Development of an Enzyme Linked Immunosorbent Assay for Direct Determination of Anticancer Drug Vitamin K3 in Serum

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Synthetic vitamin K3 (VK3, 2-methyl-1,4-naphthoquinone, or menadione) has been shown to have anticancer activity in various human cancer cells. We developed an enzyme-linked immunosorbant assay (ELISA) for determination of VK3 in fetal calf serum (FCS) without any pretreatment. The monoclonal antibody against VK3, which was secreted from an established hybridoma cell line (3A3) has been proven to have highly specific binding to VK3 in cross-reactivity analysis. The full measuring range of the assay extends from 0.39 to 50 μg/ml of VK3. Based on validation analysis, this immunological analysis is a precise, accurate, and sensitive method for determination of VK3. This simple immunological method would deserve to be applicable clinically.

Key words —— vitamin K3, monoclonal antibody, enzyme-linked immunosorbent assay

INTRODUCTION

Vitamin K is a generic term for a group of fat-soluble vitamins which has a 1,4-naphthoquinone skeleton. They have in common their physiological role as a required cofactor in the synthesis of blood clotting. Among them, vitamin K3 (VK3, 2-methyl-1,4-naphthoquinone, or menadione) (Fig. 1) is only one synthetic derivative derived from the naturally occuring vitamin K1 and vitamin K2, and is currently attracting much attention because of its strong anticancer activities. VK3 has been used in clinics in combination with chemotherapeutics and radiation for the treatment of several malignant tumors.[1] Moreover, the potential function of VK3 antineoplastic activity includes generation of superoxide,[2] free radicals,[3] semiquinone,[4] and lipid peroxide.[5] Although many methods for detection[6,7] or determination[8–13] of VK3 were investigated, immunological methods have not yet been reported. In our previous study, we developed an enzymed-linked immunosorbant assay (ELISA) for determination of plumbagin (PL, 5-hydroxy-2-methyl-1,4-naphthoquinone) (Fig. 1) using monoclonal antibody (MAb).[14] We focused on the character of MAb 3A3 which also has highly-specific binding to VK3, and exploited it in an ELISA for determination of VK3. Morever, this analytical method could directly determine VK3 in fetal calf serum (FCS) with holding their sensitivity, indicating that this ELISA system have possibility to be applied in clinical use without any pretreatment. This analysis is a first report to determination of VK3 by ELISA system. We herein describe the potential use of MAb 3A3 in an ELISA for determination of anticancer drug, VK3.

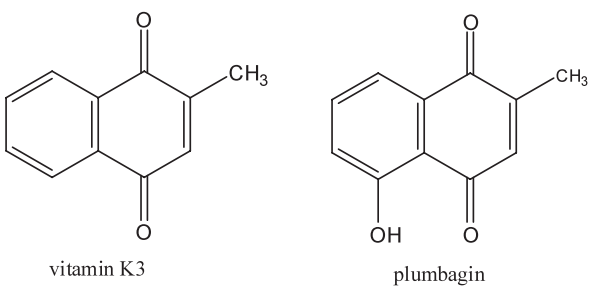

Fig. 1. Structure of Vitamin K3 and Plumbagin

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MATERIAL AND METHODS

Chemicals —— VK3 was purchased from Wako Pure Chemicals, Osaka, Japan. Ovalbumin (OVA) was provided from Sigma, Steinheim, Germany. Peroxidase-labeled anti-mouse IgG was purchased from Organon Teknika Cappel Products, West Chester, PA, U.S.A. FCS was purchased from Invitrogen, Carlsbad, CA, U.S.A. All other chemicals were standard commercial products of analytical reagent grade.

Preparation of Hapten-protein Conjugates —— The synthesis of PL-ovalbumin (PL-OVA) conjugates was performed as described in our previous study[14] with some modifications. 1-Ethyl-3-(3′-dimetylaminopropyl)-carbodiimide hydrochloride (EDC; 6 mg, 0.031 mmol) and 3′-(5-hydroxy-2-methyl-1,4-naphthoquinone-3-yl)propanoic acid (3 mg, 0.012 mmol) were added to a mixture of 35% pyridine solution (0.3 ml) and a buffer consisting of 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) and 0.9% sodium chloride (0.3 ml). The reaction mixture was added drop wise to 0.3 ml MES buffer containing 3 mg OVA, and then stirred at room temperature for 13 hr. Subsequently, the mixture was dialyzed against five changes of H_2O for 2 days at 4°C, and lyophilized to yeild 3.2 mg of PL-OVA conjugate, which was used as a coating antigen in ELISA.

Preparation of Standards —— VK3 was accurately weighted and dissolved in methanol (MeOH) to prepare a 1 mg/ml stock solution. Various concentrations of VK3 (0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 μg/ml) for standard were prepared using 20% MeOH or FCS.

Preparation of MAb 3A3 —— MAb 3A3 against PL was prepared by fusing myeloma cells (SP 2/0) with mice splenocytes as described previously.[14]

Indirect Competitive ELISA —— Indirect competitive ELISA was carried out basically by the previously-described method.[14] Briefly, PL-OVA conjugate (100 μl, 1 μg/ml) was adsorbed on the 96-well immunoplate, which was then treated with 300 μl phosphate-buffered saline containing 10% skim milk for 1 hr to reduce non-specific adsorption. 50 μl of various concentrations of VK3 in 20% ethanol (EtOH) or FCS were incubated with 50 μl of the MAb solution for 1 hr. The plate was washed three times with 0.05% Tween 20 containing phosphate-buffered saline (TPBS) and then the antibody was combined with 100 μl of 1 : 1000 diluted solution of peroxidase-labeled anti-mouse IgG for 1 hr. After washing the plate three times with TPBS, 100 μl of substrate solution, 0.1 M citrate buffer (pH 4.0) containing 0.003% H_2O_2 and 0.3 mg/ml 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Wako), was added to each well and incubated for 15 min. Absorbance at 405 nm was measured using micro plate reader (Immuno Mini NJ-2300).

The cross-reactivities (CRs) of the MAb 3A3 against various compounds were evaluated and calculated by the method of Weiler and Zenk[15] as follows:

$$\mathrm{CR}(\%) = \frac{\text{IC50 for VK3}}{\text{IC50 for compound under investigation}} \times 100$$

RESULTS AND DISCUSSION

The indirect competitive ELISA was characterized by sensitivity, specificity, precision and accuracy. Following competition, free MAb 3A3 is bound to a polystyrene micro-immunoplate precoated with PL-OVA. After washing the plate, the amount of antibodies bound to the PL-OVA conjugate was measured using a secondary antibody (peroxidase-conjugated IgG). The range of the VK3 concentration which could be detected in this assay was ranged from 0.39 to 50 μg/ml.

Subsequently, FCS was used in this assay to confirm this ELISA system could have possibility to be applied directly in clinical use. Figure 2 shows the standard curve of this system for determination of VK3. Interestingly, the system using FCS shows the same range (0.39–50 μg/ml) as the case of using 20% MeOH. This result indicates that this ELISA system could be applied directly using the serum from patients without any pretreatment though some pretreatments are generally needed to detect/determine samples from serum to remove other factor which might be effected.

The specificity of the MAb was determined by the CRs of antibodies with other compounds using the competitive ELISA and calculated by using developed by Weiler and Zenk.[15] Table 1 shows the CRs of MAb 3A3 with various compounds. This MAb shows very highly-specific binding to VK3 although it also shows high CR with PL as we previous described. The CR with PL was found to be 124.2%, whereas other tested compounds including a 1,4-naphthoquinone skeleton did not

exhibit any cross-reactivity with MAb 3A3 (less than 0.005%) indicating that the 2-methyl group on 1,4-naphthoquinones plays a key role in recognition by MAb 3A3 because high CRs were obtained only when there was 2-methyl group on 1,4-naphthoquinone (VK3, PL).

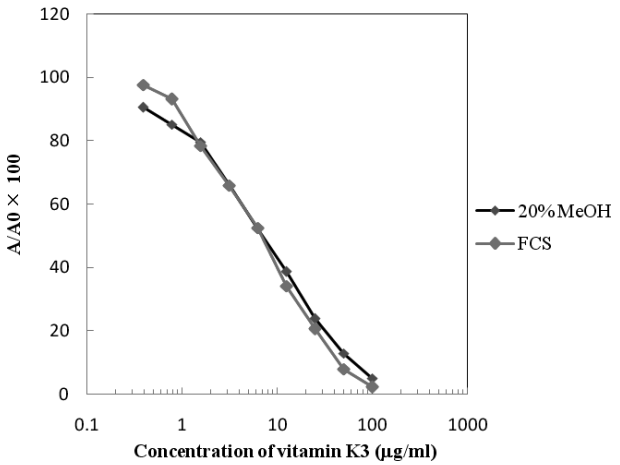

Fig. 2. Calibration Curve of Vitamin K3

Various concentrations of VK3 in 20% MeOH or FCS were incubated with MAb 3A3 (50 ng/ml) in wells precoated with PL-OVA (1 μg/ml). After washing with TPBS, the wells were incubated with peroxidase-labeled anti-mouse IgG. Absorbance was measured at 405 nm. A/A_0, A_0 is the absorbance with no VK3 present, and A is the absorbance with VK3 present.

To validate the indirect competitive ELISA, intra- and inter-assay reliabilities were studied. Intra- and inter-assay variabilities were evaluated by testing nine different VK3 concentration samples in five assays performed together on the same day and on three consecutive days, respectively. Intra- and inter-assay coefficients of variation (CV) for the precision were determined by the ratios of standard deviations (S.D.) and means from five assays. From the result of Table 2, the maximum intra-assay CV was 2.3%, while the inter-assay CV was 7.3%. All of CVs were below 10%, indicating that the assay is used for sample analysis.

In conclusion, we have developed an indirect competitive ELISA to directly determine VK3 which is well known as a derivative of vitamin K and cancer drug. The benefits of this ELISA system are mainly its cost-effectiveness, rapidity, reliability, and simplicity. Therefore, the newly developed ELISA system using MAb 3A3 could be applied to monitoring of VK3. Due to strong anticancer activity of VK3, it is necessary to monitor precisely its concentration in body fluids from the point of view of optimum usage and avoidance of side effects. Since the developed ELISA has adequate sensitivity

Table 1. Cross-reactivities of MAb 3A3 against Various Compounds

Compound	Classification	CRs (%) of MAb 3A3
Vitamin K3 (2-methyl-1,4-napthtoquinone)	1,4-Naphthoquinones	100
Plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone)	1,4-Naphthoquinones	124.2
Juglone (5-hydroxy-1,4-naphthoquinone)	1,4-Naphthoquinones	< 0.005
Lawsone (2-hydroxy-1,4-naphthoquinone)	1,4-Naphthoquinones	< 0.005
1,4-Naphthoquinone	1,4-Naphthoquinones	< 0.005
Sennoside A	Anthraquinones	< 0.005
Sennoside B	Anthraquinones	< 0.005
1-Naphthol	Naphthalenes	< 0.005
2-Naphthol	Naphthalenes	< 0.005
Ginsenoside Re	Triterpenoid saponins	< 0.005
Ginsenoside Rb1	Triterpenoid saponins	< 0.005
Glycyrrhizin	Triterpenoid saponins	< 0.005
Solamargine	Steroidal alkaloids glycosides	< 0.005
Naringin	Flavonoids glycosides	< 0.005
Hesperidin	Flavonoids glycosides	< 0.005
Rutin	Flavonoids glycosides	< 0.005
Colchicine	Tropolones	< 0.005
Artemisine	Sesquiterpene lactones	< 0.005
Berberine	Benzyl isoquinoline alkaloids	< 0.005
Ephedrine	Phenethylamines	< 0.005
Atropine	Tropanes	< 0.005
Cinnamic acid	Phenyl propanoids	< 0.005
Catechol	Catechols	< 0.005

Table 2. Intra- and, Inter-assay Precision of Vitamin K3 Analysis by ELISA

Vitamin K3 (μg/ml)	CV (%)	
	Intra-assay (n = 5)	Inter-assay (n = 3)
0.78	2.27	1.92
1.56	1.90	7.33
3.13	1.68	5.96
6.25	1.83	2.65
12.50	1.66	5.32
25.00	2.28	4.91
50.00	2.00	1.79

All values represent the mean ± S.D. for three plates and five replicate wells for each concentration within one plate for three consecutive days.

and accuracy to detect a small amount of VK3 even when it is contained in serum, it would be useful for clinical application.

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