chromosomal damage in bone marrow, was not different among the unirradiated groups, but was significantly higher in the TBI groups (Figure 1b). Among the TBI groups, the proportion of MNed reticulocytes was highest in the low folic acid diet group and did not differ between the basal and high folic acid diet groups. The folate concentration in plasma and erythrocytes was lower and higher in the low and high folic acid diet groups, respectively, compared to the basal diet group (Table II). In the liver and bone marrow, the concentration of folate was 20 ~ 50% lower in the low folic acid diet group (Table II and Figure 1a). Folate concentration was comparable in the high folic acid diet group compared to the basal folic acid group, in spite of the 20 times higher content of folic acid in the diet (2 mg/kg vs. 40 mg/kg). In the bone marrow, the decrease in the concentration of folate and increase in TBI-induced chromosomal damage corresponded well (Figure 1). In the TBI groups, the proportion of MNed reticulocytes was significantly correlated with the concentration of folate in plasma ($R = -0.72$, $p < 0.05$), erythrocyte ($R = -0.56$, $p < 0.05$), and bone marrow ($R = -0.59$, $p < 0.05$), but not in liver ($R = -0.24$, $p = 0.34$). Interestingly, the concentrations of folate in the plasma, erythrocyte and bone marrow were lowered by TBI but there was no reduction in the liver (Table II and Figure 1a). In contrast, the concentration of vitamin C in plasma was unaltered by TBI (data not shown). To confirm this finding, fresh mouse plasma was irradiated with X-ray (3 Gy) and changes in the concentration of folate and vitamin C were measured. Similar to the *in vivo* experiment, *in vitro* X-ray irradiation to plasma decreased the concentration of folate but not vitamin C (Figure 2).

Table I. Body, liver and spleen weights of mice fed various folic acid diets for 4 weeks followed by TBI (animal study).

Dietary folate	X-ray (Gy)	Body weight (g)	Liver weight (g/100g BW)	Spleen weight (g/100g BW)
Low	0	37.5±3.9	4.01±0.27	0.29±0.18
	0.5	35.2±3.1	4.14±0.17	0.21±0.04[x]
Basal	0	35.4±2.4	3.95±0.20	0.27±0.02
	0.5	37.5±2.0	4.01±0.12	0.19±0.01[x]
High	0	36.4±1.1	3.88±0.28	0.25±0.05
	0.5	37.8±0.9	4.03±0.32	0.21±0.04[x]
Significant effects as determined by two-way ANOVA:				
Dietary folate		NS	NS	NS
X-ray		NS	NS	<0.0001
Folate vs. X-ray		NS	NS	NS

Male ICR mice (4-weeks-old) were fed a low folic acid diet (0 mg/kg), basal diet (2 mg/kg) or high folic acid diet (40 mg/kg) for 4 weeks, then given TBI (0.5 Gy) and sacrificed on day 2 post-exposure. Values are the means±SD, $n = 6$; [x]Significant TBI effect (vs. respective non-irradiated diet group, $p < 0.05$).

Human study

All subjects successfully completed the study. In the placebo group, the proportion of MNed lymphocytes and the concentration of folate and homocysteine in plasma did not differ before and after the intervention (Tables III and IV). The concentration

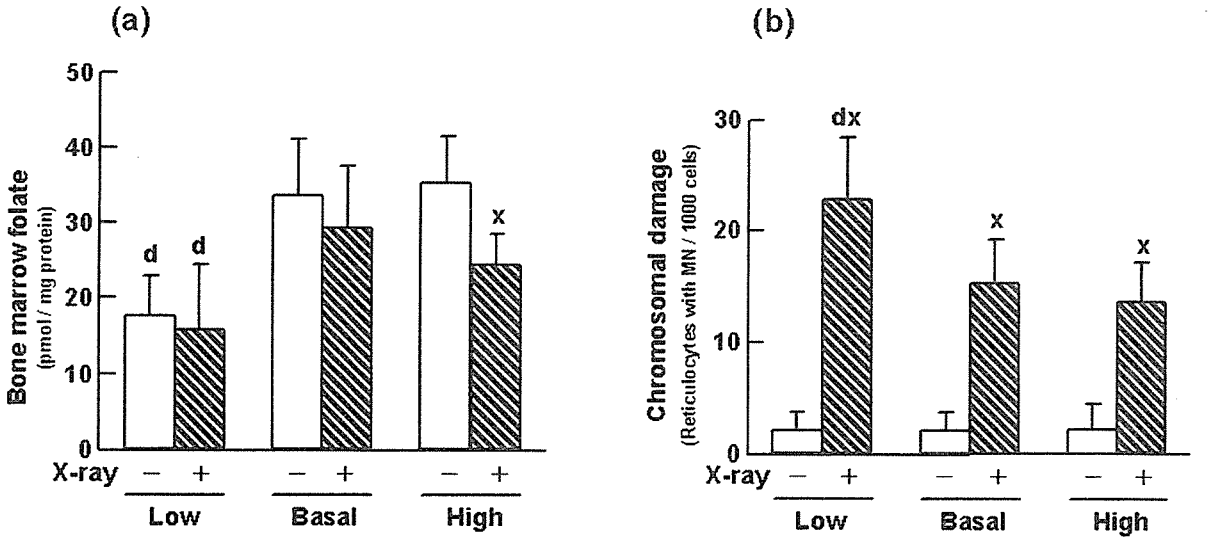

Figure 1. Folate status (a) and TBI-induced chromosomal damage (b) in the bone marrow of mice fed various folic acid diets for 4 weeks (animal study). Male ICR mice (4 weeks old) were fed a low folic acid diet (0 mg/kg), basal diet (2 mg/kg) or high folic acid diet (40 mg/kg) for 4 weeks, then given TBI (0.5 Gy) and sacrificed on day 2 post-exposure. Chromosomal damage in bone marrow was evaluated by the appearance of MNed reticulocytes in peripheral blood, determined 2 days after TBI; [d]Significant dietary effect (vs. basal folic acid diet group with same TBI dose; $p < 0.05$); [x]Significant TBI effect (vs. unirradiated (0 Gy) group with the same diet treatment; $p < 0.05$). Each column and vertical bar indicates the mean and SD, n = 6.

Table II. Concentrations of folate in the plasma, erythrocytes and liver of mice fed various folic acid diets for 4 weeks following by TBI (animal study).

Dietary folate	X-ray (Gy)	Plasma folate (nM)	Erythrocyte folate (μM)	Liver folate (pmol/mg protein)
Low	0	78.2 ± 45.7^{d}	1.06 ± 0.35^{d}	99.1 ± 25.9^{d}
	0.5	42.6 ± 10.1^{d}	0.91 ± 0.45^{d}	125.2 ± 23.1^{d}
Basal	0	136.3 ± 26.8	2.38 ± 0.62	149.8 ± 24.8
	0.5	99.3 ± 23.8	2.14 ± 0.57	161.0 ± 19.8
High	0	214.2 ± 64.4^{d}	3.26 ± 0.49^{d}	172.0 ± 40.5
	0.5	141.1 ± 46.4^{x}	2.48 ± 0.68^{x}	164.8 ± 36.2
Significant effects as determined by two-way ANOVA:				
Dietary effect		<0.0001	<0.0001	0.0001
TBI effect		0.002	0.0389	NS
Diet vs. TBI		NS	NS	NS

Male ICR mice (4 weeks-old) were fed a low folic acid diet (0 mg/kg), basal diet (2 mg/kg) or high folic acid diet (40 mg/kg) for 4 weeks, then given TBI (0.5 Gy) and sacrificed on day 2 post-exposure. Values are the means $\pm$ SD, $n = 6$. [d]Significant dietary effect (vs. basal folic acid diet group with same TBI dose; $p < 0.05$); [x]Significant TBI effect (vs. unirradiated (0 Gy) group with the same diet treatment; $p < 0.05$).

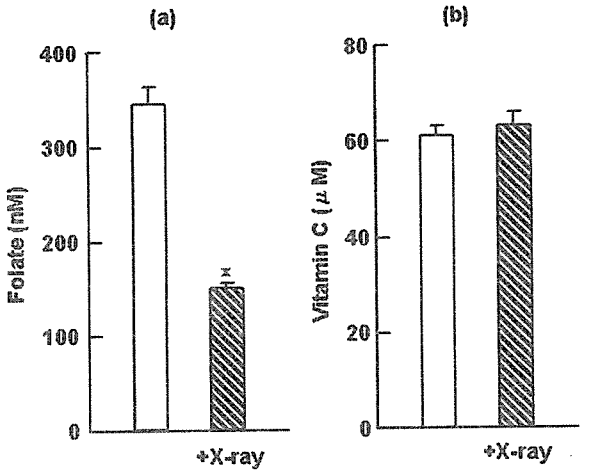

Figure 2. Concentration of folate (a) and vitamin C (b) in mice plasma with and without X-ray irradiation *in vitro*. Fresh mice plasma was irradiated with X-ray (3 Gy) and changes in the concentrations of folate and vitamin C were measured; each column and vertical bar indicates the mean and SD, n = 3; *Significant irradiation effect ($p < 0.05$).

of folate in the plasma was increased about 3-fold by folic acid supplementation, but the concentration of homocysteine, an indicator of low folate status, and the proportion of MNed lymphocytes with and without X-ray irradiation *in vitro* were unaltered by folic acid supplementation.

Discussion

Several studies have shown that folate affects chromosomal stability (Duthie et al. 2002; Fenech

Table III. Concentration of folate and homocysteine in plasma after 2 weeks' intervention (human study).

	Placebo group	Folic acid supplemented group
Folate (nM)		
Baseline (pre)	13.6 ± 5.0	10.3 ± 3.2
2 wk (post)	11.5 ± 3.1	29.4 ± 3.7
pre-post	-2.0 ± 6.7	19.1 ± 2.9*
Homocysteine (uM)		
Baseline (pre)	6.7 ± 4.0	7.4 ± 2.5
2 wk (post)	6.7 ± 3.9	7.0 ± 2.5
pre-post	-0.1 ± 0.5	-0.4 ± 1.3

Healthy male volunteers were supplemented with placebo or folic acid 800 μg/day for 2 weeks; Before and after the supplementation period, their peripheral blood was collected after an overnight fast; Values are the means $\pm$ SD ($n = 10$ in placebo group, and $n = 12$ in supplemented group); *Significant supplementation effect (vs. placebo: $p < 0.05$).

Table IV. Spontaneous and X-ray induced chromosomal damage in lymphocytes before and after 2 weeks' intervention (human study).

	Placebo group		Folic acid supplemented group	
X-ray irradiation	−	+	−	+
	(% of MNed binuleated cells in binucleated cells)			
Baseline (pre)	2.6 ± 2.1	29.6 ± 6.8	2.1 ± 1.3	36.6 ± 8.8
2-wk (post)	2.7 ± 3.4	33.1 ± 12.8	2.0 ± 0.8	35.0 ± 10.9
pre-post	-0.1 ± 3.2	3.5 ± 13.7	0.1 ± 0.9	-1.6 ± 9.9

Healthy male volunteers were supplemented with placebo or folic acid 800 μg/day for 2 weeks. Before and after the supplementation period, their peripheral blood was collected after an overnight fast. Blood samples were irradiated with and without X-ray (0.5 Gy) *in vitro*, and subjected to chromosomal analysis by micronucleus assay. Values are the means $\pm$ SD ($n = 10$ in placebo group, and $n = 12$ in supplemented group).

2001). Low folate status and gamma-ray irradiation increase chromosomal damage *in vitro* (Beetstra et al. 2005, Courtemanche et al. 2004). This evidence suggests that a low folate status increases susceptibility to X-ray-induced chromosomal damage *in vivo*. In this study, we investigated the effect of folate status on X-ray-induced chromosomal damage in animal and human studies. MN assay is not a direct marker of oxidative damage but is able to detect DNA damage better than 8-hydroxydeoxyguanosine (Kobus et al. 1993). TBI-induced chromosomal damage in bone marrow in mice can be detected at 2 days after TBI by increases in peripheral reticulocytes with MN (Umegaki et al. 1994b). This time point is also suitable to estimate the decrease in antioxidants and increase in oxidative damage in the bone marrow of mice and rats given TBI (Umegaki

et al. 1995, 2001, Umegaki and Ichikawa 1994). Therefore we evaluated various parameters on day 2 after TBI in the mouse study.

In the animal study, mice were fed either a low, basal, or high folic acid diet for 4 weeks, and then given TBI (0.5 Gy) to induce chromosomal damage in bone marrow. The folate concentration of plasma, erythrocytes, and bone marrow in the low folic acid diet group was less than 2/3 of the basal folic acid diet group but not in liver (Table II and Figure 1a). The concentration of folate in liver did not decrease with TBI at a dose of 0.5Gy, which may be due to high folate stores and more antioxidant activity in the liver compared to blood and bone marrow (Izzotti et al. 1999). It is reported that γ-ray (7Gy)-irradiated mice showed a decrease in folate levels and an increase in degraded compounds in the liver (Kesavan et al. 2003). In the non-irradiated groups, chromosomal damage in the bone marrow was comparable among the groups, suggesting that the reduction of folate status per se did not influence chromosomal damage in the bone marrow (Figure 1b). However, TBI-induced chromosomal damage in the low folate group was significantly increased 1.5-fold more than the basal and high folic acid groups. A similar increase in chromosomal damage associated with a low folate status has been reported with caffeine and sodium arsenite treatment (MacGregor et al. 1990, McDorman et al. 2002). These findings suggest that a low folate status increases susceptibility to chromosomal damage by not only chemical treatment but also X-ray irradiation, and that folate has a valuable role in chromosomal stability in the body after DNA-damaging treatment.

This animal study showed a modification of TBI-induced chromosomal damage by a low folic acid diet, but not by a high folic acid diet relative to the basal diet. As the folate concentration in the bone marrow did not differ between basal and high folic acid diet groups, it is reasonable to conclude that folate saturation in the bone marrow resulted in no further protection against TBI-induced chromosomal damage in the high folic acid diet group. In the human study, 2-week supplementation with folic acid (800 μg/day) elevated plasma folate 3-fold, but did not change lymphocyte chromosomal damage induced by X-ray irradiation *in vitro* (Tables III and IV). We detected an increase in plasma folate by folic acid supplementation, but unfortunately, we could not examine folate levels in lymphocytes due to limited samples. It is well known that folic acid supplementation to subjects with a low folate status decreases the plasma homocysteine level (Brouwer et al. 1999, Ward et al. 1997). In this study, folic acid supplementation did not decrease plasma homocysteine. Therefore, it is reasonable to speculate that the subjects had an adequate folate status, and folic acid supplementation did not increase the lymphocyte folate level. This human study was similar to the animal study with regard to showing no effect of excessive folic acid supplementation on X-ray-induced chromosomal damage. It is interesting to note the study of Blount et al. (1997), who examined the effect of 5 mg/day folic acid supplementation for 8 weeks on chromosomal damage using erythrocytes and reticulocytes in splenectomized subjects. They found that folic acid supplementation increased erythrocyte folate (5.1 times) and decreased chromosomal damage by less than half in low folate subjects, but supplementation increased erythrocyte folate (1.6 times) and did not change chromosomal damage in subjects with normal folate levels. The result is consistent with this study, suggesting that folic acid supplementation attenuates chromosomal damage induced by X-ray irradiation only in a low folate status, but not in a normal folate status. According to these findings, it is suggested that the supplementation of folic acid in a low folate status has a benefit against X-ray-induced chromosomal damage, but excess supplementation in a normal folate status has little benefit.

It would be interesting to know the mechanisms of how a low folate status increases chromosomal damage following X-ray irradiation. We propose two mechanisms: The first and major mechanism would be that folate protects against chromosomal damage during DNA synthesis and repair. It is well known that folate donates methyl groups for DNA synthesis and repair (Choi & Mason 2000). In a low folate status, the supply of methyl groups needed for DNA synthesis and repair following X-ray irradiation is limited, resulting in chromosomal damage. The second mechanism may be that folate removes ROS generated by X-ray irradiation as a radical scavenger. In this study, X-ray irradiation decreased the concentration of folate, but not vitamin C in fresh mice plasma (Figure 2). Recently, Kesavan et al. (2003) reported that γ-ray-irradiated mice showed a decrease in folate levels and an increase in degraded compounds in the liver. Folic acid has been shown to scavenge free radicals efficiently (Joshi et al. 2001). In screening tests of antihemolytic action among several antioxidant vitamins, folic acid has been shown to have similar effects to vitamin C (Stocker et al. 2003). If a compound has an antioxidative effect, it would be degraded to react with ROS, thereby protecting other substances such as DNA that exist in the same condition. The previous reports and this study suggest that folate can act as a radical scavenger, thereby contributing to protection against X-ray-induced chromosomal damage. The degradation of folate due to X-ray irradiation also suggests the enhanced requirement of folate in the body in individuals exposed to biologically significant doses

of radiation. To address these possibilities, further detailed investigation is needed.

In conclusion, this study showed that a low folate status increases susceptibility to chromosomal damage induced by X-ray irradiation, and that excessive folic acid supplementation under normal conditions saturates folate concentration in a target tissue, resulting in no further benefit against irradiation. Further investigation is needed to clarify the minimum dietary intake level of folate for protection against chromosomal damage.

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Vulnerability of folate in plasma and bone marrow to total body irradiation in mice

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Abstract

Purpose: To examine how folate status in a body is influenced by oxidative stress.
Material and methods: Mice were given total body irradiation (TBI) by X-ray, and changes in the concentration of folate were compared to those in vitamins C and E.
Results: In a time-dependent study, folate in plasma and bone marrow decreased from 5 h until 120 h post-TBI at 3 Gy. Folate in plasma and bone marrow decreased in a dose-dependent manner at 24 h. Marked decreases of vitamins C and E were also detected in bone marrow, but not in plasma even at 10 Gy of TBI. The susceptibility of plasma folate by irradiation was confirmed by an *in vitro* exposure study. Neither vitamins C and E nor folate were decreased in the liver by TBI.
Conclusion: It is suggested that folate is vulnerable to oxidative stress, and folate may need to be evaluated, particularly for TBI or radiotherapy.

Keywords: *Total body irradiation, folate, bone marrow, plasma, antioxidant*

Introduction

Oxidative stress on the body induces oxidative damage in biomolecules such as DNA and lipids. Damaged biomolecules are implicated in the process of aging and the occurrence of various diseases (Ames et al. 1993). Folate is known to be an essential cofactor for the synthesis of nucleotide and methylation of various biological substances (Moat et al. 2004). Low folate status is a risk factor for cancer (Choi & Mason 2000), megaloblastic anemia (Lindenbaum & Allen 1995) and neural tube defects (Tamura & Picciano 2006). Folate deficiency increases plasma homocysteine (Ueland et al. 1993), which is associated with oxidative stress-related diseases such as cardiovascular disease (Moat et al. 2004), arteriosclerosis (Fruchart et al. 2004), diabetes mellitus (Schalinske 2003) and neurodegenerative disease (Mattson & Shea 2003).

Reducing agent(s) is necessary throughout the folate assay procedure because it is known to be highly unstable (Pfeiffer et al. 1997). Folate has been reported to be broken down to pteridine moiety and *p*-aminobenzoylglutamic acid moiety by hydroxyl radical and ultraviolet radiation *in vitro* (Off et al. 2005, Patro et al. 2005). Decreased folate and increase of *p*-aminobenzoylglutamic acid in the liver are shown in mice given γ-ray (7 Gy) (Kesavan et al. 2003). Furthermore, the radical scavenging capacity of folate is reported to be an equivalent to that of vitamin C (Stocker et al. 2003). According to these findings, folate is thought to be involved in oxidative stress in many ways, and it is therefore interesting to elucidate folate status change due to oxidative stress.

In radiotherapy, ionizing radiation is used to kill the target cells or tissues. For example total body irradiation (TBI) at a dose of about 3 Gy is performed several times preceding bone marrow transplantation to kill the bone marrow cells of the recipient. We have reported decreased vitamins C and E and increased markers of oxidative damage in X-ray-irradiated mice and rats at a dose of about 3 Gy (Umegaki & Ichikawa 1994, Umegaki et al. 1995, Umegaki et al. 2001). We also have reported

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decreased folate in the bone marrow of mice given 0.5 Gy of TBI (Endoh et al. 2006); however, details of the findings remain unclear.

In this study we gave mice TBI and determined the time- and dose-dependent changes in the concentration of folate in plasma, liver and bone marrow, and the changes were compared to those of vitamin C and vitamin E. The results from this study may be used as a reference to estimate the folate requirement under accumulating oxidative stress or to assess folate effects on medical treatment during radiotherapy.

Materials and methods

Materials

Folinic acid calcium salt pentahydrate was from Fluka (Buchs, Switzerland). *Lactobacillus rhamnosus* (ATCC number 27773) was from the American Type Culture Collection (Rockville, MD, USA). Folic Acid Casei Medium was obtained from Becton Dickinson (Sparks, MD, USA). Rat serum was purchased from Nippon Biotest Laboratories Inc. (Tokyo, Japan). Other chemicals were obtained from Wako Pure Chemical Ltd. (Osaka, Japan).

Experimental animals and X-ray irradiation

Male ICR mice (4 weeks old) were purchased from Japan Clea (Tokyo, Japan). Mice were given TBI with X-ray in a chamber without anesthesia. X-ray irradiation was performed using a soft X-ray unit (OM-150RS, Ohmic, Tokyo) at a dose rate of 0.5 Gy/min. The beam was filtered through Cu (0.1 mm) and Al (0.2 mm). In a time-dependent study, mice were irradiated with 3 Gy using X-ray and sacrificed at various time points (0, 1, 3, 5, 24, 48, 96, 120 h). In a dose-dependent study, mice were irradiated with various X-ray doses (0, 1, 3, 10 Gy) and sacrificed at 24 h. The mice were kept in polypropylene cages with *ad libitum* access to laboratory feed and tap water. After exposure, the mice were anesthetized with sodium pentobarbital, and blood was taken from the large abdominal vein with a heparinized syringe. The blood was immediately centrifuged (RS-20IV; TOMY SEIKO Co., Ltd, Tokyo, Japan) at 1500 *g* for 15 min at 4°C to prepare plasma. The liver was immediately removed, frozen, and stored at −80°C until analysis. Bone marrow cells were prepared from the femurs and tibiae according to the method reported previously (Umegaki & Ichikawa 1994). For the analysis of folate, blood was immediately mixed with 0.5% ascorbic acid and bone marrow cell samples were similarly mixed with 0.5% ascorbic acid sodium salt. Plasma was immediately mixed with 5 volumes of 6% metaphosphoric acid for the analysis of vitamin C and 100 volumes of ethanol containing 0.15% butylated hydroxyl toluene for the analysis of vitamin E. In an *in vitro* study, freshly prepared mouse plasma was irradiated with X-ray at a dose of 3 Gy to examine changes in the concentration of folate, vitamin C and vitamin E in the sample. All procedures were in accordance with the National Institute of Health and Nutrition guidelines for the care and use of laboratory animals.

Analytical methods

Folate was analysed by microbiological assay using a 96-well microplate and glycerol-cryoprotected *Lactobacillus rhamnosus* methods as reported previously (Endoh et al. 2006). Briefly, the liver and bone marrow cell samples were homogenized or sonicated with 9 volumes of folate extraction buffer (50 mM phosphate buffer, 0.5% ascorbic acid, final pH 6.1). These samples were autoclaved at 121°C for 30 min, cooled in an ice-water bath, and centrifuged at 2000 *g* for 15 min. The supernatant (150 μl) was incubated with 100 μl rat serum conjugase and 2.79 ml folate extraction buffer at 37°C for 6 h. The folate concentration in the plasma and conjugase-treated liver and bone marrow samples was analysed by the microbiological assay (Horne 1997).

Vitamin C and vitamin E were analysed by HPLC with an electrochemical detector (Shiseido, Tokyo, Japan for vitamin C and IRICA Co., Kyoto, Japan for vitamin E) (Umegaki & Ichikawa 1994, Umegaki et al. 1999). Protein was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) and Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

Statistical methods

Data are presented as the mean ± standard deviation (SD) for individual groups. Statistical analyses of the data were performed with analysis of variance followed by a post hoc test of Fisher's Protected Least Significant Difference. These statistical analyses were performed with Stat View 5.0 (Abacus Concepts, Inc., Berkeley, CA, USA). Values of $p < 0.05$ were considered significant.

Results

Time-dependent study

Mice were irradiated at 3 Gy of X-ray and time-dependent changes in the concentration of folate, vitamin C and vitamin E in plasma, liver and bone marrow were determined until 120 h of post exposure. Body weight was not significantly influenced

by TBI up to 120 h, while relative spleen weight was decreased in a time-dependent manner (Table I). Vitamin C and vitamin E in the plasma showed no significant change due to TBI (data not shown), but folate was significantly decreased at 5 h, falling to a minimum at 24 h (53% of the nonirradiated level) (Figure 1). In the liver, three vitamins showed no clear change with TBI (data not shown). In bone marrow, vitamin C was markedly decreased at 1 h and was undetectable at 24 h. Vitamin E also decreased to a minimum at 48 h (being 12% of the nonirradiated level) (Figure 2). Similar to vitamin E, folate in bone marrow decreased to a minimum at 48 h (being 27% of the non-irradiated level).

Dose-dependent study

In the time-dependent study, significant decreases of folate in bone marrow and plasma were detected at 24 h after TBI. Thus, mice were irradiated at various doses of X-ray ranging from 1 – 10 Gy, and dose-dependent changes in folate, vitamin C and vitamin E were determined at 24 h post-exposure. In plasma, vitamin C and vitamin E showed no significant change up to 10 Gy of exposure (data not shown), but folate was significantly decreased at 1 Gy and fell to a minimum at 3 Gy (Figure 3). In the liver, folate as well as vitamin C and vitamin E showed no significant change up to 10 Gy (data only shown folate in Figure 3). In bone marrow, vitamin C was undetectable at 1 Gy, and vitamin E decreased with exposure of 1 Gy and fell to a minimum at 3 Gy (Figure 4). Similar to the changes of vitamin E, folate decreased at 1 Gy and fell to almost minimum levels at 3 Gy.

Table I. Time-dependent changes in body weight, liver and spleen weights of mice after TBI at 3 Gy.

Time after X-ray (h)	Body weight (g)	Relative spleen weight (g/100 gBW)
0	28.9 ± 1.8	0.32 ± 0.03*
1	29.2 ± 1.2	0.39 ± 0.06
3	29.5 ± 1.6	0.28 ± 0.04
5	29.3 ± 2.0	0.28 ± 0.06*
24	28.2 ± 2.2	0.19 ± 0.02*
48	28.7 ± 1.6	0.15 ± 0.03*
96	29.3 ± 2.7	0.17 ± 0.03*
120	29.4 ± 2.6	0.18 ± 0.02*

Male ICR mice (4 weeks old) were subjected to TBI via X-ray at a dose of 3 Gy, and then sacrificed at 1, 3, 5, 24, 48, 96, 120 h. Values are the means ± SD, $n = 5$; *Significant TBI effect (vs. non-irradiated diet group, $p < 0.05$).

In vitro *study*

To confirm the result of the *in vivo* study, fresh mouse plasma was directly irradiated *in vitro* at 3 Gy to determine the changes in folate, vitamin C and vitamin E. Consistent with the *in vivo* study (Figure 1), vitamin C and vitamin E in the plasma were unchanged by exposure, but folate was significantly decreased (Figure 5).

Discussion

It is reported that both oxidative stress and low folate status are involved in the occurrence of diseases such as atherosclerosis and cancer (Choi & Mason 2000, Fruchart et al. 2004). In addition, folate has unstable chemical characteristics. Clarifying the relationship between oxidative stress and folate status in the body will give some insight into understanding the

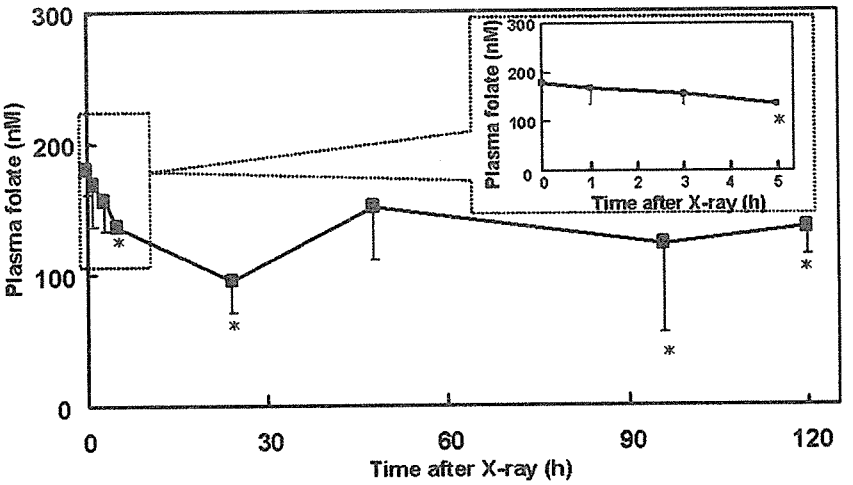

Figure 1. Time-dependent changes in folate in the plasma of mice after TBI at 3 Gy. Male ICR mice (4 weeks old) were subjected to TBI via X-rays at a dose of 3 Gy, and then sacrificed at 1, 3, 5, 24, 48, 96, 120 h for the analysis of antioxidant vitamins. Each point and vertical bar indicates the mean and SD for 5 mice. *Significantly different from non-irradiated level ($p < 0.05$).

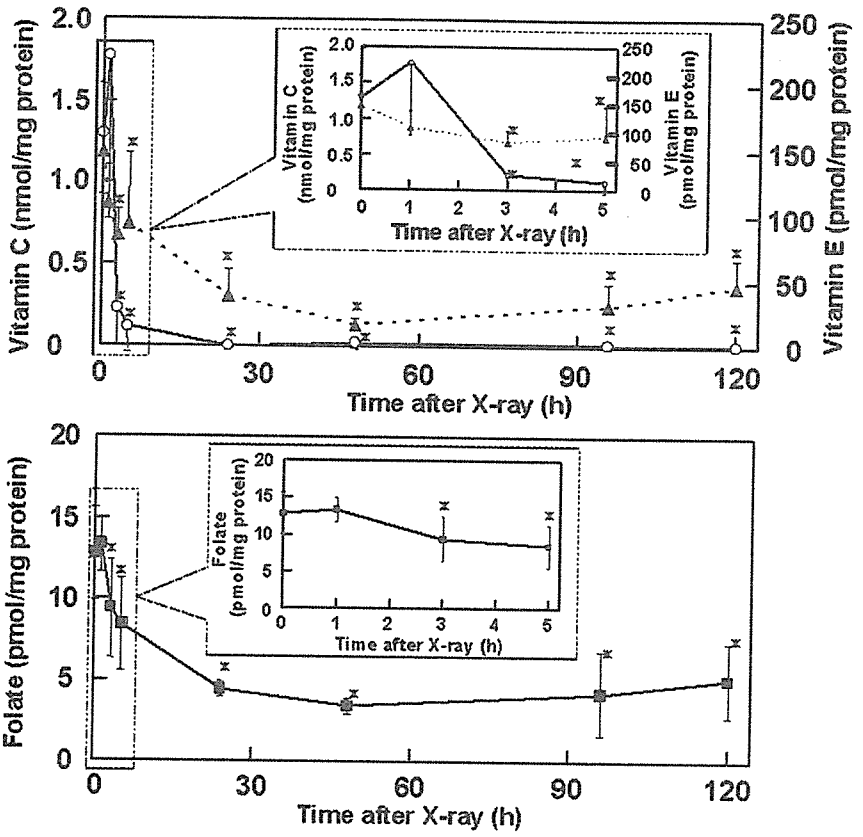

Figure 2. Time-dependent changes in folate, vitamin C and vitamin E in the bone marrow of mice after TBI at 3 Gy. Male ICR mice (4 weeks old) were subjected to TBI via X-rays at a dose of 3 Gy, and then sacrificed at 1, 3, 5, 24, 48, 96, 120 h for the analysis of antioxidant vitamins. Each point (vitamin C, circle; vitamin E, triangle; folate, square) and vertical bar indicates the mean and SD for 5 mice. *Significantly different from non-irradiated level ($p < 0.05$).

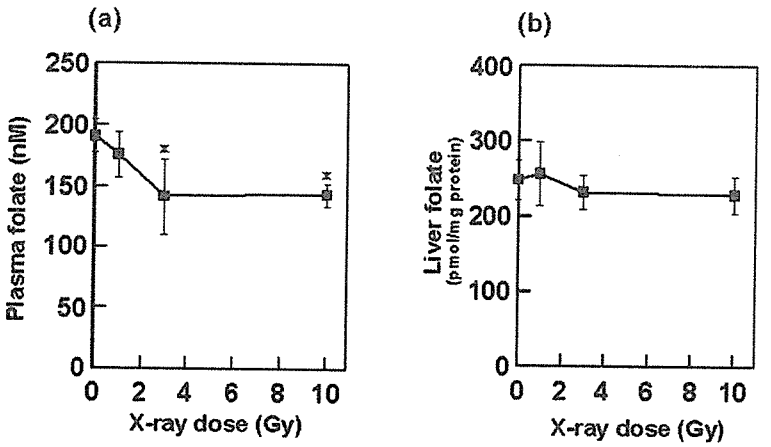

Figure 3. Dose-dependent changes in folate in the plasma (a) and liver (b) of mice after TBI at various doses. Male ICR mice (4 weeks old) were subjected to TBI via X-rays at a dose of 0, 1, 3, 10 Gy. The concentrations of vitamins were determined 24 h after irradiation. Each point and vertical bar indicates the mean and SD for 5 mice. *Significantly different from non-irradiated level ($p < 0.05$).

requirement of folate. In this study, we gave mice TBI, a well-known oxidative stress on the body, and determined the changes of folate status in the plasma, liver and bone marrow in the context of the X-ray dose and post-exposure time. We also compared the changes with those of vitamin C and

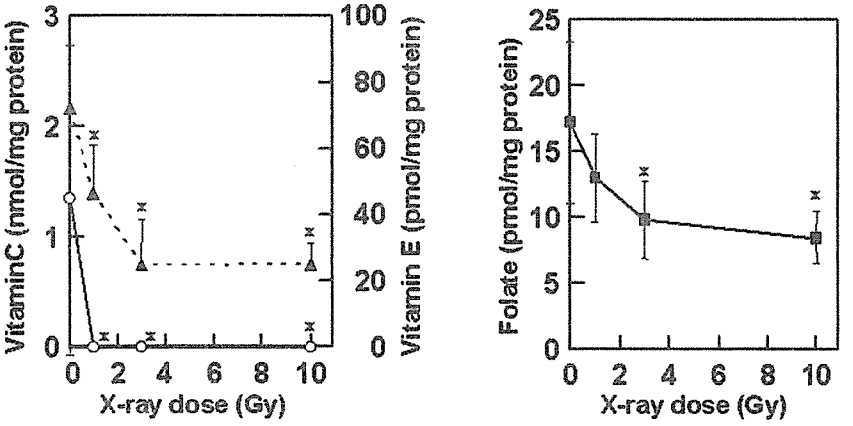

Figure 4. Dose-dependent changes in folate, vitamin C and vitamin E in the bone marrow of mice after TBI at various doses. Male ICR mice (4 weeks old) were subjected to TBI via X-rays at a dose of 0, 1, 3, 10 Gy. The concentrations of vitamins were determined 24 h after irradiation. Each point (vitamin C, circle; vitamin E, triangle; folate, square) and vertical bar indicates the mean and SD for 5 mice. *Significantly different from non-irradiated level ($p < 0.05$).

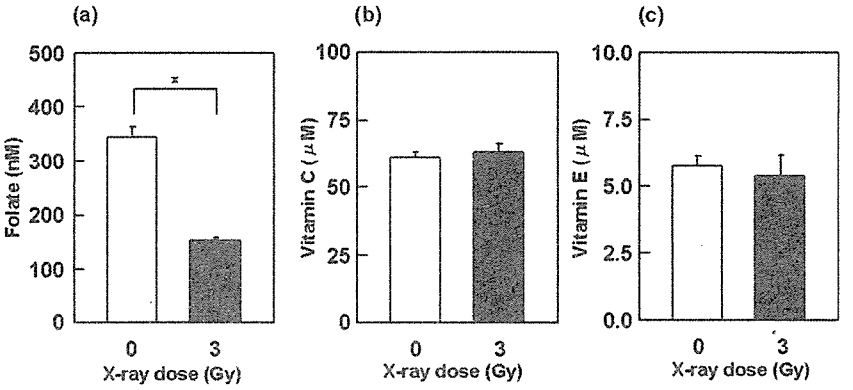

Figure 5. Concentration of folate (a), vitamin C (b) and vitamin E (c) in mice plasma with and without X-ray irradiation *in vitro*. Fresh mice plasma was irradiated with X-ray (3 Gy) and changes in the concentrations of folate, vitamin C and E were measured. *Significant irradiation effect ($p < 0.05$).

vitamin E, which we have reported previously (Umegaki & Ichikawa 1994, Umegaki et al. 1995, 2001).

Bone marrow is particularly susceptible to X-ray irradiation, and marked decreases of vitamin C, vitamin E and folate were detected. The decreases of the three vitamins showed X-ray-dose and exposure-time dependency. Similar to previous studies (Umegaki & Ichikawa 1994, Umegaki et al. 1995, 2001), TBI up to 10 Gy to mice did not decrease vitamin C and vitamin E in the liver and plasma. On the other hand, folate in the plasma was significantly decreased in this study. The results were confirmed by an *in vitro* exposure study (Figure 5). Folate is reported to be degraded by hydroxyl radical and ultraviolet *in vitro* (Off et al. 2005, Patro et al. 2005). The vulnerability of plasma folate by oxidative stress may be related with the high radical scavenging capacity of folate observed in *in vitro* studies (Joshi et al. 2001). Although the level of decrease varied among the tissues, the results in this study are consistent with the findings that X-ray- or γ-ray-irradiated mice showed a decreased folate level and an increase in degraded compounds (Endoh et al. 2006, Kesavan et al. 2003). It is therefore suggested that folate requirement is enhanced when oxidative stress is accumulated.

Folate decreased significantly in the bone marrow, but not in the liver. Previously, we reported that the degree of the decrease in vitamin C and vitamin E, and the increase in 8-hydroxydeoxyguanosine and 4-hydroxy-2-nonenal were lower in the liver than in bone marrow (Umegaki & Ichikawa 1994, Umegaki et al. 1995, 2001). We speculate that the increase of iron in the bone marrow after TBI, and high antioxidant system in the liver would underlie the

mechanisms (Umegaki et al. 2001). It has been shown that folate is degraded in the presence of iron and scavenges free radicals efficiently (Joshi et al. 2001, Shaw et al. 1989). It has also been shown that the decrease in the percentage of conjugated folate in bone marrow (from 42–10%) was lower than that in the liver (from 56–60%) in rats irradiated with X-ray (Viswanathan & Noronha 1970). Mono- and di-glutamic forms of folate are less likely to bind to folate-dependent enzymes and are catabolized (Suh et al. 2000). The form of folate between bone marrow and liver may be different, resulting in different catabolism due to TBI in this study. It has been shown that the microbiological assay used for folate measurement in this study can detect mono-, di-, tri-glutamic forms of folate (Tamura 1990), and that the cleavage of C^9-N^{10} bond of folate molecules by hydroxyl radical is a mechanism for the catabolism (Patro et al. 2005). However, it is unclear the types of damage to the folate molecules in various tissues due to TBI. Further study will be needed to clarify the underlying mechanisms.

TBI of a few gray is performed several times preceding bone marrow transplantation to kill the bone marrow cells of the recipient. Decreases in antioxidants such as vitamin E and beta-carotene in plasma were reported by TBI (Clemens et al. 1990), but little is known about folate. The results of this study suggest that folate in plasma is also decreased by TBI in humans. It is noted that folate levels affect the effectiveness and toxicity of cancer chemotherapy in animal experiments and *in vitro* studies (Whiteside et al. 2004). Sometimes, both radiotherapy and chemotherapy are performed simultaneously (Clemens et al. 1990), and the decrease in folate by radiotherapy may affect the therapeutic effects. Oxidative stress is induced in our body not only by irradiation, but also by chemical treatments or in pathological states such as diabetes mellitus. A decline in folate status increases the level of homocysteine (Ueland et al. 1993), which induces oxidative damage to the cells (Oikawa et al. 2003). Accordingly, it is suggested that folate status is involved in various diseases in many ways. It may be necessary to consider folate status during conditions involving oxidative stress, particularly during radiotherapy and to test whether excessive normal tissue morbidity following radiotherapy is related to folate status.

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日本骨粗鬆症学会 平成17年度 研究奨励賞

血中25-ヒドロキシビタミンDの新規定量法の開発と臨床応用

津川尚子　鎌尾まや　須原義智　岡野登志夫

はじめに

血中25-hydroxyvitamin D（25-OH-D）濃度はビタミンD栄養状態を最もよく反映する代謝物として骨代謝解析において欠かせない指標である。25-OH-D濃度は，従来HPLC法や競合的蛋白結合（CPBA）法で測定されてきたが，最近では特異的抗体を用いたradioimmunoassay（RIA）法や化学発光を用いた蛋白結合測定法（CLPBA）が用いられるようになった。しかし，これらの測定法はおのおのに原理が異なり，検体によっては異なる数値を与える場合がある。このことから，簡便な繁用法とは別に，標準法として真度，精度，感度がともに高い定量法の開発が望まれている。そこで，質量分析法を利用したLC/MS/MS法による25-OH-D_3，25-OH-D_2および24,25$(OH)_2D_3$濃度の高感度分別定量法を開発し，従来法による測定の妥当性を評価した。また，この方法を臨床検体に応用し，ビタミンD栄養の評価を行った。

1　方　　法

1）LC/MS/MS法による血中25-OH-D濃度測定法の確立

①血液サンプルからの抽出

ヒト血漿あるいは血清0.1mLを遠沈管にとり，

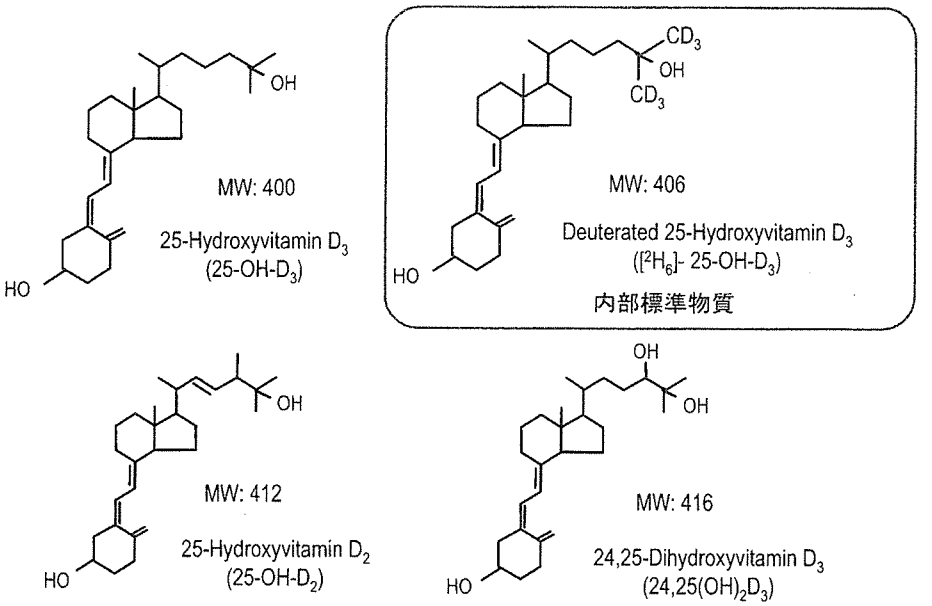

図1　ビタミンD代謝物および内部標準物質の化学構造

Key words：25-hydroxyvitamin D_3，25-hydroxyvitamin D_2，LC/MS/MS

神戸薬科大学衛生化学研究室

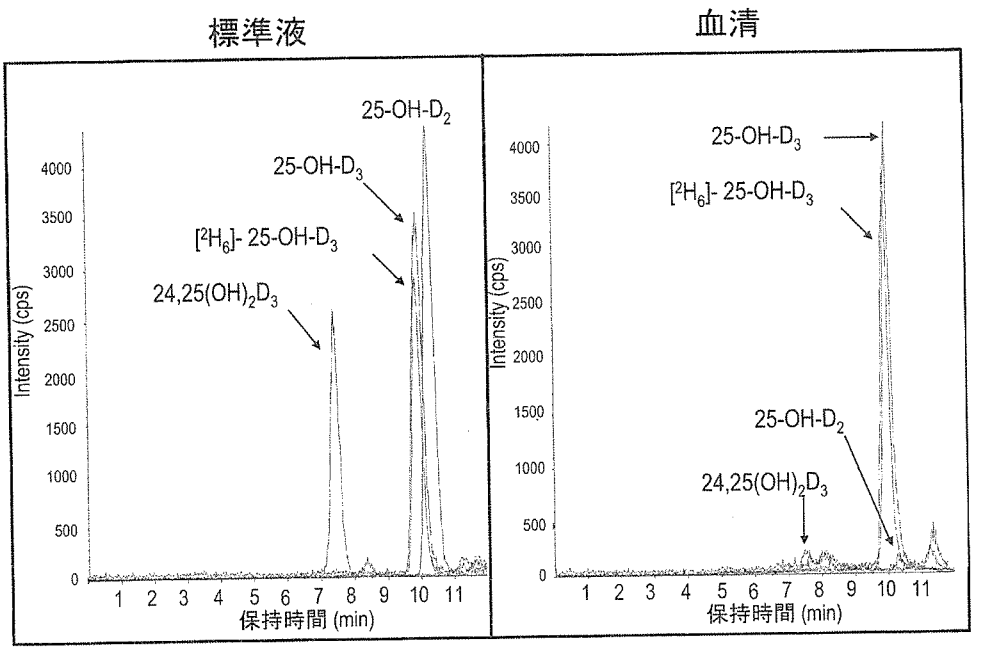

図2　LC/MS/MS分析におけるクロマトグラム

合成内部標準物質[1]として[$^2H_6$]-25-OH-D_3（側鎖26,27位メチルの水素を重水素化した化合物；図1）を2ng添加し，メタノール0.2mLを加えてボルテックスミキサーで撹拌した。3000rpmで遠心分離した後，得られた上清を，あらかじめメタノール/水（7：3，v/v）15mLで洗浄したBond Elut C_{18}に負荷し，メタノール/水（7：3，v/v）15mLで洗浄後，25-OH-D_2/D_3および24,25$(OH)_2D_3$画分をアセトニトリル/メタノール（8：2，v/v）5.0mLにより溶出させた。溶出液をロータリーエバポレーターで乾固した後，得られた残渣をメタノール100μLに溶解し，50μLを以下の条件のLC-APCI/MS/MSに適用した。別に調製したビタミンD代謝物標準溶液の分析を同時に行い，内部標準物質に対する標準ビタミンD代謝物のピーク面積比（Qs）を算出し，検量線を作成した。検体の分析から同様におのおののピーク面積比（Qt）を算出し，検量線よりLC-APCI分析時の濃度Aを求め，以下の計算により血中濃度を算出した。

血中濃度（ng/mL）=A×20/50

②ビタミンD代謝物標準液の調製

25-OH-D_3，25-OH-D_2，24,25$(OH)_2D_3$（図1）を100ng/mL含む標準原液を調製し，この溶液から10～200ng/mLの範囲で段階的な希釈溶液を調製した。一方，重水素ラベルした[$^2H_6$]-25-OH-D_3を100ng/mL含む内部標準液を別に調製した。これと先の標準希釈液を等容量で混合し，25-OH-D_3，25-OH-D_2，24,25$(OH)_2D_3$を5～100ng/mLおよび内部標準物質50ng/mLを含む標準系列を得た。

③HPLC条件

ポンプ：LC-10AD（島津製作所社製），オートインジェクター：SIL-10AD（島津製作所社製），カラム：CAPCEL PAK C_{18} UG120（4.6×250mm，5μm，資生堂社製），移動相：メタノール：水（95：5，v/v），流速：0.5mL/min

④APCI-MS/MS装置およびMS検出条件

装置：API-3000（アプライドバイオシステムズ社製），MS検出条件：Precursor ion/product ion（*m/z*）：25-OH-D_3（*m/z*：401.4/257.0），25-OH-D_2（*m/z*：413.4/355.4），24,25$(OH)_2D_3$（*m/z*：417.4/363.1），[$^2H_6$]-25-OH-D_3（*m/z*：407.4/263.4）

⑤定量精度の確認

市販のヒトコントロール血清（和光純薬社製）を用いて，IntraおよびInter assayを行い，定量

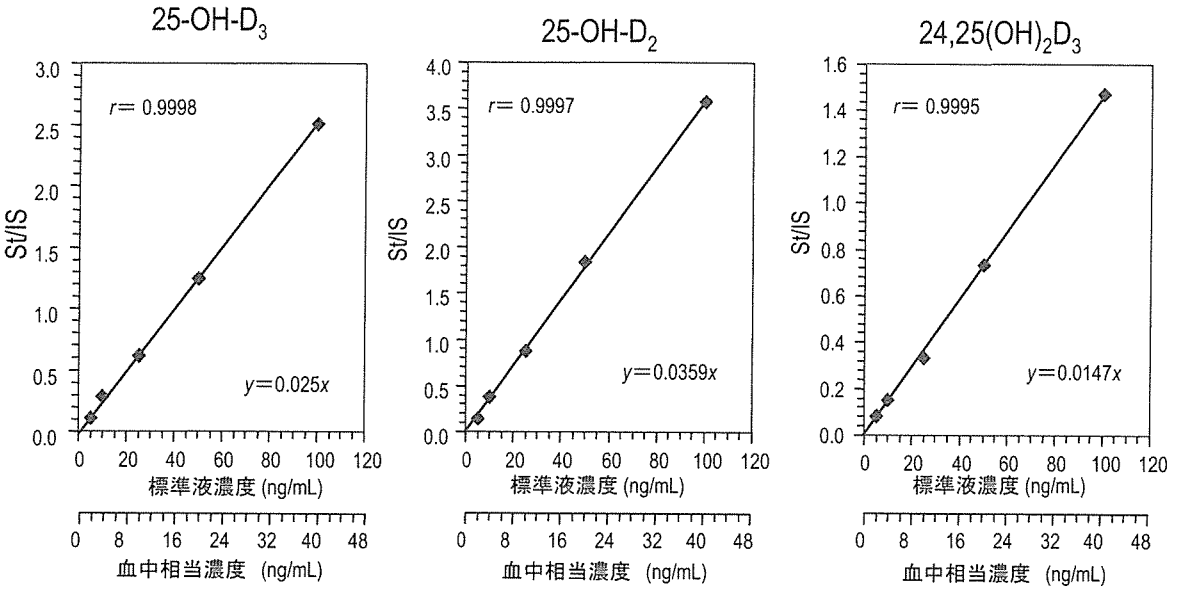

図3　ビタミンD代謝物の標準検量線

表1　標準血清を用いたビタミンD代謝物濃度の精度評価

代謝物		濃度	RSD%
25-OH-D_3	Intra assay (n=10)	20.0±1.1 (ng/mL)	5.7
	Inter assay (n=5)	18.6±0.5 (ng/mL)	2.5
	Recovery (n=5)	103.8±4.3 (%)	4.1
25-OH-D_2	Intra assay (n=10)	2.5±0.1 (ng/mL)	4.5
	Inter assay (n=5)	2.7±0.1 (ng/mL)	5.1
	Recovery (n=5)	99.3±2.2 (%)	2.2
24,25$(OH)_2D_3$	Intra assay (n=10)	2.8±0.3 (ng/mL)	11.4
	Inter assay (n=5)	2.5±0.3 (ng/mL)	9.9
	Recovery (n=5)	98.8±5.1 (%)	5.2

平均値±標準偏差

精度を確認した。

2) RIA法との比較

長野県在住の高齢者を中心とする日本人女性278名（62.1±11歳）の血漿中ビタミンD代謝物濃度を，繁用法である市販の25-OH-D RIAキット（DiaSorin社製）を用いた測定値と比較した。使用したヒト血漿検体は成人病診療研究所の白木正孝先生よりご供与いただいた。

3) 統計解析

MedCalc9.0.1.1を用いて解析した。

2　結　　果

図2に示すように，標準液ならびに標準血清において25-OH-D_2，25-OH-D_3，24,25$(OH)_2D_3$および内部標準物質は単一ピークとして検出された。標準溶液に対して作成した検量線は，5～100ng/mLの範囲で良好な直線性を示した(図3)。また，検出限界はいずれも血漿中濃度として1ng/mLであり十分な感度が得られた。

市販のヒトコントロール血清（和光純薬社製）を用いて，Intra assayおよびInter assayを行ったところ，表1に示すように十分な精度が得られ

表2　LS/MS/MS 法および RIA 法で測定されたヒト血漿中ビタミン D 代謝物濃度

LC/MS/MS				RIA (ng/mL)
25-OH-D_3 (ng/mL)	25-OH-D_2 (ng/mL)	24,25$(OH)_2D_3$ (ng/mL)	Total (ng/mL)	
21.2±6.5	0.8±1.4	1.1±0.6	23.1±6.9	20.4±6.5

n=278

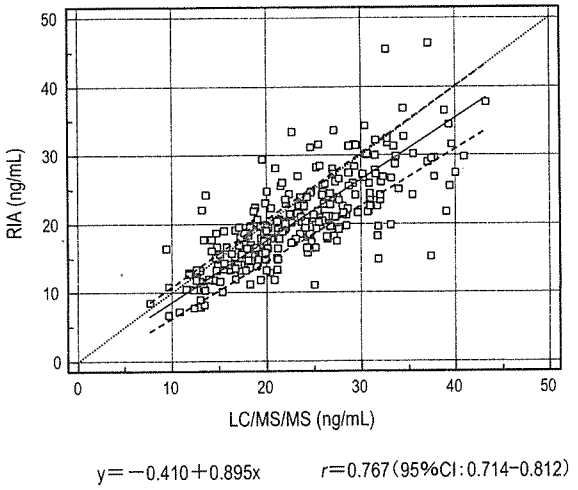

図4　Passing & Bablock 回帰分析

た。また，各ビタミン D 代謝物を 20ng/mL 添加して添加回収率を求めたところ，良好な回収率が得られた（**表 1**）。血清の 3 段階希釈試験で得られた回帰直線の相関係数は 25-OH-D_3：0.9999，25-OH-D_2：0.9967，24,25$(OH)_2D_3$：0.9997 であった。以上のことから，本法における定量は十分な精度・真度であると判断した。

本法と従来法の測定値の比較を行うため，健常ヒト血漿 278 検体を用いて DiaSorin 社製 25-OH-D RIA キットによる測定値との比較を行った。それぞれの測定法から得られた平均±SD を**表 2** に示す。LC/MS/MS 法で分別定量された 3 種の D 代謝物濃度の平均値を比較したところ，血中 25-OH-D_2 および 24,25$(OH)_2D_3$ 濃度は 25-OH-D_3 濃度の約 3〜5%程度で存在していることを確認した。RIA 法による 25-OH-D 濃度は 25-OH-D と 24,25$(OH)_2$D の合計値として得られるため，両測定法の比較では LC/MS/MS 法の濃度として 25-OH-D_3，25-OH-D_2，24,25$(OH)_2D_3$ の合計値を用いた。Passing & Bablock 回帰分析で両測定法の関係を評価した結果（**図 4**），相関係数 r=0.767（95%CI：0.714〜0.812），直線回帰式 y(RIA)=−0.410+0.895x（LC/MS/MS）が得られ，LC/MS/MS 法による測定値はやや高値を示すものの両測定法には良好な相関関係が得られた。次に，ビタミン D の不足・欠乏の指標であり 25-OH-D 濃度とは逆相関することが知られる血中 PTH 濃度との関係を比較した結果，両者はほぼ同等の逆相関関係を示し，両測定法によるビタミン D 不足の評価に差異はないと判断された（**図 5**）。また，両測定法による 25-OH-D 濃度は骨吸収マーカーの NTX とも有意に負相関することを確認した（LC/MS/MS：p=0.007，RIA：p=0.005）。

次に，LC/MS/MS 法で測定された 25-OH-D_3 濃度と 24,25$(OH)_2D_3$ 濃度の関係について検討した。24,25$(OH)_2D_3$ は 25-OH-D_3 の異化代謝物であり，両者が正相関することが知られているが，今回の対象者においても LC/MS/MS 法で測定された両代謝物濃度は有意な正相関関係を示すことを確認した（**図 6**）。25-OH-D 濃度と PTH 濃度に有意な負相関があることから，PTH 濃度と 24,25$(OH)_2D_3$ 濃度間にも有意な負の相関関係を認めた（p=0.003，r=0.179）。一方，PTH は腎臓の 1α-水酸化酵素の誘導と 24-水酸化酵素の抑制作用を示すことが知られることから，24,25$(OH)_2D_3$/25-OH-D_3 比と血中 PTH 濃度の関係を解析した結果，両者は弱いながら有意な負の相関関係を示すことを確認した（p=0.011）。

3　考　　察

血中 25-OH-D の濃度は，従来 HPLC 法あるいは CPBA 法で測定されていた。しかし，これらの測定法は 0.5mL 程度の検体が必要で抽出・精製も比較的煩雑なため，熟練した技術と測定に長

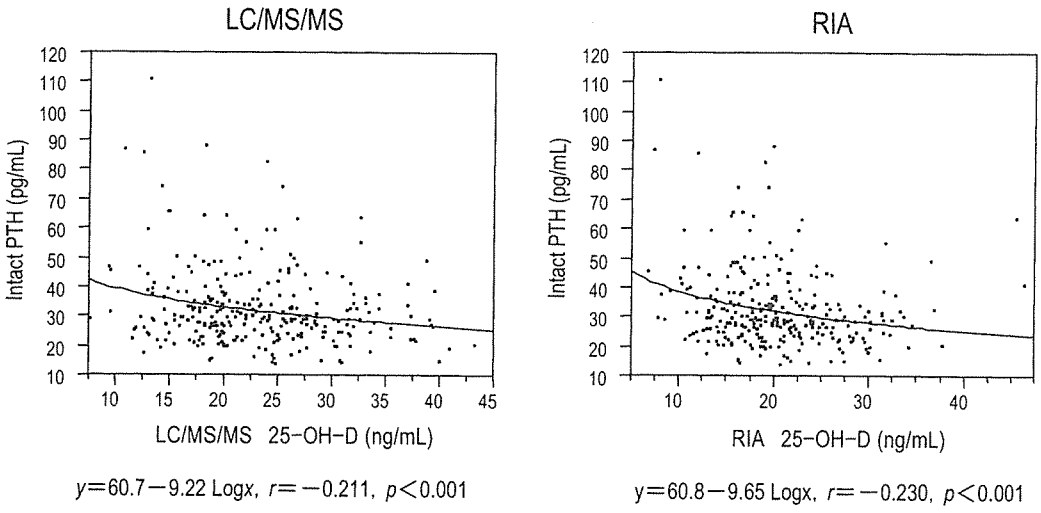

図5　LC/MS/MS 法および RIA 法による血中 25-OH-D 濃度と PTH 濃度との関係

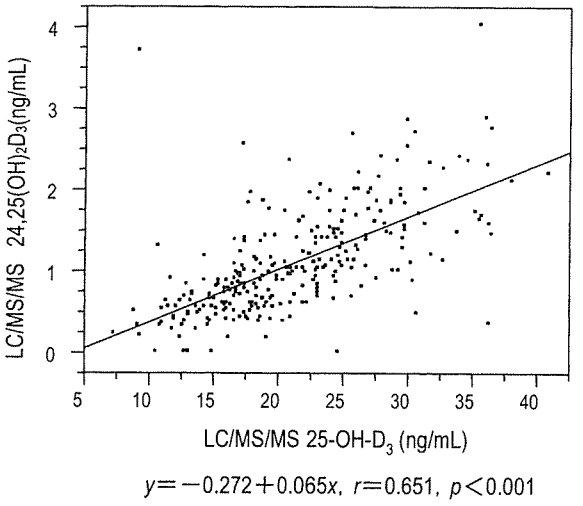

図6　血中 25-OH-D_3 濃度と 24,25$(OH)_2D_3$ 濃度の相関

時間を要することが難点であった。そのため，最近では RIA 法や酵素免疫（EIA）法や，CPBA 法と化学発光を利用した chemiluminescence protein-binding assay（CLPBA）法[2]，EIA 法と化学発光を利用した自動測定法[3]が利用されている。これらの方法はいずれも抗体やビタミン D 結合蛋白質（DBP）を利用した生物化学的方法であるが，検体中に含まれる種々の要因によってしばしば測定値に変動が生じることがある。標準法として物理化学的検出法である GC-MS 法[4]が用いられているが，今回新たに高精度質量分析法を用いた LC/MS/MS 法を開発し，本法が感度，精密度，正確度ともに十分な精度を備えた方法であることを確認した。このことから，本法は今後 HPLC 法や GC-MS 法に代わる標準法として利用できることが期待できる。RIA 法との比較においては，両者は良好な相関性を示し，両測定方法の妥当性を評価することができた。また，本法はビタミン D の栄養状態を十分評価できる方法であることも確認した。

25-OH-D_3，25-OH-D_2，24,25$(OH)_2D_3$ 濃度の同時分別定量は繁用法には期待できない LC/MS/MS 法のメリットである。抗体や DBP を用いる生物化学的検出法を用いる測定法の場合，25-OH-D_2 と 25-OH-D_3 に対する反応性の違いが問題となり，D_2 サプリメント服用者などにみられる 25-OH-D_2 濃度が高い血漿では測定法によって過大あるいは過少評価される場合がある[5~8]。わが国では米国のように D_2 をサプリメントとして利用するということはほとんどないが，シイタケなど D_2 含有食品の摂取が比較的多いために血中での存在量を把握しておく必要がある。今回の対象者の 25-OH-D_2 濃度の平均値は 25-OH-D_3 濃度の約 3%程度で測定値にはほとんど影響しない濃度であるものの，その範囲は 0～15.1ng/mL であったことから，一部の検体において分別定量による評価が必要となる可能性が示唆され

る。また，24,25(OH)$_2$D$_3$濃度については，副甲状腺機能や腎機能との関連も報告されており[9,10]，これらのD代謝物濃度の簡易分別定量が，D栄養とカルシウム代謝，骨代謝との関連性の臨床評価に応用できるものと期待される。

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Development of Novel Serum 25-Hydroxyvitamin D Determination Method and Its Clinical Application

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25-hydroxyvitamin D (25-OH-D) can be used for the evaluation of vitamin D nutritional status. However, many reports have also pointed out the marked inter-laboratory variability in the serum 25-OH-D measurement, making it difficult to define the optimal serum 25-OH-D concentration for the maintenance of bone health. Therefore, we have developed a precise and reliable method to determine 25-hydroxyvitamin D (25-OH-D$_2$/-D$_3$) in human plasma using HPLC-tandem mass-mass spectrometry with atomospheric-pressure chemical ionization (LC/MS/MS).

Osteoporosis Japan

日本人高齢女性における血中ビタミンK濃度と骨折との関係

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はじめに

ビタミンK(VK)は多くの血液凝固因子の合成において重要な役割をもつことが知られる。一方VKは，オステオカルシンやマトリックスグラ蛋白あるいはプロテインSのようなVK依存性蛋白質を，γ-カルボキシル化することによって骨代謝において重要な役割を果たす[1,2]。低フェロキノン(PK)摂取は，閉経後女性の大腿骨頸部骨折のリスクを増大させるとともに，大腿骨，脊椎における低BMDと関連があると報告されている[3〜6]。わが国では骨粗鬆症治療にVK_2が使用されているが，PKやメナキノン(MK-4, MK-7)を中心とするVK栄養が骨粗鬆症予防に果たす役割については十分な検討がなされていない。そこで，骨粗鬆症予防におけるVKの栄養効果を検討するため，日本人成人女性を対象に血中PKおよびMK-4，MK-7濃度を測定し，骨代謝関連指標との関連について調査した。

1　方　　法

対象者は30〜88まで歳の日本人女性398名(30〜49歳：52名，50〜69歳：216名，70歳以上：130名)，平均年齢62.5歳である。骨粗鬆症以外の骨代謝疾患をもつ女性および活性型VD，VK，VK拮抗薬，エストロゲン，ビスフォスフォネート，ステロイドなどの骨代謝関連薬を服用している対象者は除外した。また，BMIが16.5以下の対象者は除外した。

測定項目は血中PK，MK-4，MK-7濃度，PTH，ucOC，Ca，P，BAP濃度，Alp活性，尿中NTX/Cr，DPD/Cr，U-Ca/Cr，BMI，L_{2-4}BMDおよび椎体骨骨折頻度である。統計解析には，JMP 5.0.1Jを用いた。

2　結果と考察

日本人高齢者女性398人の血漿中PK，MK-4，およびMK-7濃度はそれぞれ1.57±1.22，0.10±0.22と6.47±9.65ng/mLであった。他の血漿および尿中生化学パラメータは正常範囲内にあり，加齢に伴って血清ucOC濃度と尿中NTX，DPD濃度は増加し，L_{2-4}BMDは減少した。対象者のうち脊椎骨折有病者は72名(18.1%)であった。

血中PKとMK-7濃度は年齢層30〜49歳および70歳以上に比べて50〜69歳で有意に高く，血中ucOC濃度と逆相関した。**図1**に，VK濃度と脊椎骨折との関係を評価するロジスティク回帰分析の結果を示す。PK濃度の増加と骨折有病率の減少とに有意な関係を認めた。MK-7濃度の増加は有意ではないが骨折有病率を減少させる傾向にあったが，MK-4濃度は骨折有病率と

Relationship between Plasma Vitamin K Concentration and Bone Fracture in Japanese Elderly Women
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Key words : Vitamin K, Bone fracture, Phylloquinone, Japanese women

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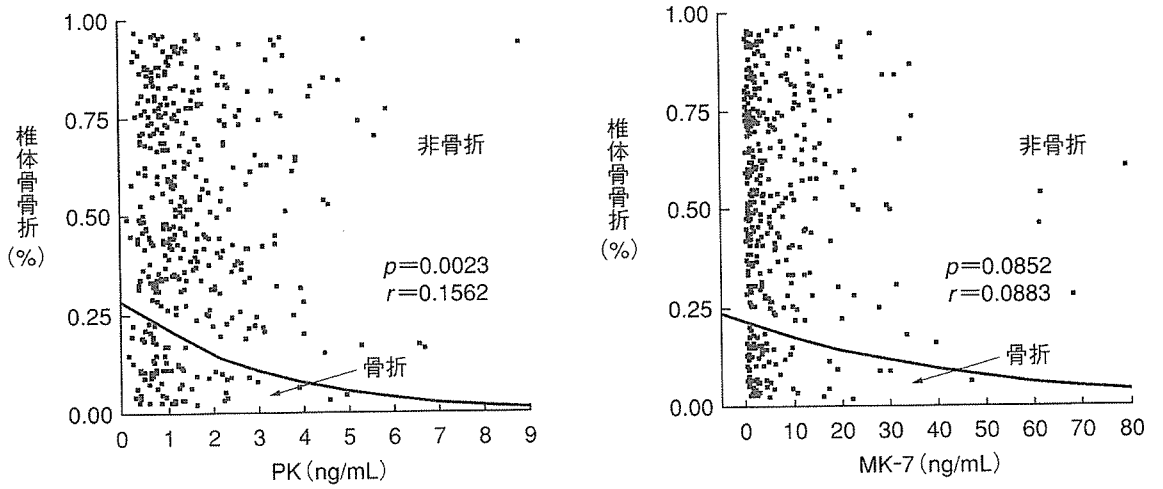
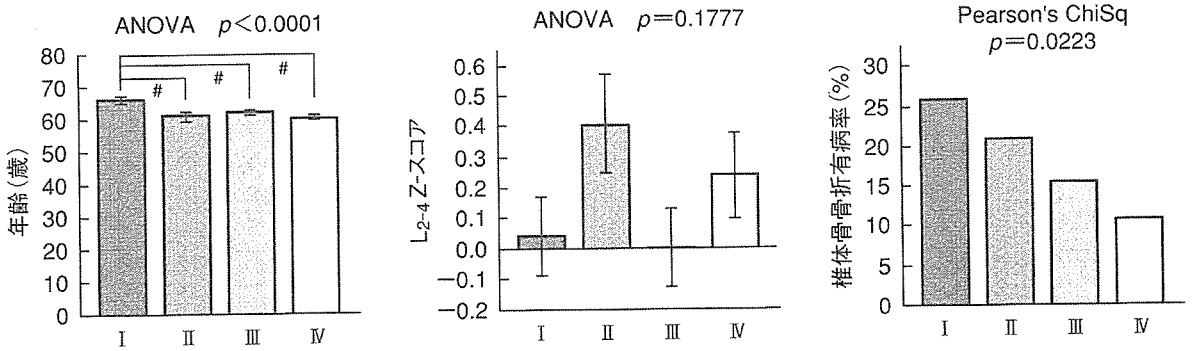


図1　ロジスティック回帰分析による血中ビタミンK濃度と椎体骨骨折の関係



群	オッズ比	95% 信頼区間	p値
Ⅰ：低 PK 高 ucOC	2.9	(1.4～6.2)	0.0041
Ⅱ：低 PK 低 ucOC	2.2	(1.0～5.0)	0.0505
Ⅲ：高 PK 高 ucOC	1.5	(0.7～3.6)	0.3096
Ⅳ：高 PK 低 ucOC	1.0		

図2　Ⅰ, Ⅱ, Ⅲ, Ⅳ群における年齢，骨密度，骨折有病率

関連しなかった。

次に，対象者をPK濃度とucOC濃度の中央値(PK：1.18ng/mL, ucOC：3.73ng/mL)で4群(Ⅰ群：低PK高ucOC群，Ⅱ群：低PK低ucOC群，Ⅲ群：高PK高ucOC群，Ⅳ群：高PK低ucOC群)に分割して骨折有病率を比較した(**図2**)。Ⅰ群の年齢は他の3群に比して有意に高く，Ⅱ, Ⅲ, Ⅳ群間の年齢に差異はみられなかった。低ucOCとして特徴づけられるⅡ群とⅣ群は高いL_{2-4} BMDおよびL_{2-4}BMD Z-scoreを示す傾向にあった。しかしながら，脊椎骨折有病率はⅠ群からⅣ群に向けて低下し，BMD，年齢とは無関係であった。また，Ⅳ群に対するⅠ群，Ⅱ群の脊椎骨折のオッズ比は有意に高く，VKの栄養状態が低下すると骨折の危険性が高くなることが示唆された。