later. At time 0 or 0.5, 1, 2, 4, or 24 h after hemorrhage, rats were killed, and livers were removed and snap-frozen in liquid nitrogen for extraction of total cellular RNA (\(n = 5\) livers for each time point). Immediately before the slaughter, 0.4 ml of blood was withdrawn from the arterial catheter for measurement of TNF activity by cytotoxicity against L929 cells (24). An endotoxin-treated rat, killed 2 h after 100 g/kg iv endotoxin, was used as a positive control (Escherichia coli lipopolysaccharide, serotype 0111:B4; Sigma Chemical, St. Louis, MO). The time course of changes in TNF-\(\alpha\) mRNA was detected by reverse transcription (RT) and polymerase chain reaction (PCR), as previously described (28, 29). Briefly, the first strand of cDNA was synthesized by reverse transcriptase (MVML-T, GIBCO BRL, Rockville, MD) and pooled. The resulting cDNA samples were adjusted to PCR buffer conditions and were run for PCR simultaneously. The primers for TNF-\(\alpha\) were 5'-TCAGCCTCTTCTC-
ATTCTGTCG and 5'-GTGCAAGCATGTGTTGTGTT (15). A threetemperature step PCR cycle program was carried out with an OmniGene Temperature Cycler (0.5 min annealing at 54°C, 1 min extension at 72°C, and 1 min denaturation at 94°C). The length of the PCR product was 203 base pairs and was checked by electrophoresis with 1 kb DNA Ladder (GIBCO BRL). After PCR, the products were quantitated by HPLC as previously described (28). Twenty-five to thirty-five PCR cycles were run to determine the dependency of the number of PCR products on cycle numbers. Linearity between the amount of amplified cDNA and that of applied RNA was checked in the preplateau exponential phase. The relative multiple-increase changes in TNF-α mRNA at each time point after hemorrhage compared with the level of normal untreated rats were determined after normalization for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The primers for GAPDH were 5'-TCCTTGGAGGCATGTAGGCCAT (28).

Experiment 2. First, to detect the changes in mean arterial blood pressure (MABP) and heart rate (HR), fifteen chronically cannulated rats were divided into three groups and were subjected to either a 10 ml/kg hemorrhage or sham hemorrhage on two successive days; these were the HH group (10 ml/kg hemorrhage on days 1 and 2), the SH group (sham-hemorrhage on day 1 and a 10 ml/kg hemorrhage on day 2), and the SS group (sham-hemorrhage on days 1 and 2). The changes in MABP and HR were recorded on day 2 by connecting the arterial cannula to a hemodynamic monitor (Life Scope II, Nihon Kohden, Tokyo, Japan) through a high-pressure transducer (Gould, Oxnard, CA). Another 75 cannulated rats were divided into the three (HH, SH, and SS) groups for detection of the changes in hepatic TNF-α mRNA. Rats were killed and livers were removed on day 2 at time 0 or at 0.5, 1, 2, and 4 h for extraction of RNA (n = 5 for each group). The time course of changes in hepatic TNF-α mRNA was detected by the method described in experiment 1.

Experiment 3. Ten chronically cannulated rats underwent two sequential 10 ml/kg hemorrhages spaced by 48 and 72 h (n = 5 for each group). Rats were killed and livers were removed 1 h after the second hemorrhage for extraction of RNA. The increases in TNF-α mRNA were detected by the method used in experiment 1 and were compared with the results in experiment 2.

Statistics. Data were analyzed by two-way ANOVA, corrected for repeated measures over time. Differences between HH, SH, and SS groups at the individual time points were analyzed by the Newman-Keuls test after two-way ANOVA. Differences between control, single, and repeated 10 ml/kg hemorrhage were also analyzed by the Newman-Keuls test after two-way ANOVA. Significant differences are indicated (P < 0.05).

RESULTS

Experiment 1. One blood sample taken 2 h after 20 ml/kg hemorrhage showed 0.2 U/ml TNF activity, but all the other samples showed no detectable TNF activity (<0.1 U/ml). Thus the changes in TNF activity were not significant after either size of hemorrhage. On the contrary, the blood sample taken 2 h after endotoxin treatment showed >64 U/ml.

When reverse-transcribed RNA from an endotoxin-treated rat was subjected to an increasing number of PCR cycles, a period of exponential increase in PCR products was followed by a period of saturation between 25 and 35 cycles. At 29 cycles, good linearity between the amount of reverse-transcribed RNA and that of the resulting PCR product was acquired over a range of 0.0063–0.2 µg. When 0.2 µg of reverse-transcribed RNA from hemorrhaged rats was amplified by RT-PCR at 29 cycles, the peak areas of the PCR products were within the linear range. All further PCR experiments were performed using 0.2 µg of reverse-transcribed RNA at 29 cycles. Reproducibility of PCR was good, and the PCR products for TNF-α and GAPDH were well quantitated by HPLC (data not shown). Both 15 ml/kg and 20 ml/kg hemorrhage led to rapid and significant increases in TNF-α mRNA in the liver (Fig. 1). The increases peaked 1 h after hemorrhage and then fell to near control levels by 24 h. After 10 ml/kg hemorrhage, however, the increase in TNF-α mRNA was small and not significant compared with the pre-hemorrhage level.

Experiment 2. MABP and HR remained constant in the SS group throughout the experiment. However, in the HH and SH groups, 10 ml/kg hemorrhage caused a prompt decrease in both MABP and HR (Fig. 2). By 1 h after hemorrhage, MABP and HR recovered to the prehemorrhage level and stabilized. The responses of MABP and HR were similar in the HH and SH groups, and there were no significant differences between these two groups at any time point. The changes in TNF-α mRNA after hemorrhage were small and not significant in the SS and SH groups, whereas the changes were significant in the HH group; the peak level in the HH group was ~70% of that after single 15 ml/kg hemorrhage (Fig. 2, bottom). The differences between the HH and SH groups were significant at 0.5 and 1 h.

Experiment 3. When two sequential 10 ml/kg hemorrhages were spaced 48 h apart, the increase in TNF-α mRNA at 1 h was still significant, whereas when two sequential hemorrhages were spaced 72 h apart, the increase in TNF-α mRNA was small and not significant compared with the prehemorrhage level (Fig. 3).

DISCUSSION

In traumatized patients, control of bleeding is sometimes difficult, and bleeding may occur repeatedly. In such cases, the responses of the neuroendocrine sys-
system, including the HPA axis and the sympathoadrenal system, are augmented, even if hemorrhage size is small (17, 18). To our knowledge, the response of the cytokine network to repeated hemorrhage has not yet been described. In this study, single 10 ml/kg hemorrhage did not induce a significant increase in hepatic TNF-α mRNA. When the same size of hemorrhage was repeated 24 h later, however, TNF-α mRNA showed a higher peak, and the increase after hemorrhage was significant. These facilitative effects of the first hemorrhage persisted for \( \geq 48 \) h. These results show that the cytokine network also shows a potentiated response when moderate hemorrhage is repeated.

It is now widely believed that TNF-α is a crucial factor in the development of the systemic inflammatory response syndrome (SIRS) and organ failure after surgical insult (4, 12). Many studies have demonstrated elevated circulating levels of TNF-α in patients with clinically overwhelming infections and in animals subjected to intravenous injections of live bacteria or endotoxin (6, 27). However, in clinical SIRS and even in animal models, increases in serum TNF-α concentrations are often modest or absent (2, 7). Our previous study also showed that neither hemorrhagic shock (MABP = 40 mmHg for 60 min) nor 20 ml/kg hemorrhage induces significant changes in serum TNF activity (29). It seems that circulating levels of TNF-α do not always reflect the magnitude of insult. One of the reasons for these results is that induction of TNF-α is usually transient. Its mRNA is unstable because of an adenine/uracil-rich sequence in the 3'-untranslated region, and both protein and message are easily degraded when its role is finished (1). Another reason is that TNF-α mainly acts at local sites in an autocrine and/or paracrine manner. Interestingly, a recent study has shown that gene expression of α2-acid glycoprotein in the liver correlates well with intrahepatic TNF-α abundance but not with circulating TNF-α levels in cecal ligation and puncture models of rats (2). Liver is a major site for synthesis of TNF-α as well as of acute phase proteins and blood coagulation factors (10, 22). Although TNF-α mRNA levels may not accurately reflect protein secretion, they correlate with TNF synthesis in general (1, 6). On the basis of these findings, we detected the changes in hepatic TNF-α mRNA in this study.

**Fig. 2.** Time course of changes in mean arterial blood pressure (MABP; top), heart rate (HR; middle) and hepatic TNF-α mRNA (bottom) in three groups characterized by type of 10 ml/kg hemorrhage on 2 successive days: HH (hemorrhage on days 1 and 2), SH (sham hemorrhage on day 1 and hemorrhage on day 2), and SS (sham hemorrhage on days 1 and 2). Values are means ± SE (n = 5). *P < 0.05 between HH and SH groups by Newman-Keuls test after ANOVA.

Responses of the mammalian body to external stimuli are exquisite and complicated, and both feedback inhibition and facilitation can occur after repeated hemorrhage. The changes observed after the second hemorrhage are the result of a complex interplay of the facilitative and feedback effects elicited by the first stimulus (17, 18). For example, the dog shows an inhibition of adrenocortical response after two sequential 10 ml/kg hemorrhages spaced 90 min apart, whereas it shows a facilitative response after two 7.5 ml/kg hemorrhages spaced 24 h apart (13, 18). Which phenomenon, inhibition or facilitation, is dominant is influenced by many factors, including magnitude of hemorrhage, rate of hemorrhage, interval of sequential hemorrhages, anesthesia, and dietary factors (18). When a delay of 24 h is selected between two small hemorrhages, this period provides an adequate time for resolution of the feedback effects after the first hemorrhage and reveals the residual facilitative effects in the HPA axis (18). We also adopted an interval of 24–72 h between two sequential 10 ml/kg hemorrhages in this study. Another important factor is an interval between cannulation and hemorrhage. We began the hemorrhage experiments on the morning of the 4th day after

**Fig. 3.** Relative multiple increase changes in TNF-α mRNA 1 h after single and repeated 10 ml/kg hemorrhage. Pre, prehemorrhage level; SH and HH, levels at 1 h in SH and HH groups of exp 2, respectively; 48 and 72, levels 1 h after 2 sequential 10 ml/kg hemorrhages spaced 48 and 72 h, respectively. Values are means ± SE (n = 5). *P < 0.05 vs. prehemorrhage level; #P < 0.05 vs. SH level, both by Newman-Keuls test after ANOVA.
anesthesia and cannulation, whereas experiments were performed immediately after anesthesia and cannulation in most of the other studies detecting the changes in cytokines. In such an acute setting, the first stimuli of anesthesia and surgical intervention may exert complex influences on the following treatment and may obscure a net effect of the treatment. Accordingly, chronically cannulated rats like ours seem to be more adequate for analysis than acutely prepared models.

"Two-hit theory" has been proposed in the development of organ failure after surgical insult (12). According to this theory, priming of monocytes/macrophages is achieved after the first insult. When the second attack is delivered in this state, even if it is not so severe, primed cells produce excessive inflammatory mediators and bring about organ failure. Although excessive production of TNF-α may harm the body, induction of TNF-α to external stimuli is originally a teleological response. For example, mild endotoxin administration results in the release of TNF-α into the local tissue milieu, where TNF-α initiates and orchestrates many of the beneficial responses aimed at improving antimicrobial function and reducing tissue damage (21). The physiological significance of cytokine induction after hemorrhage is unknown, but one possibility is activation of the blood coagulation system to avoid further blood loss. Although vascular endothelial cells are antithrombogenic in the normal state, they lose their antithrombogenic properties when they are perturbed by cytokines (10). We have shown that clotting time shortens and plasminogen activator inhibitor (PAI) activity increases after 20 ml/kg hemorrhage and that both tissue factor mRNA and PAI-1 mRNA are induced in the liver (28, 30). The augmented responses of hepatic TNF-α mRNA to repeated hemorrhage may prepare the body for prompt hemostasis.

The mechanism whereby augmented responses of hepatic TNF-α mRNA occur to repeated hemorrhage is also unknown. In this study, the responses of MABP and HR were similar in the SH and HH groups, and no significant differences were noted between these two groups. Although there is a possibility that we could not detect small hemodynamic differences by monitoring MABP and HR, blood volume is completely restored within 24 h after 10 ml/kg hemorrhage even without reinfusion (14), and no change in blood volume was detected in experiments using a similar hemorrhage and reinfusion protocol (McCloud and Gann, unpublished observations cited in Ref. 17). Therefore, it is unlikely that the potentiation in hepatic TNF-α response was due to differences in the hemodynamic response. After repeated exposure to endotoxin, suppressed TNF-α production by macrophages is observed (16), and this "endotoxin tolerance" is reported to be associated with alterations in guanine nucleotide binding protein activity and in arachidonic acid turnover (8, 23). Tolerance to myocardial ischemia is also reported, and this phenomenon seems to be associated with alterations in receptor subtypes and with phosphorylation of intracellular enzymes (20). The augmented response of hepatic TNF-α mRNA to repeated hemorrhage may also result from alterations in intracellular signaling pathways at several levels. Recently, it has been shown that gene expression of TNF-α is transcriptionally regulated by nuclear factor-kB (NF-kB), a transcription factor that is activated by a variety of conditions, including oxidant stress and endotoxin (9, 25). In response to these stimuli, IκB, an inhibitory protein, is removed from the latent NF-kB, and the active transcription factor is then translocated from the cytoplasm to the nucleus, where it binds to a specific cis-acting regulatory element in target genes. Interestingly, in vitro studies have shown that NF-kB is activated by TNF-α (25) and that dexamethasone treatment diminishes the basal nuclear level of NF-kB (9). Changes in nuclear factor as a consequence of prior stimulation might play a role in the augmented response of hepatic TNF-α mRNA to repeated hemorrhage. Further study is needed to elucidate the precise mechanism.

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