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Experimental Study on the Onset of Traumatic Disseminated Intravascular Coagulation: Changes in the Coagulofibrinolytic System, Blood Cytokine Levels and Histopathological Findings Following Hemorrhage in Rats

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In order to produce an experimental animal model of disseminated intravascular coagulation (DIC) related to traumas, rats were subjected to hemorrhage and the onset of DIC was investigated in terms of platelet count, coagulofibrinolytic activity, cytokine levels, and histopathological findings following hemorrhage. Biological invasion, including trauma and hemorrhage, induces systemic inflammatory response syndrome (SIRS) through inflammatory cytokines produced and released in the body. When these cytokines affect the vascular endothelium, tissue factor is released and exogenous coagulation is instituted. We investigated the effects of hemorrhage in rats, based on an animal experiment report of marked cytokine production occurring after hemorrhage. Changes in platelet count and the coagulofibrinolytic system showed values corresponding to DIC on the basis of Matsuda's DIC diagnostic standards. Histopathological findings characteristic of DIC together with these changes enabled the onset of DIC to be identified. Cytokine levels also increased significantly. Similar results were obtained in rats administered endotoxin, and as a consequence, the onset of DIC was confirmed. This simple and easy hemorrhage method has the advantage of allowing invasion to be determined quantitatively as well. We anticipate that this method will be useful as a trauma-related DIC animal model.

Introduction

Patients suffering from a serious trauma frequently exhibit coagulofibrinolytic abnormality and occasionally develop disseminated intravascular coagulation (DIC). The onset of DIC varies according to the primary disease and DIC has complicated underlying pathophysiological properties[1)]. Although early diagnosis and early treatment are desirable, especially for DIC following a trauma, many aspects of the pathogenic mechanism of DIC, including the time when the pathological state of trauma changes to DIC, have yet

to be elucidated.

A disease called systemic inflammatory response syndrome (SIRS) has been suggested to explain a state of biological systemic reaction to invasion recently[2]. This has been understood to be a syndrome occurring as a result of inappropriate or excessive biological response. Reactions of mediators, mainly inflammatory cytokines, induced by stimuli including infection, tissue damage and anoxia, have been reported to play the leading role in the pathogenic process[3]. In DIC, cytokines act on vascular endothelial cells to produce and release tissue factor and this institutes an increase in coagulation[4]. It has been proposed that DIC is part of the SIRS process[5]. We confirmed that the DIC resulting from a multiple trauma in the present patient corresponded to SIRS in all aspects[6].

To conduct a pathological study of traumatic DIC, an experiment was carried out to establish a trauma-related DIC animal model. Many reports have demonstrated increased blood inflammatory cytokine levels due to occurrences, such as trauma, hemorrhage, burn and infection, in humans and experimental animals[7)~9)]. Hence, we investigated the pathogenesis of DIC by determining changes in the coagulofibrinolytic system and examining histopathological findings following experimentally induced hemorrhage in rats, in accordance with a report of markedly increased cytokine production following hemorrhage[7]. A comparison was made with an endotoxin-administered rat DIC model[10].

Materials and Methods

The control and treatment of experimental animals used for this study was approved by the Institutional Committee.

Male Sprague-Dawley rats weighing 350 to 450 g (Jcl: SD retire, Clea Japan Inc., Otsu, Japan) were used for the experiment. After the rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), a polyethylene tube was inserted to indwell in the unilateral carotid artery for exsanguination and blood sampling.

Table 1 Changes in hemoglobin, platelet count, fibrin degradation product and antithrombin Ⅲ after hemorrhage and endotoxin administration

Coagulofibrinolytic system		Control	Hemorrhage			Endotoxin	
			4~6h	24h	n	4~6h	n
Hb	(g/dl)	16.33±0.247	11.49±0.45	7.92±0.43	21	12.86±0.42	14
Plt	($10^3/\mu l$)	90.30±1.55	70.22±2.34**↓	71.21±4.47*↓	21	21.16±5.99**↓	14
FDP	(μg/ml)	< 5	> 40**↑	> 40**↑	21	> 40**↑	11
Fibr	(mg/dl)	134.89±4.09	182.00±19.44*	215.33±29.88*	16	38.81±12.89**	11
AT Ⅲ	(mg/dl)	103.68±3.04	111.87±8.59	134.44±11.51	16	62.09±4.56**	11

Plt: platelet, FDP: fibrin degradation products, Fibr: fibrinogen, AT Ⅲ: antithrombin Ⅲ, Values are expressed as the mean±SEM, n: numbers of experimental animals., *$p<0.05$, **$p<0.01$, significantly different from the control.

Table 2 Changes in blood levels of interleukin-8 and tissue necrosis factor after hemorrhage and endotoxin administration

Cytokine	Control	Hemorrhage			Endotoxin	
		4~6h	24h	n	4~6h	n
IL-8 (ng/ml)	0.22±0.88	0.78±0.84*	0.09±0.01**	8	1.52±0.69*	8
TNF (pg/ml)	< 16.0	21.14±3.38*	20.14±3.03*	8	536.56±5.64**	11

Values are expressed as the mean±SEM, n: numbers of experimental animals., *$p<0.05$, **$p<0.01$, significantly different from the control.

The hemorrhage was made by bleeding of 30% of a predetermined total volume (calculated to present 8% of body weight) over a 15 minutes period through the carotid artery catheter[7]. At 4～6 and 24 hours after the hemorrhage the blood was sampled and the coagulofibrinolytic system and cytokine levels were measured. Interleukin-8 (IL-8) and tumor necrosis factor (TNF) levels

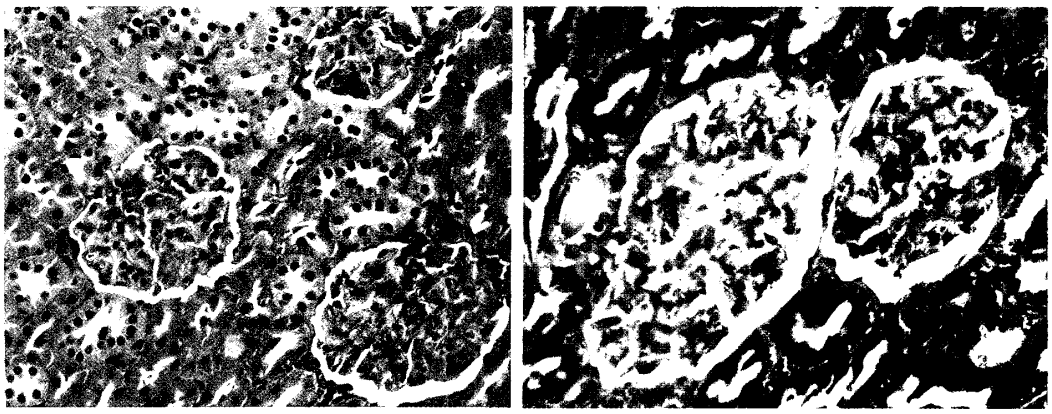

Fig. 1

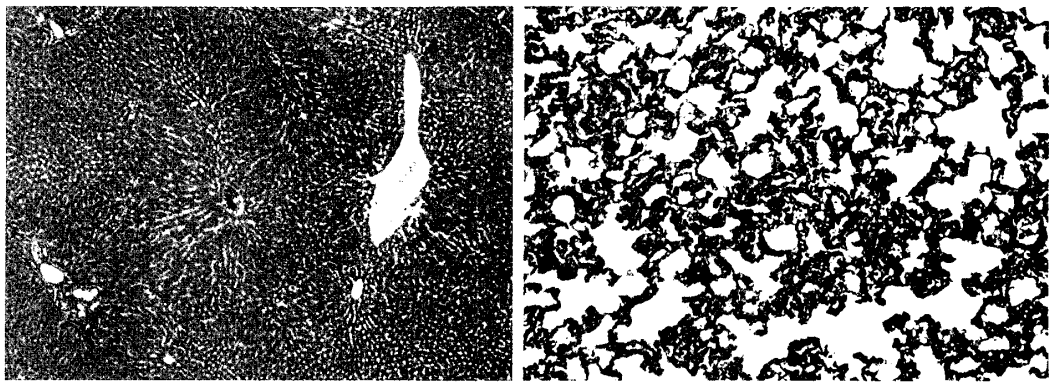

Fig. 2

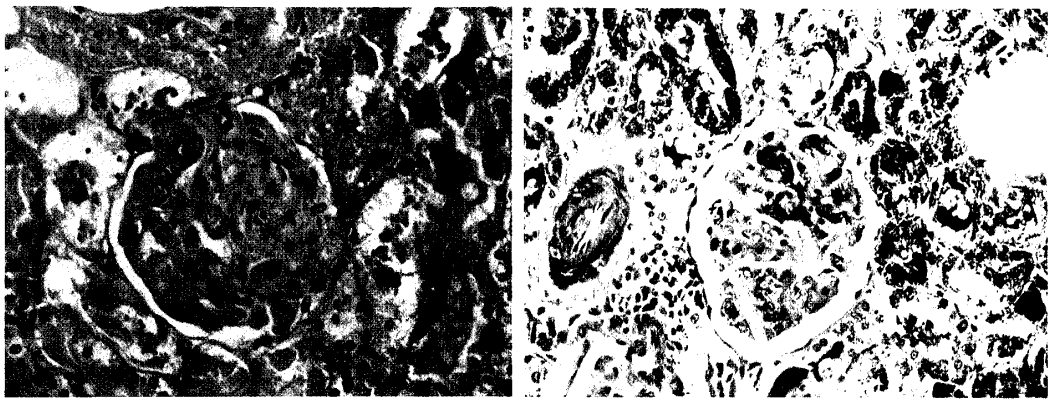

Fig. 3

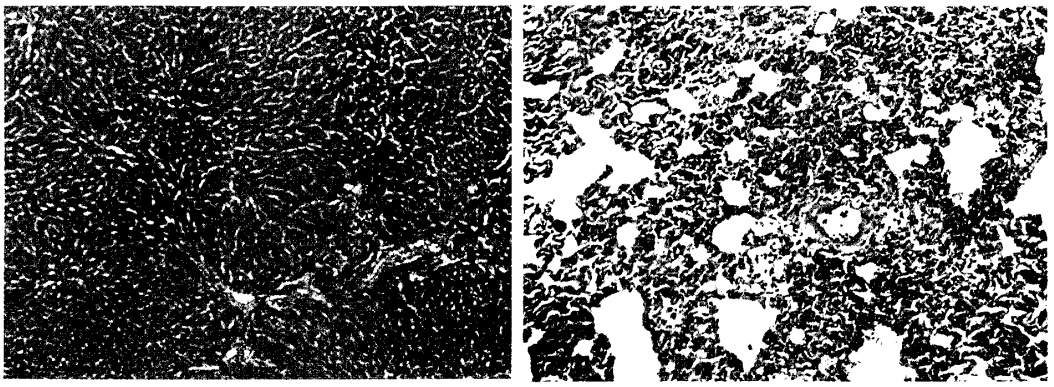

Fig. 4

were determined as inflammatory cytokines by measurement kit, EIA method PRF 081 (Panapharm Laboratories Co., Kumamoto, Japan) and rat TNF-αELISA kit (Biosource International Inc., Camarillo, California), respectively, and the coagulofibrinolytic systems were measured by serum fibrin/fibrinogen degradation products (FDP; FDP-E, MBL Co., Nagoya, Japan), platelet count (electric resistance method) and plasma fibrinogen (cyanmethohemoglobin method) and antithrombin III (AT III; spectrophotometry with coupler, SAT III, Daiichi-kagakuyakuhin, Tokyo, Japan). After the end of the experiment, the lung, kidneys and liver were isolated and fixed in 10% formalin and stored. Thin tissue sections were stained by hematoxylin-eosin (HE) and by phosphotungstic acid hematoxylin (PTAH) for fibrin and investigated histopathologically. Rats intravenously administered endotoxin have been established previously as a DIC animal model[10]. Effects of intravenous administration (10 mg/kg) of endotoxin (LPS, *E.coli*, DIFCO Laboratories, Detroit, Michigan) on the coagulofibrinolytic system, blood cytokine levels, and histopathological images were assessed for this endotoxin administration group as the comparative control of the hemorrhage group in the same way as in the hemorrhage.

All measurement values were expressed as mean ± standard error. For testing the significant difference between the groups, variance was tested using an F test and its uniformity was identified. A difference in the mean value between the groups was tested using the Student's t test with no correspondence. $p<0.05$ was determined to be the level of significant difference.

Results

Changes in platelet count and the coagulofibrinolytic system (Table 1)

Platelet count declined significantly ($p<0.05$) and FDP increased very significantly ($p<0.01$) following hemorrhage. No decrease in fibrinogen or AT III was observed. Following endotoxin administration, a significantly decreased platelet count ($p<0.01$), significantly increased serum FDP ($p<0.01$), and significantly decreased fibrinogen and AT III levels ($p<0.01$ and $p<0.05$, respectively) were observed.

Changes in cytokine levels (Table 2)

Both IL-8 and TNF levels increased significantly following hemorrhage ($p<0.05$). Following endotoxin administration as well, significantly increased IL-8 and TNF levels were observed ($p<0.05$).

Histopathological changes

Fibrin thrombus, tubular necrosis and tissue hemorrhage were observed in the kidney following hemorrhage (Fig. 1). Hemorrhages from tissue were noted in the lung and liver (Fig. 2). Histopathological findings obtained following endotoxin administration were similar to those following hemorrhage (Figs. 3, 4), but tended to be affected to a greater degree.

Discussion

In order to establish a trauma-related DIC animal model, the pathogenesis of DIC in rats subjected to hemorrhage was investigated. In DIC, fibrin is formed in the systemic microcirculatory

Fig. 1 The histopathological findings of renal tissue, 6 hours after hemorrhage (left: HE ×80, right: PTAH ×80)

Fig. 2 The findings of the liver and lung 6 hours after hemorrhage (left: liver HE ×80, right: lung HE ×80)

Fig. 3 The findings of renal tissue, 6 hours after endotoxin administration (left: HE ×80, right: PTAH ×80)

Fig. 4 The findings of the liver and lung 6 hours after endotoxin administration (left: liver HE ×80, right: lung HE ×80)

system in the presence of a basic disease by which the coagulofibrinolytic system is likely to be activated. Tissue factor is mainly involved in this disease. The tissue factor is produced when cytokines released at the time of biological invasion act on vascular endothelial cells, and exogenous coagulation reaction is initiated. This coagulation reaction is increased by dysfunction of the protein C-protein S inhibitory system and fibrinolysis inhibition via plasminogen activator inhibitor type I[11)~13)]. The coagulofibrinolytic system is further activated by the plasminogen activator produced by injured tissue, leading to the occurrence of hemorrhage[14)]. Furthermore, cytokines activate neutrophils, and act on tissue impairment by releasing elastase, free radicals and nitric oxide[15)~17)], and as a consequence, this, coupled with intravascular coagulation, causes multiple organ failure.

Since increased production of tissue factor via inflammatory cytokines is involved in the central mechanism of increased DIC coagulation factor under invasion, as mentioned above, we noted reports on animal experiments which described increased cytokine production following invasion[7)~9)]. We conducted an experiment in accordance with a reported rat experiment in which IL-1 production by peripheral blood monocytes increased to five times the normal value following hemorrhage of 30% of the total blood volume[7)].

TNF[18)]and IL-1[19)]released mainly by the monocyte and macrophage system following biological invasion, such as trauma, hemorrhage and shock, are involved in inflammation and immune reaction. IL-1 and TNF act on vascular endothelial cells in adjacent tissue causing them to release IL-8, liberate leukocytes, and induce adhesive factor expression[20)]. IL-6, induced by IL-1, plays an important role in the immune response[21)]. Via more than one network set up intercellularly, these cytokine signals regulate and control their mutual production and biological activity[22)]. In the present experiment, IL-8 and TNF were assessed with regard to these cytokine activities.

No international DIC diagnostic standards have been established, and various problems relating to the evaluation of DIC have not yet to be resolved although early diagnosis of the disease is desirable. It is internationally well-known that the "Ministry of Health and Welfare DIC Diagnostic Standards" has been established. Matsuda et al[23)] investigated the reciprocity of the main factors among these and proposed DIC diagnostic standards based on a combination of platelet count and FDP values[23)]. The results were consistent for all of 131 patients in the leukemia group on the basis of the Standard; and consistent for 91.5 % of 59 patients in the non-leukemia group. Increased FDP and decreased platelet count values following hemorrhage corresponded to DIC, strongly suggesting the onset of DIC. Cytokines IL-8 and TNF levels also increased significantly, which suggested the onset of DIC. Histopathological findings corresponding to DIC were obtained as shown below.

Histopathological findings of DIC include multiple thrombi, hemorrhage, ischemic lesion, and tissue injury[24)25)].

Multiple thrombosis formation

The most characteristic pathological finding of DIC is micro thrombi. Micro thrombi include platelet thrombi, fibrin thrombi, hyaline thrombi and globular thrombi, which are distinguished according to their constitution and time of formation. Platelet thrombi consisting of abundant platelets are formed at an early stage, after which fibrin thrombus rich in fibrin are formed, and gradually transform to hyaline thrombi uniform with no structure. The incidence of micro thrombi in DIC classified by the organ was highest for the kidneys (83%), and following this, the lung (76%) and spleen (51%), and in systemic

organs including the heart and liver[26]. After hemorrhage in the rats of the present experiment, fibrin thrombi were observed in the kidneys and lung. Similar histopathological findings were obtained for the endotoxin administration group as well, despite some differences in severity.

Hemorrhage

DIC is characterized by a tendency for various organs of the whole body to hemorrhage along with thrombi formation[14]. Dotted or macular hemorrhage is observed in many cases and frequently occurs in the skin, serous membrane, endocardium, and mucous membrane. The present experimental results showed tissue hemorrhage in the lung and liver.

Ischemic lesions

Frequent manifestations in DIC include parenchymal degeneration and focal necrosis due to circulatory injury with thrombi formation of microcirculation. Tubular necrosis in the kidney was observed in the present experiment. No essential difference was observed between the hemorrhage and endotoxin administration groups.

Tissue injury

The coagulofibrinolytic activity increases through the production and release of tissue factor by cytokines in DIC. Simultaneously, the cytokines activate neutrophils to make them release elastase, free radicals and NO and to induce adhesive factor expression and cause neutrophils to adhere to the vascular endothelium. The released elastase, free radicals and NO cause vascular endothelial or tissue injury[17].

The histopathological findings found in the present study were tissue images characteristic of DIC. The onset of DIC was identified in the hemorrhage rats from these findings and the changes in the coagulofibrinolytic system and blood cytokine levels.

In the rats administered endotoxin, the onset of DIC was identified based on changes in the platelet count and the coagulofibrinolytic system and those in blood cytokine levels. Moreover, these changes tended to be greater than in the hemorrhagically invaded rats under the present experimental conditions.

Conclusions

1. The onset of DIC was identified by examining the findings concerning the platelet count, the coagulofibrinolytic system, the blood cytokine levels and histopathological findings following trauma-related hemorrhage of rats.

2. The relationship between the pathogenic mechanism of DIC and SIRS was assessed.

3. Results for the rats following endotoxin administration similar to those for the rats after hemorrhage were obtained and the onset of DIC was identified in the endotoxin-administered animals as well.

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外傷性DICの発症に関する実験的研究—ラットにおける出血性侵襲後の凝固線溶系，血中サイトカインレベルの変化と組織病理学的所見—

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外傷に関連したDICの実験動物モデルを作製する目的で，ラットに出血性侵襲を加え，血小板・凝固線溶系，サイトカインレベルおよび組織病理学的所見について，DICの発症を検討した．外傷，出血等の生体侵襲は，生体に産生，遊離される炎症性サイトカインを介してSIRSの病態を発現させるが，このサイトカインが血管内皮に作用する時，組織因子を遊離して，外因性凝固反応が始動されることになるので，出血により顕著なサイトカインの産生の見られた動物実験の報告に準拠して，ラットに出血性侵襲を加えた時の影響について検討した．血小板・凝固線溶系の変化は，松田のDIC診断基準によりDICに相当する数値であり，DICに特徴的な組織病理学的所見と併せて，DICの発症を確認し得た．サイトカインも有意な増加が認められた．エンドトキシン投与ラットにおいても同様な結果が得られてDICの発症を確認した．この出血性侵襲の方法は簡便であり，侵襲を量的に規定できることも利点であり，外傷に関連したDIC動物モデルとしても使用し得ると考える．