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Changes of Tissue Factor Activity on Inflammatory Stimulus and Aging in Rat

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Tissue factor (TF), a principal initiator of the vertebrate coagulation cascade, is expressed in organ tissues, cells and blood. TF is known to be induced in endothelial cells, monocytes and macrophages by inflammatory stimuli and in many pathologic conditions. By using the modified method for *in vivo* TF activity assay, we found that turpentine oil injection as an inflammatory stimulus also induced the TF activity in lung and brain tissues of rats. And the age-related increase in TF activity was observed in healthy rat brain tissue.

Key words : Brain tissue factor, Lung tissue factor, Inflammatory stimulus, Age-related increase of tissue factor

INTRODUCTION

Tissue factor (TF, also referred to as thromboplastin, tissue thromboplastin, or coagulation factor III) is a cell surface receptor of coagulation factor VII (FVII) and is the principal initiator of the vertebrate coagulation cascade (Nemerson, 1988; Davie *et al.*, 1991; Ruf *et al.*, 1994). Vascular damage exposes blood to cells expressing TF, which forms a calcium and phospholipid-dependent high-affinity complex with FVII. Binding to TF promotes activation of zymogen FVII to the serine protease FVIIa. The TF/ FVII(a)/phospholipid/Ca^{2+} complex mediates the cleavage of FIX and FX, thus activating both the intrinsic and the extrinsic pathways (Nemerson and Pitlick, 1972; Osterud and Rapaport, 1977).

Constitutively high levels of TF are found in certain organs (e.g., brain, lung, placenta) whereas other cells, such as blood monocytes and vascular endothelial cells, when quiescent, contain only trivial amounts of TF (Gonmori and Takeda, 1975, 1976). These cells, however, can be modulated by inflammatory stimuli to synthesize TF: within 2 to 6 hours after exposure to such agents, cells may generate up to a 1000-fold increase in cell-associated procoagulant activity. Variety of agonists such as lipopolysaccharide, lymphokines, tumor promotors and immune complexes which cause the induction of TF in cultured endothelial cells have been reported (Schorer and Moldow, 1988).

TF is found in atherosclerotic plaques (Ichikawa *et al.*, 1996; Kato *et al.*, 1996; Toschi *et al.*, 1997) and can be induced in endothelial cells, monocytes and macrophages in pathologic conditions such as infections, vascular lesions or immunologic diseases (Rivers *et al.*, 1975; Levy and Edgington, 1980; Colucci *et al.*, 1983; Carson and Johnson, 1990; Key *et al.*, 1990). Blood coagulation is activated in cancer cells (Colucci *et al.*, 1980; Naito *et al.*, 1983), and recently it has been found that TF is expressed highly in human gliomas (Hamada *et al.*, 1996) and in certain type of acute leukemia (Bauer *et al.*, 1989).

Various assay methods for TF have been developed. Anti-TF antibodies or specific binding of labeled FVII is used to quantify TF molecules (Zeldis *et al.*, 1972; Broze, 1982). TF is measured by procoagulant activity using one stage or two stage clotting assay (Nemerson, 1968; Hogg, 1983). Enzymatic assay is also available, which is in two stage, and FXa generated by TF and clotting factors is measured by its ability to cleave synthetic substrate (Hische *et al.*, 1981; Bolhuis *et al.*, 1982; Shands, 1983; Kato and Uchida, 1988; Fukuda *et al.*, 1989). Enzyme-linked immunosorbent assay methods for TF have been developed (Koyama *et al.*, 1994; Wada *et al.*, 1994; Kobayashi *et al.*, 1995).

The researchers have detected TF antigen in plasma and TF induction or inhibition in cultured endothelial cells, monocytes and macrophages. We introduced the method for detecting the TF activities quantitatively in the organs like brain and lung which show high TF activities in normal conditions, to investigate the TF activitiy changes in infectious conditions in an animal model. We have modified the one stage clottting assay method to measure the prothrombin time using rat plasma, which is simple and reproducible assay method for TF activity in tissues. In this paper we report that TF activities in rat brain and lung tissues were induced by inflammatory stimulus such as turpentine oil and that

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an age-related increase of TF activity was shown in healthy rat brain.

We designed the method for detecting the TF activities quantitatively in the organs like brain and lung which show high TF activities in normal conditions, to investigate the TF activitiy changes in infectious conditions in an animal model.

MATERIALS AND METHODS

Materials

All the reagents used were reagents grade, and healthy male Sprague-Dawley rats were used for the assay.

Determination of the tissue factor activity *in vitro*

One-stage clotting assay: Prothrombin time (Thompson and Harker, 1983) was measured to determine the TF activity using citrated plasma from rats. Blood was taken from heart using syringe containing 3.13% sodium citrate, of which final concentration after taking blood was 10%. Plasma was prepared by centrifugation at 2, 500 rpm for 15 min. In a plastic tube prewarmed at 37°C water bath, 100 µl of plasma, 100 µl of TF solution diluted to the proper concentration with saline solution and 100 µl of 25 mM $CaCl_2$ were taken and coagulation time after the addition of $CaCl_2$ was measured. It was executed in duplicate and the average was taken.

Standard curve: From the measurements of the prothrombin time assayed on the lung tissues from 25 rats and the brain tissues from 17 rats over several times against the pooled up plasma from 9 rats, standard curves were made. It was 100% TF activity when the clotting time was 18 sec for the lung TF, and 30 sec for the brain TF. Considering that the clotting time without TF was 0% TF activity, clotting acceleration percentage was calculated according to the Eq. 1 where RT is clotting time without TF (recalcification time), PT is clotting time with TF (prothrombin time) and A is 18 for the lung TF, and 30 for the brain TF.

$$\text{Plasma clotting acceleration (\%)} = \frac{\text{RT-PT}}{\text{RT-A}} \times 100 \quad \text{Eq. 1}$$

Standard curves were drawn from the acceleration (%) on the Y axis and the unit of TF in log scale on the X axis. The amount of TF that produced 50% of clotting acceleration was arbitrarily defined as one unit of TF.

Protein determination: Protein concentration was determined according to the Lowry's method (Lowry *et al.*, 1951) using bovine serum albumin as a standard protein.

Determination of the tissue factor activities in rat brain and lung tissues

Microsomal fractions of the tissues were used as crude tissue factor. Brain or lung tissue of Sprague-Dawley rats was taken and one g of each tissue was homogenized with 4 ml of 0.15 M NaCl solution for 2 min with homogenizer in ice bath. The homogenate was centrifuged at 2,000 rpm for 20 min (Sorvall RT 6000), and an aliquot (2.5 ml) of the supernatant 1 (S_1) was centrifuged at 31,000 rpm (105,000×g), 4°C for 1 hr (Sorvall OTD 65B ultracentrifuge, rotor T 865) to give the supernatant 2 (S_2) and the microsomal fraction. The fraction was suspended in 2.5 ml of 0.15 M NaCl to give the suspension (S_3) which was used as the TF stock solution. 100~200 times dilution of this stock was used for assay which gives 5~10 units.

We defined age of rats from body weight. For three groups of rats weighing 122~169 g (avr. 143±16.1), 203~227 g (avr. 218±9.0) and 333~385 g (avr. 360±20.5), 6 animals each group, brain TF activities were measured as above.

Statistical significance of the data in each group was calculated by t-test.

Turpentine oil treatment in rat

One ml/kg body weight of turpentine oil was injected subcutaneously to each group of 3 rats, weighing 200~250 g. Brain and lung tissues were taken after the removal of blood from heart using syringe containing 3.13% sodium citrate, in 1 and 2 days of injection. TF stock solutions were prepared as above and TF activities were detected.

RESULTS

Calibration of tissue factor activity

The clotting time (plasma recalcification time) of control to which saline solution was added instead of TF ranged mostly from 120 sec to 200 sec. When tissue factor was added, the clotting time (prothrombin time) was reduced compared to that of the control. But the prothrombin time was not reduced to less than 18 sec for the lung TF, and 30 sec for the brain TF (Fig. 1, 2). According to the clotting acceleration (%) calculated based on the Eq. 1, the standard curve was made against TF unit in log scale on the X axis (Fig. 3). The amount of TF which accelerated the prothrombin time by 50% was arbitrarily defined as one unit of TF.

Quantitative preparation of crude tissue factor

TF activities of each tissue factor preparation step of the lung and the brain tissues were calculated considering that the TF activity of supernatant 1 (S_1) as 100%. The TF activity yield of microsomal fraction of the lung tissues was 79.7% and that of the brain tissues was 95.3%. And specific activity was increased

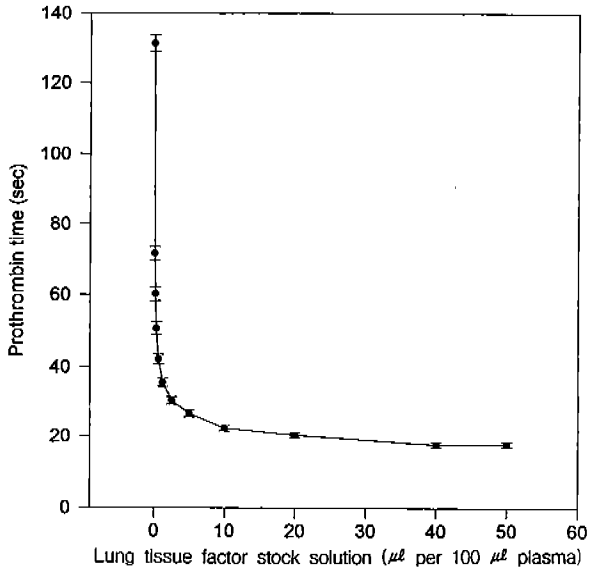

Fig. 1. Prothrombin time in rat plasma by lung tissue factor of rats. Amount of TF added in the reaction mixture containing 100 μl of plasma was expressed as μl TF stock solution, and prothrombin time was the average ± S.E. for the TF from 25 rats. Prothrombin time was not shortened to less than 18 sec.

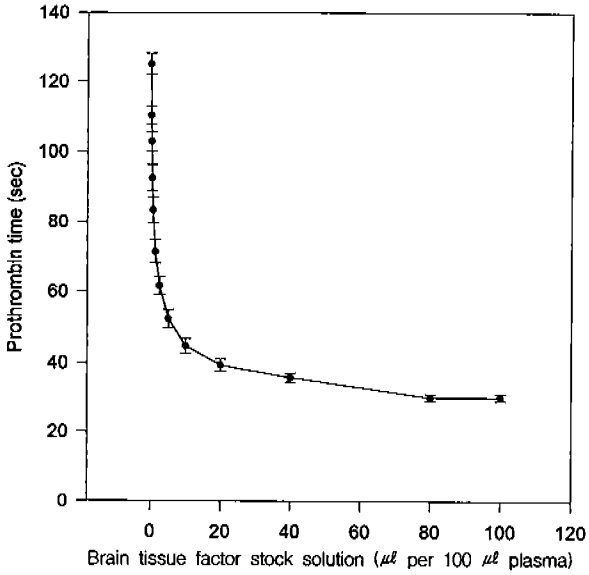

Fig. 2. Prothrombin time in rat plasma by brain tissue factor of rats. Amount of TF added in the reaction mixture containing 100 μl of plasma was expressed as μl TF stock solution, and prothrombin time was the average ± S.E. for the TF from 17 rats. Prothrombin time was not shortened to less than 30 sec.

about 5 folds for the lung tissues, and about 4 folds for the brain tissues (Table I). This suspension of microsomal fraction (S_3) was used as TF stock and diluted as needed when it's used to determine the activity.

Induction of tissue factor by turpentine oil in rat brain and lung tissues

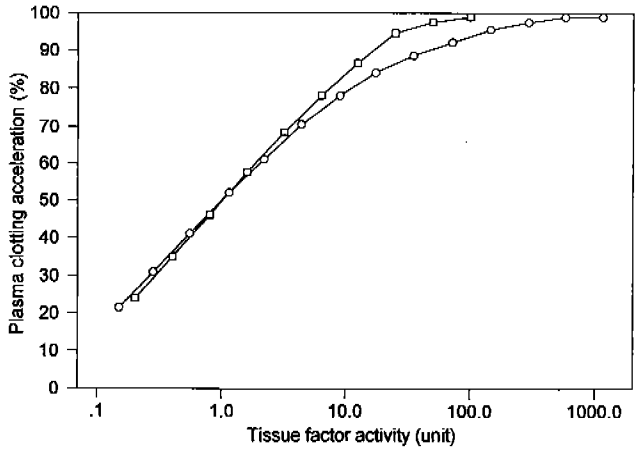

Fig. 3. Standard curves for tissue factor activities of rat lung and brain tissues. It was considered as 100% activity when the prothrombin time with TF was 18 sec (lung, ○) or 30 sec (brain, □), and the plasma recalcification time without TF as 0% activity. The amount of TF which gave 50% acceleration of prothrombin time on the standard curve was arbitrarily defined as one unit of TF.

Table I. Preparation of crude tissue factor from rat lung and brain tissue

Preparation step[a]	Total TF activity (unit)	Total protein (mg)	Specific activity (unit/mg protein)	Purification (fold)	Yield (%)
Lung					
S_1	166.3×10^3	608.1	273.5	1.00	100.0
S_2	1.5×10^3	229.8	6.5	0.02	0.9
S_3	132.5×10^3	99.2	1335.7	4.88	79.7
Brain					
S_1	95.5×10^3	255.8	373.2	1.00	100.0
S_2	3.8×10^3	91.8	39.7	0.11	3.9
S_3	91.0×10^3	61.5	1481.5	3.97	95.3

[a] S_1: supernatant at 2,000 rpm, S_2: supernatant at 105,000×g and S_3: suspension of the microsomal fraction from 5 g of rat lung or brain taken from 4 rats (350±10 g).

After the subcutaneous injection of turpentine oil, TF activities in the lung and the brain tissues increased by about two folds in one day and decreased to about 0.7 folds in two days compared to the control group (Table II). We used turpentine oil which is known to induce inflammation locally, and we could find that the lung and the brain TF were induced by turpentine oil injection.

Effect of age on the tissue factor activities in rat brain

We realized that TF activity was also influenced by age (body weights) of rats. Three groups of rats, of which body weights ranged 122~169 g (avr. 143 g± 16.1), 203~227 g (avr. 218±9.0) and 333~385 g (avr. 360±20.5), were compared its brain TF activity (Table III). TF activity (unit/mg protein) of each group was 448.2, 806.4 and 1296.8. It was increased significantly

Table II. Effect of turpentine oil injection on the tissue factor activities in rat lung and brain tissues

Tissue	Days after injection[a]	TF activity (unit g tissue)	Activity change (fold)
Lung	control	25.6×10^3	1.00
	1	56.0×10^3	2.19
	2	16.8×10^3	0.66
Brain	control	8.8×10^3	1.00
	1	17.6×10^3	2.00
	2	6.0×10^3	0.68

[a]One ml turpentine oil per kg body weight was injected to each group of 3 rats weighing 200~250 g.
TF used for assay was 2,000 rpm supernatant (S_1) of tissue homogenates from 3 rats (pooled up).

Table III. Effect of body weight on the tissue factor activity in rat brain

Group	Body weight (g) (Average[a])	No. of animals	TF activity[b] (U/mg protein)
1	122~169 (143±16)	6	448.2±64.8
2	203~227 (218±9)	6	806.4*±122.2
3	333~385 (360±21)	6	1296.8**±108.2

[a]Average body weight ± S.D.
[b]Brain tissue factor activity of preparation S_3. Mean±S.E.
Significantly different from group 1 at *$p<0.05$ and **$p<0.02$

at $p<0.05$, according to its body weight increment in three groups.

DISCUSSION

Human and bovine TF has been purified to homogeniety from microsomal fraction of saline homogenates (Williams, 1964; Williams, 1966; Bjorklid *et al.*, 1973), from lipoprotein fraction of acetone powder (Nemerson, 1969; Pitlick and Nemerson, 1976) or by affinity chromatography (Bach *et al.*, 1981; Broze *et al.*, 1985; Kang and Niemetz, 1988). However these purification procedures could not be adopted for quantitative analysis methods for TF in tissues. So, we obtained the microsomal fraction quantitatively from saline homogenates of rat lung or brain tissue through two steps, and its suspension was used as TF source for TF activity assay. TF activity recovery of the suspension was 79.7% for the lung and 95.3% for the brain tissues (Table I); the TF activity yield of the brain was better than that of the lung.

From the standard curve (Fig. 3) the TF activity could be read in unit, and it would be more reliable in the linear fraction of the curve which is about 0.6~10 unit. The standard curve was drawn with the TF activity unit on the X axis in log scale and clotting acceleration % on the Y axis.

For TF activity unit definition, Levy and Edgington (1980) used rabbit brain thromboplastin standard 36 mg/ml as 100 units for determining the mice TF activity. Andoh *et al.* (1990) used standard human brain TF preparation which gave 14~15 sec as 100 U/ml for determining human endothelial cell TF activity. For standard curves, we used the TF preparation from the same tissues as TF to be measured, because the standard curves were not exactly same in different tissues as shown in those of rat brain and lung TF (Fig. 3). Williams (1964) assigned 100 unit of TF for lung microsomes giving the clotting time of 11~12 sec. Instead of using the clotting time for defining TF unit, plasma clotting acceleration (%) was used in our study, considering that there is individual difference in plasma recalcification time of rats and it also varies daily depending on the experimental condition. The clotting acceleration (%) by TF against the clotting time of control was calculated by Eq. 1.

It has been reported that lipopolysaccharide (LPS) induces TF activity in cultured endothelial cells, monocytes or macrophages (Collucci *et al.*, 1983; Bom *et al.*, 1991), and most of the induction studies has been carried out in cultured cells. In *in vivo* study, endotoxin injection induced disseminated intravascular coagulation (DIC) in rabbit (Warr *et al.*, 1990). We injected turpentine oil which is known to give rise to local inflammation. In cultured cells the effect of the LPS injection showed in 2~6 hrs (Schorer and Moldow, 1988). Since the response was presupposed to be slow in whole animals than in cultured cells, we measured TF activity in one and two days of turpentine oil injection. The TF activity increased in one day in both lung and brain tissues and decreased in two days as shown in Table II. The reason why it was decreased below that of the control in two days is not clear at this monent. In order to find the point of maximum effect it would have been better to detect the TF activity at shorter intervals. It was reported that when cell membrane was damaged physically or chemically, TF activity increased and this was due to the antibody-mediated complement activation (Carson and Johnson, 1990). At present, we cannot explain the mechanism how local inflammation affect the TF activity in brain and lung tissues.

The TF activity of brain was increased as the age increases (age, based on the body weight) in three groups weighing 143 g, 218 g, 360 g on the average as shown in Table III and the result was quite understandable because there is more thrombotic disposition in aged animals than in young ones. This result reminds us that it is desirable to have control group of same body weight (age) as test group in *in vivo* assay.

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