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Enhancement of the Fibrinolytic Activity in Plasma by Oral Administration of Nattokinase[1]

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Abstract. The existence of a potent fibrinolytic enzyme (nattokinase, NK) in the traditional fermented food called 'natto', was reported by us previously. It was confirmed that oral administration of NK (or natto) produced a mild and frequent enhancement of the fibrinolytic activity in the plasma, as indicated by the fibrinolytic parameters, and the production of tissue plasminogen activator. NK capsules were also administered orally to dogs with experimentally induced thrombosis, and lysis of the thrombi was observed by angiography. The results obtained suggest that NK represents a possible drug for use not only in the treatment of embolism but also in the prevention of the disease, since NK has a proven safety and can be massproduced.

Introduction

Fibrinolytic therapy by oral drug administration was investigated by Sumi and co-workers [1, 2] 10 years ago in an animal model where enteric-coated urokinase (UK) capsules were given to normal and experimental dogs with saphenous vein thrombosis. Our previous findings indicated that intravenous (i.v.) administration did not show any clear thrombolytic effect, but that oral administration enhanced the fibrinolytic activity, serving as a treatment to lyse the thrombi in a mild but maintained way.

The underlying mechanism of such fibrinolytic therapy by oral administration was then confirmed by basic research to involve absorption of the administered UK across the intestinal tract, and release into the blood of endogenous plasminogen activator which originated from the liver and/or endothelial cells [3–6]. The enteric coated UK capsules (60,000 U/day for 7 days) also exhibited a clinical efficacy against cerebral thrombi [7, 8]. Moreover, more effective results were obtained in double-blind tests at multicenter trials employing a dose of 120,000 U/day for 7 days [9].

Nevertheless, some concern about fibrinolytic therapy by oral drug administration still remained. The greatest problem was that of the cost of the fibrinolytic enzymes, while another involved their low stability in the intestinal tract. It thus became desirable to evaluate several other fibrinolytic enzymes in addition to oral UK, in a search for a practical agent to use in fibrinolytic therapy by oral administration. Good results were not obtained in oral trials with streptokinase (SK) [10] or the fibrinolytic enzyme *Lumbricus rubellus* protease (LRP) [11] from the earthworm, since there was some evidence of internal hemorrhage within the intestinal tract. During these series of experiments, we examined the effect on the circulatory system of several foods including liquor (more than 173 kinds of natural food were studied), and we unexpectedly discovered a fibrinolytic enzyme in the Japanese fermented food 'natto' with a potency matched by no other enzyme. The isolated fibrinolytic

[1] Part of these data was presented at the 9th International Congress on Fibrinolysis, Amsterdam, 1988; Abstract by H. Sumi, H. Hamada and H. Mihara, A novel strong fibrinolytic enzyme (nattokinase) in the vegetable cheese 'natto', p. 67.

enzyme resembled plasmin. It showed a potent fibrinolytic activity and was named nattokinase (NK) [12]. NK originates from the microorganism, *Bacillus natto,* and can be produced in large quantities. It is extremely cheap and comparatively stable to temperature, acid and alkali. It is also easy to purify. In terms of drug use, the most important fact may be that natto has been widely used in Japan in the daily diet for over 1,000 years, amply justifying its safety in the oral form.

The present study demonstrates that oral administration of natto and NK can enhance the fibrinolytic activity in the plasma for long periods of time and that this is related to tissue plasminogen activator (TPA) as an endogenous plasminogen activator.

Materials and Methods

Preparation of Nattokinase

NK was extracted by the following procedure [12]. Four liters of distilled water were added to 5 kg of natto obtained from the National Federation of Cooperatives on Natto, and stirred at room temperature for 1 h. The extract was treated with 25% v/v ethanol. After removal of the materials which floated at the top of the supernatant, the extract was filtered through gauze and centrifuged for 10 min at 3,000 rpm. The supernatant obtained by the above procedure was then lyophilized (approximately 2.13 cu/mg of protein using human plasmin as standard). The molecular weight was approximately 20,000 daltons and the protein did not react with UK or TPA antibody by the Ouchterlony or ELISA methods.

Enteric-coated capsules of NK were prepared in the same manner as described previously [2]. Each capsule contained 250 or 650 mg of NK, respectively. Placebo capsules were prepared similarly but without the active ingredient.

Experimental Thrombus Model in the Dog

These experiments were carried out as reported previously [2, 13], using mongrel dogs (males, BW 10.1–10.6 kg). The experimental thrombosis was induced by infusion of bovine fibrinogen and bovine thrombin via the external saphenous vein of the dogs.

Four capsules (250 mg NK/capsule) or placebo capsules were administered orally to the dogs and the fibrinolytic activities in the plasma were estimated after the duodenum had been reached. The thrombosis was evaluated by angiography [13] by inserting a catheter into the femoral arteries and injecting 4 ml of angiographin for 2 s. Angiograms were obtained before thrombus formation and at 2.5, 5, 12, 18 and 24 h after thrombosis.

Oral Administration to Humans and Determination of Fibrinolytic Activities

Twelve healthy Japanese volunteers (6 men and 16 women, aged between 21 and 55 years) were given 200 g of natto or boiled soybeans (control group) once before breakfast, or 2 enteric-coated capsules containing NK (650 mg/cap) 3 times a day after meals. Blood was collected periodically with 1/10 its volume of 3.8% sodium citrate as anticoagulant. Blood collection from the NK capsule group was always performed at 10:00 a.m. The enzymatic activities in the plasma were determined from the whole blood clot lysis time (WBCLT) employing the method of Chohan et al. [14]. The plasma was diluted with 10 times its volume of 0.12 *M* sodium acetate buffer. The euglobulin lysis time (ELT) was measured by the method of Milstone [15] using a clot lysis time recorder (Ricoh Shoji Co., Ltd., Japan). The euglobulin fibrinolytic activity (EFA) was determined by the standard fibrin plate method of Astrup and Müllertz [16] by spotting onto the plate the fraction obtained from euglobulin by the method of Kluft et al. [17]. The activity was expressed as the lysis area obtained with 0.03 ml of euglobulin solution at 37°C for 18 h. Bovine fibrinogen was purchased from Armour Co., and bovine thrombin from Mochida Pharmaceutical. Determinations of the degradation products from fibrin/fibrinogen (FDP) in the serum were performed by the latex aggregate method [18] using an FDPL kit purchased from Teikoku Laboratories. Estimation of the TPA antigen in the plasma was carried out by the method of Bergsdorf et al. [19] employing an ELISA kit from Biopool Laboratories with melanoma TPA (single-chain form) as standard.

Results

Before investigating the effects of NK, a pre-examination was performed. That is, 200 g wet weight of the natto marketed in Japan was given to 12 healthy volunteers and the fibrinolytic activity in their plasma was evaluated periodically. As a control, the same amount of boiled soybeans was given to the same human subjects after a 2-week interval. As shown in table 1, in contrast to the control experiment showing only some variability in the data ($p > 0.1$), a clear shortening of the ELT and elevation of the EFA were confirmed after a single administration of natto. The enhancement of the plasma fibrinolytic activity was maintained for a long time ($p < 0.005$; values from 2 to 8 h).

Capsules containing 1.3 g of NK each, were administered after meals 3 times a day. Blood was collected at 10:00 a.m. every morning and the fibrinolytic parameters were measured in the serum and plasma. The results obtained with the reaction system (WBCLT), which included several plasma inhibitors, indicated that there was no significant difference between the values before administration of NK ($851 \pm 1{,}032$ min) and the values on the 8th day. The only value of note was that observed during the 2nd day (623 ± 766 min), but it was not significantly different ($p > 0.5$). As illustrated in figure 1, the EFA increased gradually from the 1st to the 8th day after NK administration. Also, the FDP level in the serum was

Table 1. Fibrinolytic activity in the plasma after ingestion of natto

	ELT, h	EFA, mm^2
Time after ingestion of natto		
0 h	31.5 ± 6.2	0
2 h	16.4 ± 8.6*	8.4 ± 5.1*
4 h	16.7 ± 6.6*	15.2 ± 3.0*
8 h	19.3 ± 12.0*	5.8 ± 4.1*
12 h	27.4 ± 10.3	1.9 ± 5.2*
24 h	31.9 ± 8.9	0.8 ± 0.6
Time after ingestion of boiled soybeans		
0 h	32.2 ± 6.3	0
2 h	33.4 ± 9.0	0
4 h	35.2 ± 4.8	0
8 h	36.1 ± 5.5	0
12 h	34.6 ± 7.3	0
24 h	34.6 ± 7.7	0.4 ± 0.2

Healthy male volunteers were given 200 g each of natto or boiled soybeans. Plasma was collected and the ELT and EFA were measured. Each value represents the mean ± SD (n = 12).

* $p < 0.005$: significantly different from the control.

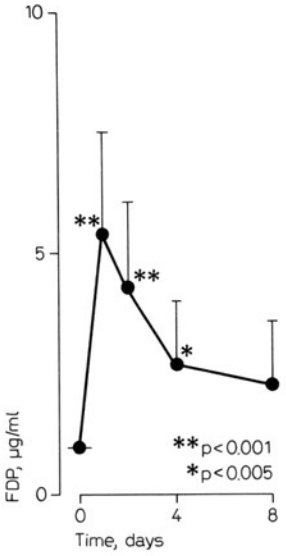

Fig. 2. Effect of oral NK on the amount of serum FDP. The amounts of FDP in the serum were measured periodically after oral administration of NK. Each value represents the mean ± SD (n = 7).

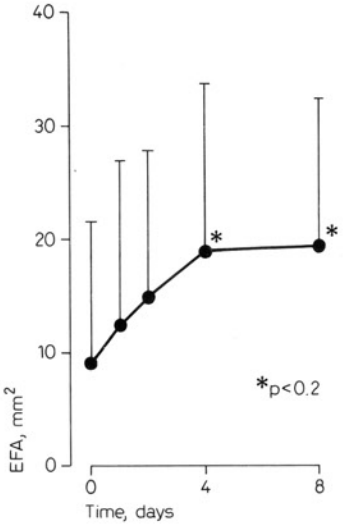

Fig. 1. Effect of oral NK on the fibrinolytic activity of the euglobulin fraction. Healthy volunteers (6 males and 1 female) were administered enteric-coated capsules containing 1.3 g of NK 3 times a day after meals. Blood was collected periodically and the EFA was measured by the standard fibrin plate method. The activity is expressed as the lysis area (mm^2) after 18 h at 37 °C. Each value represent the mean ± SD (n = 7).

statistically significantly higher ($p < 0.001$) on the 1st day of NK administration in comparison with pre-administration (fig. 2). The amount of TPA antigen, which is well known as one of the factors related to the fibrinolytic activity in the blood, was also significantly different ($p < 0.05$) over the long term (fig. 3).

Although the efficacy of NK in the dog was demonstrated only in a small number of animals, lysis of the experimental thrombi was observed. In contrast with the group of 6 dogs treated with placebo, whose ELT values did not change more then 60 ± 10 min from 2.5 to 12 h after administration, in the group of 3 dogs treated with oral NK a tendency for shortening of the ELT values (fibrinolytic enhancement) was observed (30 ± 18, 41 ± 13, 54 ± 11 and 55 ± 9 min after 30 min, 1, 3 h and 6 h, respectively, after administration). Concerning lysis of the embolisms, angiographic studies revealed that in comparison with the control group, where even at 18 h after administration there was no evidence of lysis, the NK group revealed complete recanalization of the blood circulation within 5 h of administration. The efficacy of NK for thromboembolysis was confirmed.

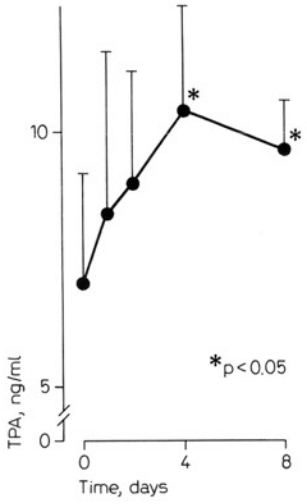

Fig. 3. Effect of oral NK on the amount of TPA in the plasma. After administration of oral NK, the amount of TPA was measured periodically Each value represents the mean ± SD (n = 7).

Discussion

TPA and pro-UK have been developed for the treatment of embolism because of their efficacy and their stronger affinity to fibrin deposits than those displayed by SK and UK. They are currently being used clinically by i.v. infusion. Also, other fibrinolytic enzymes with a long half-life due to gene manipulation, and chemically modified enzymes such as APSAC (anisoylated plasminogen-streptokinase activator complex) [20, 21] are being developed. In practice, however, these enzymes have failed to confirm the expected level of thrombolysis. Thrombolysis with these enzymes requires extremely large quantities and long periods of administration. One of the reasons is the fact that their half-life is very short (less than 20 min), and soon after administration, these enzymes are degraded or eliminated in the kidney and liver. If the enzyme is changed by chemical modification, or by gene manipulation, it becomes a foreign protein immunologically, as are SK and other molecules. It is also known that when UK is administered, immediately after completion of the administration, a rebound effects occurs and a transient ELT prolongation is induced [6]. There is a basic limitation to the supplement of such exogenous enzymes from outside of the body.

On the other hand, there are methods whereby endogenous fibribolytic enzymes within the body can serve to promote the lysis of the thrombus. With oral administration of NK, as with oral UK, rather than supplementing the enzyme from the outside, the human body utilizes the available endogenous plasminogen activator, and this can be considered an effective biological treatment. Furthermore, it can also be used for disease prevention.

The data in table 1 and figures 1–3 show that both natto and NK can enhance fibrinolysis for a comparatively long period of time. Previously, Fearnley et al. [22] demonstrated a diurnal fibrinolytic rhythm by the blood clot lysis time test. In our cases, although the plasma fibrinolytic activity was lowest at 6:00 p.m., the difference in each during the 24-hour period tested was not sufficiently great ($p > 0.1$). In contrast, the activation by natto and NK were of a much higher degree ($p < 0.005$ at 2–8 h). The data in figure 2 demonstrate a transient effect on the serum FDP. In healthy humans, it appears that the increase in FDP may have been caused by lysis by plasminogen activator and consumption of thrombi in the vascular system. The enhancement of the plasma fibrinolytic activity was thought to involve absorption of NK across the intestinal tract, as with orally administered UK. Although other fibrinolytic enzymes in natto, besides NK, were not examined in our study, Ohkuro et al. [23] have reported that when natto bacteria were fed to mice and rats, a mucopolysaccharide-degrading lysozyme was absorbed and delivered into the blood.

When evaluating the effects of oral administration on fibrinolytic enzymes, the amount of TPA antigen in the plasma is very important. As shown in figure 3 the TPA level was also significantly increased and remained so for a long period of time. Since NK does not display a TPA antigenicity, these results probably indicate that small amounts of NK are absorbed from the plasma or lymph and induce synthesis of a TPA type plasminogen activator in the liver or vascular endothelial cells.

Oral UK, SK and LRP have been used for approximately 10 years, which is a much shorter history than that of NK, since natto has been part of the daily diet in Japan for over 1,000 years with a fully proven safety. Traditionally, natto's various effects on heart and vascular disease are well known [24], and they are thought probably to be due to the presence of NK. Thus, NK itself may represent the best natural agent for use in oral fibrinolytic therapy. As demonstrated previously [25], UK can accelerate the activity of mi-

tomycin C. Also, recent oral administration results obtained by a Chinese research group [26] with LRP containing the fibrinolytic agent '912' have shown a very good effect for treatment of one type of cancer. Studies on the possible usefulness of NK as an anticancer agent and on its further characterization are now in progress.

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