Disorders of Hemostasis

Role of fibrinolysis in tissue-factor-induced disseminated intravascular coagulation in rats - an effect of tranexamic acid

We clarified the role of fibrinolysis in tissue-factor (TF)-induced rat disseminated intravascular coagulation (DIC) using tranexamic acid (TA). TA suppressed the elevation in D-dimer levels normally observed following TF-induced DIC, and an increase in organ dysfunction was seen. Enhanced fibrinolysis plays an important role in preventing the development of organ failure in TF-induced DIC.

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We recently reported that there is considerable difference in the pathophysiology of TF- and lipopolysaccharide (LPS)-induced DIC in rats. Briefly, we found a marked elevation in the level of D-dimer, without any organ dysfunction or much glomerular fibrin deposition (GFD) after administration of TF. In contrast, we observed a markedly prolonged period of elevated activity of plasminogen activator inhibitor (PAI), severe organ failure, as well as a markedly prolonged period of GFD, following LPS administration.[1-2] In the current study, we attempted to clarify the role of fibrinolysis in TF-induced rat DIC using TA, a known antifibrinolytic agent,[3] as well as to establish why organ failure does not develop in TF-induced rat DIC.

Male Wistar rats were obtained from Nippon SLC. One model of experimental DIC was induced by a sustained 4-hr infusion of 3.75 U/kg TF into the tail vein. Blood was withdrawn at 4, 8, and 12 hrs (rat numbers: n=7, 8 and 8, respectively). Another model of experimental DIC was induced by a sustained 4-hr infusion of 30 mg/kg LPS into the tail vein. Again, blood was withdrawn at 4, 8 and 12 hrs (rat numbers: n=7, 8 and 10, respectively). TA (50 mg/kg) was administered to rats from 30 min before the infusion of TF after which TA infusion was continued for another 4 hr, and blood was withdrawn at 4, 8 and 12 hrs (rat numbers: n=7, 10 and 12, respectively). After staining the renal tissue specimens with phosphotungsten acid hematoxylin, each sample was histologically examined.

Changes within the various parameters examined for the TF group, the LPS group, and the TF+TA group, are shown in Table 1. The three groups demonstrated similar platelet counts throughout the experiment. Plasma levels of fibrinogen decreased rapidly in the TF and LPS groups. A lesser degree of depression of plasma fibrinogen was observed in the TF+TA group than in the TF group. Plasma levels of TAT[4] increased sharply up to 4 hrs, after which a rapid decline was noted in all three groups. Plasma levels of D-dimer (detection limit <0.06 μg/mL) had increased sharply by 4 hrs in the TF group, whereas in the LPS group the plasma D-dimer increase was modest and followed by a slow decline. The rise of plasma D-dimer was completely suppressed in the TF+TA group. Plasma levels of creatinine further increased after infusion in the LPS group. No elevation of creatinine was apparent during the experiment in the TF group. Interestingly, in the TF + TA group, an incremental increase in plasma creatinine levels, similar to that observed in the LPS group, was seen. A similar trend was observed for the plasma levels of alanine aminotransferase (ALT). Marked GFD was a specific finding in the LPS group, whereas, mild and temporary GFD was a specific finding in the TF-induced DIC group. In the TF+TA group, GFD gradually increased from 4 to 12 hrs. While no rats in the TF-induced DIC model died during the experiment, some rats in the LPS-induced DIC model did so: 1/8 (12.5%) and 4/10 (40.0%) rats died in the 8 hr LPS and 12 hr LPS groups, respectively. In the TF+TA group, similar numbers of rats died: 2/10 (20.0%) and 4/12 (33.3%) rats died in the 8 hr TF+TA and 12 hr TF+TA groups, respectively.

Similar degrees of hemostatic activation were achieved in all three types of experimental DIC, since similar changes in plasma thrombin-antithrombin (TAT) levels were observed throughout the experiment. One of the most striking results was a sharp increase in the levels of D-dimer in TF-induced DIC; this increase was almost completely suppressed by exposure to TA. Interestingly, changes in D-dimer levels followed a similar time course in the TF + TA and LPS groups. In addition, increased GFD was observed with the addition of TA to the TF-induced model of DIC. It is reasonable to conclude that a decline in plasma D-dimer due to treatment with TA limits the thrombolysis of microthrombi that normally occurs in TF-induced DIC. While no rats died following TF-induced DIC, co-administration of TA with TF resulted in mortality rates similar to those observed following LPS-induced DIC. This result was probably due to the development of organ failure in the TF+TA model. In this study, a substantial dose of TA was administered in the DIC model, and we should mention that such treatment is

Table 1. Changes in hemostatic parameters, plasma levels of creatinine, ALT and GFD.

Parameter	*pre*	*4 hr*			*8 hr*			*12 hr*		
		TF	*TF+TA*	*LPS*	*TF*	*TF+TA*	*LPS*	*TF*	*TF+TA*	*LPS*
	(n=7)	*(n=7)*	*(n=7)*	*(n=7)*	*(n=8)*	*(n=8)*	*(n=7)*	*(n=8)*	*(n=8)*	*(n=6)*
PLT(×10³/μL)	692±34	279±22	274±19	223±11	268±31	246±25	252±15	210±28	185±17	221±23
Fbg(mg/dL)	234.1±7.9	<50.0	129.4±23.7°	<50.0	151.5±17.9	171.5±12.6	<50.0°	309.1±54.8	281.9±26.3	89.9±17.6*
DD(μg/mL)	<0.06	11.06±2.94	0.48±0.09°	1.98±0.47°	0.34±0.12	0.19±0.06	1.72±0.20*	0.21±0.08	0.30±0.13	0.97±0.24*
TAT (ng/mL)	2.8±1.0	100.9±16.2	109.2±18.5	125.0±14.7	20.2±6.4	24.3±9.4	38.9±12.6	7.9±3.1	10.8±4.8	19.5±10.8
Cr(mg/dL)	0.19±0.02	0.25±0.07	0.59±0.10*	0.38±0.06*	0.21±0.08	0.65±0.11°	0.64±0.13°	0.19±0.04	1.19±0.23°	1.05±0.16°
ALT (U/L)	45.7±3.2	54.3±4.7	62.5±4.1*	60.9±4.2*	48.9±4.8	115.7±12.5°	189.3±18.4°	47.1±11.6	214.8±54.6°	315.2±99.5°
GFD(%)	0.0±0.0	10.8±0.8	28.6±4.8*	77.1±5.6°	3.8±0.9	48.9±8.2°	74.7±5.3°	0.0±0.0	65.4±9.5°	71.4±7.8°

*Parameters were measured in 7 rats in the LPS group at 8 hr, because 1 of 8 rats died in this group. Parameters were measured in 6 rats in the LPS group at 12 hr, because 4 of 10 rats died in this group.Parameters were measured in 8 rats in the LPS+TA group at 8th hr, because 2 of 10 rats died in this group.Parameters were measured in 8 rats in the LPS+TA group at 12 hr, because 4 of 12 rats died in this group. PLT, platelet count; Fbg, fibrinogen; DD, D-dimer; TAT, thrombin-antithrombin complex; Cr, creatinine; ALT, alanine aminotransferase; GFD, glomerular fibrin deposition. Pre: no infusion TF: tissue factor (3.75 U/kg/4 hr), TF+TA: TF with tranexamic acid(50 mg/kg/4.5hr) , LPS:lipopolysaccharide (30 mg/kg/4 hr) *: p<0.05 compared with the TF group **: p<0.01 compared with the TF group.(mean±S.E.)*

formally contraindicated in most cases of clinical DIC. In conclusion, enhanced fibrinolysis plays an important role in preventing the development of organ failure in a model ofTF-induced DIC.

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Disorders of Hemostasis

Duplication of exon 13 causes one third of the cases of mild hemophilia A in northern Italy

A rearrangement of exon 13 in the factor VIII gene has been identified as the causative mutation in 32% of Northern Italian patients with mild hemophilia A. We have demonstrated that all share a common haplotype, thus suggesting that the mutation likely occurred in a single ancestor. To date, no predominant mutation has been identified in mild hemophilia A, therefore it would be extremely useful to carry out more extensive studies to ascertain whether the mutation is confined to northern Italy.

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Hemophilia A (HA) is an X-linked recessive disease, caused by wide-spread mutations in the factor VIII (FVIII) gene (*http://europium.csc.mrc.ac.uk*). Direct high performance liquid chromatography (DHPLC) screening detected a causative mutation in 9 of the 18 mild (FVIIIC≥5%) HA patients among a cohort of 31 patients referred to us from northern Italy. Since direct sequencing of the whole FVIII gene of 3/9 uncharacterized patients revealed no nucleotide alterations, and since these patients had high residual FVIII:C (>8%) activity, we hypothesized that there could be a mutational mechanism which: 1) skips the routine screening techniques; 2) is not as disruptive as the intron 1[1] and intron 22 inversions;[2] 3) does not cause severe damage to the FVIII protein. An unusual duplication of exon 13[3-4] was previously characterized in a patient treated at our Center. The duplication is the result of an unequal crossing-over between two non-homologous regions of misaligned X-chromosomes. Figure 1A shows that the break points occur on the two X chromosomes within FVIII gene intron 13 and intron 12. In the recombined chromosome the two normally oriented exons 13 are separated by an AT rich region of 19 tandemly duplicated nucleotides (Figure 1B). We hypothesized that the mRNA generated could be elongated or produced in two forms by alternative splicing.

To our knowledge, this patient is still an isolated case. We, therefore, studied the possibility that this rearrangement may also have been present in some of the uncharacterized patients with mild HA. Based on the FVIII genomic DNA sequence (*http://genome.ucsc.edu*; X-chromosome positions 151650711-151791079, which we assumed as nucleotide 1 and nucleotide 140369, respectively) we designed a pair of oligonucleotides encompassing the breakpoint region of the recombined X-chromosome: PAL13F 5'- TCAGTTTGGAAGTATTTTTC -3', extending from nt 77539 to 77558 of intron 13 and PAL13R 5'-TGTGTAC-TAAAGTATTGAGA-3', extending from nt 74331 to 74350 of intron 12. The polymerase chain reaction (PCR) was generated in standard conditions, and carried out for 30 cycles (94°C for 1 min, 55°C for 20 sec, and 72°C for 2 min), resulting in the amplification of a 337 bp fragment only in the patients with the mutation. Intra-test control consisted in the simultaneous amplification of exon 8 (*data not shown*).

We examined the 9 uncharacterized patients by PCR and found that 6 of them, including the 3 in whom direct gene sequencing had been negative, were in fact positive for this rearrangement. These findings prompted us to study our whole series of patients with mild HA, using the exon 13 duplication as first screening in the patients who had not yet been investigated. The overall frequency of this mutation was 32% (10/31 patients).

As seen from the pedigree analysis, all patients were apparently unrelated. HA was familial in 5 cases and sporadic in the remaining ones. In 3/5 sporadic cases, the two available generations consisted of mothers, all of whom proved to be carriers, and their sons. The recurrence of the same breakpoint (*data not shown*), as established by direct sequencing of all fragments, coupled with the fact that 7/10 positive patients were born in our region (Liguria), led us to hypothesize that this duplication could have spread from a single, ancestral event. As a matter of fact, data from bi-allelic and multi-allelic FVIII intragenic polymorphisms showed that all patients with mutations had the same haplotype (BclI/IVS18, + allele; XbaI/IVS22, - allele; VNTR IVS 13, 20 CA repeats).

Therefore, the mutation may have arisen as a single event and was then disseminated throughout the northern Italian population by a founder effect. The lack of kinship among the families as well as the fact that about 50% of the cases were apparently sporadic, observed from the reported family history, are two features that seemingly contrast with these results. However, we must remember that most positive patients had a FVIII:C>8%, that they had been diagnosed in adulthood following trauma and/or surgical procedures, and that they could have had undiagnosed ancestors. Moreover, for the same reasons we were only able to examine the members of two generations. The body of information we obtained allows us to make some remarks. Firstly, conventional mutation screen-