Relationship between vitamin K dependent coagulation factors and anticoagulants (protein C and protein S) in neonatal vitamin K deficiency

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Abstract

To determine the relationship between vitamin K dependent coagulation factors and natural anticoagulants, namely protein C and protein S, in various degrees of vitamin K deficiency, plasma values for clotting activity, protein induced by vitamin K absence (PIVKA-II), protein C antigen, gammacarboxy protein C antigen, and protein S antigen including total and free fractions and activity of protein C were measured in 66 full term and healthy breast fed neonates who did not receive vitamin K supplement at birth. The 66 neonates were divided into a control group (17 cases) and a low group (49 cases) according to their values for clotting activity—that is, $\geq 20\%$ or $< 20\%$ during the first six days of life—and vitamin K was immediately given when the neonates showed values $< 20\%$. In the low group clotting activity gammacarboxy protein C, free protein S, and protein C activity was significantly decreased to a minimum on day 2 or 3, and increased in parallel after vitamin K administration. Furthermore, they were positively correlated with one another and inversely correlated with the PIVKA-II concentrations. These findings suggest that simultaneous gammacarboxylation of coagulation factors and proteins C and S acts to maintain both coagulation and anticoagulation activities in parallel at various concentrations of vitamin K. The breast milk intake in the group with low values of clotting activity was significantly lower than that in the control group during the first three days of life. Therefore, mothers should be advised to try to increase the breast milk secretion, for example, by providing frequent sucklings to infants in the first days of life.

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Vitamin K is required for the post-translational gammacarboxylation of glutamic acid residues in the vitamin K dependent proteins,¹ including not only coagulation factors II, VII, IX, and X but also protein C² and protein S,³ which are the important inhibitors of coagulation.^{4–7} Vitamin K deficiency results in the biosynthesis of abnormal non-gammacarboxy forms of these proteins

which are functionally defective. Dysfunction of these coagulation factors and inhibitors are clinically well known and seen in such diseases as haemorrhagic disease of the newborn^{8–10} and thromboembolic disorders including neonatal purpura fulminans.^{11–15} As vitamin K deficiency in the early neonatal period causes haemorrhagic diseases rather than thromboembolic disorders, vitamin K deficiency has been viewed exclusively from its relevance to coagulation factors.

We report our study of the changes of protein C and protein S as well as of coagulation factors from the occurrence of vitamin K deficiency to its correction with vitamin K administration. Furthermore, the relationship between the breast milk intake and vitamin K deficiency in neonates was also investigated.

Subjects and methods

SUBJECTS

Sixty six healthy, full term (range 38–41 weeks) neonates with a normal birth weight (range 2755–3970 g) aged 0 to 6 days were studied. There was no history of maternal anticonvulsant or anticoagulation treatment. They fed exclusively on breast milk except for supplements of 5% glucose solution, and did not receive vitamin K prophylaxis at birth. Informed consent was obtained from all participating parents.

SCREENING FOR HYPOPROTHROMBINAEMIA AND VITAMIN K ADMINISTRATION

A test for clotting activity (Normotest, Nyegaard and Co) was used as the screening test for hypoprothrombinaemia because this test accurately reflects total coagulant activity of factors II, VII, and X and requires only 0.01 ml capillary whole blood. The test was performed every day from 0 to 6 days of age. As the values for clotting activity in patients with vitamin K deficiency haemorrhage were less than 10%,¹⁶ we regarded values $< 20\%$ as an indication of being at risk of haemorrhagic disease of the newborn and immediately gave 2 mg of vitamin K₂ syrup (Eisai Ltd). Sixty six neonates were divided into a low (49 cases) and a control group (17 cases) according to whether their values were $< 20\%$ or $\geq 20\%$. The low group was further divided into three subgroups: low-1 (12 cases), low-2 (19

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cases), and low-3 (18 cases) showing values <20% on day 1, 2, and 3 of life, respectively.

In order to overcome the effect of packed cell volume on coagulation activity, the clotting factor value was corrected using a correction factor: $(100-H_n)/(100-H_s)$, where H_n is the normal mean packed cell volume (haematocrit) value (0.45 is used here) and H_s , the packed cell volume of a sample. We use the corrected value of the clotting activity throughout the study except for the screening test.

PREPARATION OF PLASMA SAMPLES

Blood sampling for the following assays was performed on days 0, 3, 4, and 6 in the control group, whereas in the three low groups it was performed on day 0, immediately before (on day 1 in low-1, on day 2 in low-2, and on day 3 in low-3 group) and one day after vitamin K administration, and on day 6 of life.

Venous blood was collected into siliconised tubes containing 1/10th the volume of 3.8% sodium citrate, centrifuged at 2500 *g* for 20 minutes at 4°C, and the supernatant plasma was stored at -70°C. The clotted samples

were discarded, and the samples in the low-1 group were excluded from the following measurements because the number of samples on day 1 was only 1.

ASSAY METHODS

Protein induced in vitamin K absence (PIVKA-II) was measured by enzyme linked immunosorbent assay (ELISA) using monoclonal antibody against PIVKA-II (Eitest monoP-II; Eisai Ltd).¹⁷ The lowest limit of plasma PIVKA-II is 0.06 arbitrary units (AU)/ml (1 AU corresponds to 1 µg of purified prothrombin).

Total protein C antigen was quantitated by sandwich ELISA (Asserachrom protein C, Boehringer Mannheim Yamanouchi). The lowest limit of this assay is 0.1% of the normal adult concentration.

Gammacarboxy protein C was also measured by one step sandwich ELISA (TD-82; Teijin Ltd)¹⁸ using two monoclonal anti-protein C antibodies, one of which recognised a gammacarboxyglutamic acid domain related conformational change induced by metal ions, and the other was directed against the activation peptide. Briefly, diluted plasma samples, peroxidase-conjugated antibody against gammacarboxyglutamic acid domain and antibody (against the activation peptide) coated polystyrene beads were mixed and incubated. After washing, the beads were immersed in a substrate solution containing hydrogen peroxide. Then, the reaction was stopped by addition of acetic acid, and absorbance of 650 nm was read using a spectrophotometer (Shimadzu). The lowest limit of this assay is 10% of the normal adult concentration.

Protein C activity was measured by an activated partial thromboplastin time method (Stacrot protein C; Diagnostica Stago) using Protac, an activator of protein C. The lowest limit is 5% of the normal adult value.

Total protein S antigen was determined by conventional Laurell rocket immunoelectrophoresis using anti-protein S antibody as previously reported.¹⁹ Free protein S antigen was measured by one step sandwich ELISA (TD-84; Teijin Ltd),²⁰ using polyclonal anti-protein S antibody and peroxidase-conjugated monoclonal anti-protein S antibody that recognised the C4bp binding site. The lowest limit of this assay is 5% of the normal adult value.

MEASUREMENT OF BREAST MILK INTAKE IN NEONATES

The volume of breast milk intake was measured every day from 0 to 6 days after birth by weighing neonates before and after feeding and a 1 g gain in weight was assumed to be 1 ml of milk intake.

STATISTICAL ANALYSIS

All data analyses were performed using IBM 9377 and SAS Statistical Software. Student's *t* test and Wilcoxon's rank sum test were used to analyse normally distributed and non-normally distributed data, respectively. Regression analysis was performed by the

Figure 1 Serial clotting activity and PIVKA-II for the control group and two groups shown to have low values for clotting activity on day 2 or 3 (low-2 group and low-3 group respectively); results are mean (SE). Note the rise in clotting activity and fall in PIVKA-II in the groups with low values after vitamin K administration. The control group had no vitamin K, whereas low-2 and low-3 groups had it on day 2 and 3, respectively; ****p*<0.01, ***p*<0.001 as compared with the value on day 0 within the same group.

method of least squares, and the correlation coefficient (r) was calculated.

Results

TOTAL COAGULATION ACTIVITIES AND PIVKA-II
The values for clotting activity in the control group decreased slightly to a minimum (mean 43.2%, range 28–75%) on day 2, and then rose gradually (fig 1). In the low-2 and low-3 groups, they decreased markedly on day 2 and on day 3, respectively, and rose rapidly after oral administration of vitamin K₂. The mean value in the low group immediately before vitamin K administration was 14.5% (range 10–24%).

All infants in the control group showed PIVKA-II concentrations of 1.5 AU/ml or less during the first six days of life (fig 1). However, the concentrations in the low group were 0.6 AU/ml or less at birth, then

increased to the maximum on day 2 (mean 8.1 AU/ml, range 0.3–22.1 AU/ml) in the low-2 group and on day 3 (mean 11.2 AU/ml, range 0.7–29.1 AU/ml) in the low-3 group, and decreased gradually with a half life of about 48 hours.

CHANGES IN CONCENTRATIONS OF ANTICOAGULANTS (PROTEIN C AND PROTEIN S)

The changes in protein C activity and gammacarboxy protein C values were essentially the same as those found for the clotting activity. In the low group, they decreased to a minimum on day 2 (mean 10.9% in protein C activity, 12.8% in gammacarboxy protein C) or on day 3 (mean 7.0% in protein C activity, 16.0% in gammacarboxy protein C), while in the control group no significant decreases were observed (fig 2). The values for protein C antigen showed no significant changes in any of the groups.

Both total and free protein S concentrations in the control group gradually increased with age but the ratio of free to total protein S remained almost the same, ranging from 62.0 to 67.8% (fig 3). In the low group, there were significant decreases in free protein S and in the ratio of free to total protein S on day 2 (mean 14.8% and 50.9%) in the low-2 group and on day 3 (mean 16.0% and 49%) in the low-3 group, but there was no significant decrease in total protein S (fig 3).

RELATIONSHIP BETWEEN COAGULATION FACTORS AND PROTEIN C AND PROTEIN S

All data from the three groups (excluding the data after vitamin K administration) were used for the analyses of relationship between coagulation and anticoagulation factors. Values for clotting activity, protein C activity, gammacarboxy protein C, and free protein S in all groups were positively correlated with one another, and they were inversely correlated with logarithmic values of PIVKA-II (table). Namely, the values for clotting activity and anticoagulant (protein C) activity were correlated not only with the degree of gammacarboxylation of each precursor (PIVKA-II and gammacarboxy protein C, respectively), but also with each other (fig 4). The free protein S concentration was also correlated with all of them.

BREAST MILK INTAKE AND VITAMIN K DEPENDENT COAGULATION ACTIVITIES

The cumulative breast milk intake in both control and low groups was very low until 2 days of age (mean volume on day 2 was 90 ml in the control, and 39 ml in the low group) and increased rapidly on day 3 (mean 318 ml in the control and 161 in the low group; fig 5). The breast milk intake in the low group was significantly lower than that in the control group during the first three days of life (Wilcoxon's rank sum test).

Figure 2 Decrease in values of protein C activity and gammacarboxy protein C in the low-2 and low-3 groups and their recovery after vitamin K administration; results are mean (SE). The control group had no vitamin K, whereas low-2 and low-3 groups had it on day 2 and 3, respectively; * $p < 0.01$, ** $p < 0.001$ as compared with day 0 within the same group.

Figure 3 Serial changes in values of total and free protein S and ratio of free to total protein S; results are mean (SE). The control group had no vitamin K, whereas low-2 and low-3 groups had it on day 2 and day 3, respectively; * $p < 0.05$, ** $p < 0.01$ as compared with day 0 within the same group.

Correlation between clotting activity, PIVKA-II, protein C, and protein S (figures are correlation coefficients)

	PIVKA-II	Protein C activity	Gamma-carboxy protein C	Free protein S
Clotting activity	-0.837****	0.793****	0.710****	0.554****
PIVKA-II		-0.661****	-0.541****	-0.335**
Protein C activity			0.799****	0.648****
Gamma-carboxy protein C				0.684****
Free protein S				

**** $p < 0.0001$, ** $p < 0.01$.

Discussion

Vitamin K deficiency in the early neonatal period causes two types of haemorrhagic disease²¹: early haemorrhagic disease of the newborn occurring during the first 24 hours and classic disease occurring at 2 to 5 days of age. Although both types may occur in infants born to mothers taking drugs such as anticonvulsants, warfarin, and antituberculous drugs, idiopathic cases are more frequently observed, especially in classic haemorrhagic disease of

the newborn. Breast feeding is considered to be an important aetiological factor in idiopathic disease because of the relatively low concentration of vitamin K²² and low excretion of breast milk during the first few days of life.²³

We used the following criteria for vitamin K deficiency: (1) PIVKA-II concentration was more than 1.3 AU/ml²⁴ and (2) the PIVKA-II concentration and value for clotting activity corrected after the administration of vitamin K. We found only 9.1% (1/11) of neonates in the control group and 78.3% (18/23) of neonates in the low group showed vitamin K deficiency from two to three days after birth. Furthermore, vitamin K deficiency was related to the amount of breast milk intake. These findings seem to represent the typical features of classic haemorrhagic disease of the newborn and confirm the previous observations that vitamin K storage in newborn infants is inadequate to maintain their requirement after birth and that the diet is the main source of vitamin K in this period.²³⁻²⁷

The values for protein C antigen and protein C activity in the control group were notably lower than those found in adults, as previous investigators reported.²⁸⁻³⁰ Furthermore, in vitamin K deficiency, protein C activity and gamma-carboxyl protein C decreased to near the values observed in patients with homozygous protein C deficiency,¹²⁻¹⁴ while the decrease in total protein C antigen was less. These findings indicate that only gamma-carboxy protein C is functionally active, and decarboxylated protein C (PIVKA-PC) is present in vitamin K deficiency. Thus, the measurement of gamma-carboxy protein C is useful in the estimation of protein C activity and for evaluation of patients with vitamin K deficiency.

Protein S is synthesised not only in liver cells, but also in endothelial cells³¹ and megakaryocytes,³² and serves as a cofactor of activated protein C. In human plasma, two forms of protein S are present in an equilibrium state³³; in adults, approximately 40 to 50% of total protein S is in the free form and the remaining 50 to 60% is complexed to C4b binding protein (C4bp), a regulatory component of the complement system. Only the free form can act as a cofactor of activated protein C.¹² The ratio of free to total protein S in neonates is considered to be high because of the low values of C4bp,^{34 35} although the total protein S concentration is much lower than that of adults. Our findings are in agreement with those in previous reports in this respect. In addition, our study demonstrated that the concentration of free protein S decreased markedly in vitamin K deficiency, whereas total protein S showed little change. A similar observation was made in neonates with haemorrhagic disease of the newborn by Mikami *et al.*³⁶ These findings indicate that a considerable portion of free protein S is converted to the complex form of protein S in vitamin K deficiency, which probably results from the change of C4bp binding properties due to the conversion of gamma-carboxy protein S to PIVKA-PS.

Figure 4 Pairwise correlation analyses of clotting activity, PIVKA-II, protein C, and protein S. Correlation analyses were made using all data from the three groups, except for the data after vitamin K administration.

Figure 5 Cumulative breast milk intake from 0 to 3 days of life; results are mean (SE). The milk intake in the group with the low values for clotting activity is significantly lower than that in the control group; * $p < 0.05$, ** $p < 0.01$ as compared with control group of the same age.

More remarkable findings are that there was a close correlation between the coagulant (clotting factors) and anticoagulant (protein C) activities, each of which was also correlated with the degree of gammacarboxylation of its precursor (PIVKA-II and gammacarboxy protein C, respectively). These observations imply that the simultaneous gammacarboxylation of both coagulation factors and proteins C and S serves to sustain the balanced activities of coagulation and anticoagulation at various concentrations of vitamin K. This may explain the facts that thromboembolic disorders have scarcely been reported in vitamin K deficiency, even though protein C and protein S activities are as low as the value found in severe protein C and protein S deficiency, and that coagulation abnormalities due to vitamin K prophylaxis are hardly observed in neonates.

The breast milk intake affected vitamin K

status and a low intake, which was frequently seen in normal breast fed neonates during the first days of life, caused a deficiency of protein C and protein S as well as hypoprothrombinaemia. Therefore, we must take this into consideration in assessing the activities of these proteins, especially in making a diagnosis of congenital protein C or protein S deficiency. Moreover, mothers should be advised to suckle infants frequently in the first days of life in order to increase the breast milk secretion.³⁷

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