原著

カツオ魚肉のビタミン B_{12} 含量と各種加熱調理が魚肉ビタミン B_{12} 含量に及ぼす影響

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Characterization of Vitamin B_{12} in Skipjack Meats and Loss of the Vitamin from the Fish Meats by Various Cooking Conditions

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Vitamin B_{12} contents of skipjack meats were assayed by the microbiological method authorized in the Standard Tables of Food Composition in Japan (5th Edition). The dark muscles contained substantial amounts (158.5±16.3 μg/100 g) of vitamin B_{12} relative to the ordinary muscles (dorsal portion 9.9±0.6 μg/100 g ; ventral portion 8.4±0.6 μg/100 g). A corrinoid compound was purified from the dark muscles and characterized, then it was confirmed that the dark muscles contain true vitamin B_{12}. Various heat-cooking treatments (boil, steam, sauté, fly, and microwave) did not give significant losses (2.3~14.8%) of vitamin B_{12} from the treated fish meats. The results indicate that skipjack meats (especially dark meat) are excellent vitamin B_{12} sources.
Key words: Skipjack, fish meats, dark muscle, vitamin B_{12}, heat-cooking

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緒　言

日本は世界有数の魚介類の消費国であり，魚介類から24.6%ものタンパク質を摂取しており，畜肉類の16.6%を大きく上回っている[1)]．また，魚介類はその他の栄養素（脂質，ビタミン，ミネラル）のよい供給源ともなっており[1)]，魚介類が日本人にとって重要な食品であることを示している．

ビタミン B_{12} (B_{12}) は主に動物性食品に含まれており，一部の藻類や微生物が関与する発酵食品（糸引き納豆[2)]，テンペ[3)]，後発酵茶[4)5)]）を除き植物性食品にはほとんど含まれていない[6)]．米国では，畜肉や牛乳が B_{12} のよい供給源であるが[7)]，我が国では魚介類が主要な供給源となっており[8)]，栄養学的に重要である．しかし，畜肉[9)]や牛乳[10)]に比べ，魚肉の B_{12} 含量や化学的性質，ならびに加熱調理による B_{12} の損失についての知見は非常に少ない．そこで，比較的大型で血合肉も食する赤身魚としてカツオに着目し，魚肉各部位の B_{12} 含量ならびに血合肉中に含まれるコリノイド化合物の同定を行った．また，カツオは，生で刺身やタタキとして食する場合が多いが，ま

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ぐろなどの赤身魚のモデルとして各種加熱調理が魚肉 B_{12} 含量に及ぼす影響を検討した．

実験方法

1．試料

カツオ（春獲り，生，6 匹，重量 1.5 ～ 2.0 kg）は，高知県で水揚げされたものを高知市内の市場で購入し実験に用いた．カツオの重量を測定後，内臓を除き 3 枚におろした．その後，5 匹の魚肉を血合肉と普通肉に分け，普通肉はさらに背肉と腹肉に分け，それぞれの重量を測定した．カツオ魚肉の各部位の一部は分析に供するまで－20℃の冷凍庫で保存した．

残りのカツオ魚肉は，普通肉と血合肉を分けずにフードプロセッサーで破砕・均質化した後，50 g を正確に秤量し，魚肉パテ（縦 5 x 横 5 x 高さ 2 cm）を調製した．この魚肉パテを用いて直ちに各種加熱調理の実験を行った．

ブリ，サワラ，ゴマサバの魚肉（切り身）は，高知市内の市場で購入した．

2．ビタミン B_{12} の抽出・定量法

カツオ魚肉に含まれる B_{12} の定量は，五訂日本食品標準成分表で採用されている分析マニュアル[11]に準じて *Lactobacillus delbrueckii* subsp. *lactis*（旧名 *L. leichimannii*）ATCC7830 を用いた微生物学的定量法で行った．なお，定量に用いる培地は，日水製薬株式会社製のライヒマニ保存用培地，ライヒマニ接種用培地，ライヒマニ用 B_{12} 定量基礎培地を用いた．

凍結したカツオ魚肉各部位を解凍後，フードプロセッサーにより破砕・均質化した．この均質化した魚肉各部位 2 g を正確に秤量し，定法によりシアン化カリウムを含む酢酸緩衝液（pH4.5）中で B_{12} を加熱抽出した．

また，本定量菌は，B_{12} 以外にデオキシリボースやデオキシリボヌクレオチドにも B_{12} 活性を示すため，分析マニュアル[11]に記載された以下の方法で補正した．

上記 B_{12} 抽出液の一部に 1 mol/*l* 水酸化ナトリウム溶液を加え，pH を 11 ～ 12 に調整後，オートクレーブ（121℃，30 分）処理を行い，B_{12} を分解させた．その後，1 mol/*l* 塩酸溶液で pH を 6 に調整・定容し，この溶液をアルカリ耐性因子画分として B_{12} 抽出液と同様に微生物学的定量法でみかけの B_{12} 含量を測定した．

B_{12} 抽出液画分に検出された B_{12} 含量からアルカリ耐性因子画分に検出されたみかけの B_{12} 含量を差し引き，試料中の正味の B_{12} 含量を算出した．

3．カツオ血合肉からのコリノイド化合物の単離方法

カツオ血合肉（約 600 g）をフードプロセッサーで破砕・均質化した後，0.5 g シアン化カリウムを含む 10 mmol/*l* 酢酸緩衝液（pH4.5）4 *l* を加えドラフト内で 30 分間加熱抽出した．室温まで冷却後，8000 g，10 分間の遠心分離を行い，上澄み画分を抽出液とした．

あらかじめエタノールで洗浄後，蒸留水で平衡化させたアンバーライト XAD4 樹脂（約 1 kg）をガラスカラム（5.0 x 50 cm）に充填した．上記抽出液を当該カラムにかけコリノイド化合物を樹脂に吸着させた．蒸留水 1 *l* でカラムを洗浄後，80%（v/v）エタノール 2 *l* で樹脂に吸着したコリノイド化合物を溶出させた．溶出液は，エバポレーターで乾固した後，30 ml の蒸留水に溶解させた．この時生じた不溶性画分は，遠心分離により除去した．

あらかじめエタノールで洗浄後，蒸留水で平衡化させたコスモシル 140C18-OPN 樹脂をガラスカラム（2.4 x 10 cm）に充填した．上記溶出液を本カラムにかけコリノイド化合物を樹脂に吸着させた．蒸留水 100 ml でカラムを洗浄後，10%（v/v）エタノール 100 ml と 20%（v/v）エタノール 100 ml を用いて樹脂に吸着したコリノイド化合物を溶出させた．溶出された赤色画分を回収し，エバポレーターで乾固した後，少量の蒸留水に溶解させた．この時生じた不溶性画分は，遠心分離により除去した．

この赤色溶液をシリカゲル 60 の薄層クロマトグラフィー（TLC）用アルミニウムプレートに負荷し，展開溶媒 2-プロパノール / アンモニア水 / 蒸留水（7/2/1）を用いて室温・暗黒下で展開させた．展開した TLC プレートを風乾後，赤色スポットをハサミで切り取り，80%（v/v）エタノール 20 ml を加え 4℃で一晩放置することで赤色化合物を再抽出した．抽出液は減圧下，35℃にて遠心エバポレーターで乾固した後，100 μl の蒸留水に溶解させた．

上記赤色溶液 100 μl を高速液体クロマトグラフィー（HPLC）［カラム，Wakosil-II 5C18RS（ϕ 4.6 x 150 mm）；移動相，1%（v/v）酢酸を含む 20%（v/v）メタノール溶液；カラム温度，35℃；流速，1.0 ml/min；検出波長，278 nm］で分離後，溶出液を 1.0 ml ずつフラクションコレクターで分画した．赤色画分を減圧下 35℃にて遠心エバポレーターで乾固させた後，50 μl の蒸留水に溶解させ，精製標品として実験に用いた．

4．コリノイド化合物の同定方法

カツオ血合肉より精製したコリノイド化合物溶液ならびに標準の B_{12} 溶液（20 μmol/*l*）をシリカゲル 60 の TLC アルミニウムプレートに負荷し，展開溶媒 I［1-ブタノール /2-プロパノール / 蒸留水（10/7/10）］ならびに展開溶媒 II［2-プロパノール / アンモニア水 / 蒸留水（7/2/1）］を用いて室温･暗黒下で展開させた．展開した TLC プレートは風乾後，各赤色スポットの R_f 値を測定した．

また，カツオ血合肉より精製したコリノイド化合物溶液ならびに標準の B_{12} 溶液（20 μmol/*l*）5 μl をそれぞれ HPLC［カラム，Wakosil-II 5C18RS（ϕ4.6 x 150 mm）；移動相，1%（v/v）酢酸を含む 20%（v/v）メタノール溶液；カラム温度，35℃；流速，1.0 ml/min；検出波長，278 nm］で分析し，各画分の保持時間を測定した．

5．調理条件

上記の方法で調製したカツオ魚肉パテを用いて以下の条件下で加熱調理した．

ゆでる(Boil)は魚肉パテ1個を水道水200 ml中で6分間加熱した．焼く(Sauté)は魚肉パテをホットプレート(180℃)で7分30秒加熱した．揚げる(Fly)は，魚肉パテ1個を食用油200 ml(180℃)中で1分30秒間加熱した．蒸す(Steam)は，魚肉パテ1個を蒸し器で4分間加熱した．電子レンジ加熱(Microwave)は，魚肉パテを耐熱性ラップで包み電子レンジで1分間加熱した．

各種条件下で加熱調理した後，魚肉パテの重量と煮汁がある場合は煮汁の容量を測定した．加熱調理前後の魚肉パテそれぞれ2 gを正確に秤量し，上述した方法で B_{12} を抽出・定量した．同じ加熱調理実験を3回行った．また，調理前後で魚肉パテ中の水分含量が変化する可能性があるため，調理後の魚肉パテ1個の重量も測定し，調理後の魚肉パテ1個に含まれる B_{12} 含量を求め調理前の魚肉パテ1個に含まれる B_{12} 含量に対するパーセントで示した．

結果および考察

1．カツオ魚肉各部位のビタミン B_{12} 含量

五訂日本食品標準成分表で採用されている *L. delbrueckii subsp. lactis* ATCC7830を用いた B_{12} 定量法でカツオ魚肉(普通肉背部，普通肉腹部，血合肉)に含まれる B_{12} 含量を測定した(表1)．可食部100 gあたりの B_{12} 含量は，普通肉の背部と腹部でほぼ同レベルであり，五訂日本食品標準成分表[6]のカツオ(春獲り，生)および(秋獲り，生)の B_{12} 含量(それぞれ8.4 μgと8.6 μg)とよく一致していた．B_{12} が魚肉の血合肉に高濃度で存在することは古くから知られているが[12]，カツオの血合肉(平均値158.5 μg/100 g)は，他の魚の血合肉(ブリ，平均値48.3 μg/100 g；サワラ，平均値54.8 μg/100 g；ゴマサバ，平均値47.2 μg/100 g)と比べても B_{12} を高濃度含有していた．また，カツオ魚肉全体に含まれる全 B_{12} 含量の約74%が血合肉に含まれていることが明らかとなった．

なお，本定量菌は，B_{12} 以外にデオキシリボースやデオキシリボヌクレオチド(アルカリ耐性因子)にも B_{12} 活性を示すため，アルカリ耐性因子量を補正したが，カツオ血合肉には，B_{12} 含量の0.6%以下のアルカリ耐性因子量

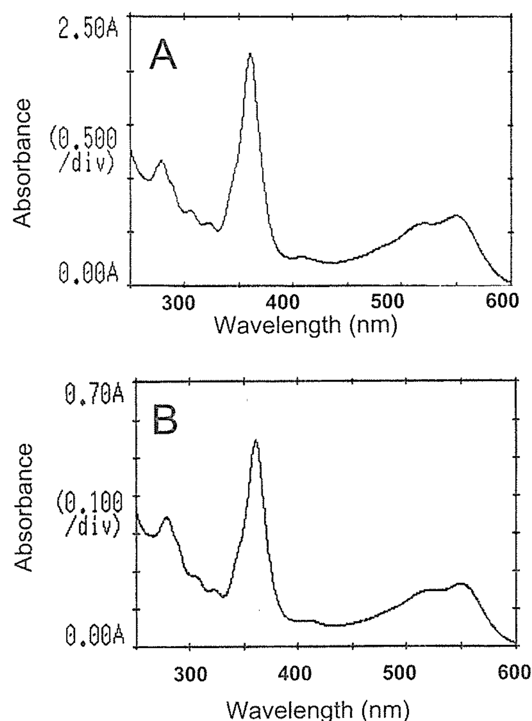

図1. Ultraviolet-visible spectrum of the purified corrinoid compound from skipjack dark muscle. (A) Authentic B_{12} (cyanocobalamin) solution (0.1 mmol/*l*). (B) A portion of the purified compound was dissolved in 0.1 ml of distilled water. These spectra were obtained with a Shimadzu spectrophotometer (UV-16000) at room temperature (25 ℃). Super micro-quartz cuvettes (0.1 ml, *d* = 1 cm) were used.

表1. Vitamin B_{12} contents of skipjack meats. Total B_{12} was extracted by boiling at acidic pH and assayed by the microbiological method with *Lactobacillus delbrueckii* sub. *lactis* ATCC 7830 according to the method described in the Japanese Standard Tables of Food Composition. Since *L. delbrueckii* ATCC 7830 can utilize both deoxyribosides and deoxyribonucleotides (known as an alkali-resistant factor) as well as B_{12}, the amount of true B_{12} was calculated by subtracting the values of the alkali-resistant factor from the values of total B_{12}.

	Vitamin B_{12} contents		Weight (g)
	(μg/100 g)	(μg/total muscle tissue)	
Ordinary meat			
Dorsal portion	9.9 ± 0.6	38.0 ± 0.6	390.2 ± 20.1
Ventral portion	8.4 ± 0.6	29.3 ± 1.5	346.7 ± 12.9
Dark meat	158.5 ± 16.3	190.3 ± 28.2	120.7 ± 13.2

Mean ± SE (n = 5)

が検出されたに留まり，普通肉背部および腹部においては検出限界以下であった．

2. カツオ血合肉に含まれるコリノイド化合物の単離・同定

五訂日本食品標準成分表で採用されている B_{12} 定量菌 *L. delbrueckii* subsp. *lactis* ATCC7830 は B_{12} 以外のコリノイドにも感応するため，本定量法で測定した結果，B_{12} が多量に含まれていると評価されても，ヒトに生理活性を有しないシュード B_{12} である場合が淡水産の藻などにおいて報告されている [13]．そこで，カツオ血合肉中に多量に検出される B_{12} が真の B_{12} であるかどうかを確認するために，カツオ血合肉からコリノイド化合物を単離･同定した．カツオ血合肉抽出液から各種クロマトグラフィーを用いて HPLC で単一のピークになるまで赤色化合物を精製した．カツオ血合肉より精製した赤色化合物の紫外・可視吸収スペクトル分析を行った結果（図 1），コリノイド化合物特有の吸収スペクトルを示した．シリカゲル 60TLC 分析と逆相 HPLC 分析において，カツオ血合肉より精製したコリノイド化合物と標準の B_{12} の挙動が完全に一致したことから（表 2），カツオ血合肉には真の B_{12} が多量に含まれていることが明らかとなった．

3. カツオ魚肉のビタミン B_{12} 含量に及ぼす各種加熱調理の影響

カツオ魚肉パテを用いて各種加熱調理による魚肉 B_{12} の調理損失について検討した結果を表 3 に示す．焼く（Sauté）・蒸す（Steam）・揚げる（Fly）では，カツオ魚肉 B_{12} の残存率は 94.5 ～ 97.7% と調理損失はほとんどなかった。また，ゆでる（Boil）において煮汁中への溶出は 3.4% 程度に留まり，よい残存率を示した．電子レンジ加熱（Microwave）による調理損失が最も大きかった．

B_{12} の供給源である畜肉や牛乳の調理損失について，焼く・ゆでる・揚げるなどの加熱調理による B_{12} の残存率は牛肉各部位で 61 ～ 88%，豚肉各部位で 76 ～ 90% と報告されている [14]．また，牛乳中の B_{12} は加熱調理により顕著に減少し，電子レンジ 3 分加熱および直火 30 分の加熱で約 50% の B_{12} が消失する [10]．加熱調理の条件が異なるため単純に残存率を比較して評価することはできないが，畜肉や牛乳に比べ，カツオ魚肉中の B_{12} の調理損失は少ないと考えられる．

以上の結果からカツオ魚肉（特に血合肉）は，調理方法に関係なく B_{12} のよい供給源となりえることが明らかとなった．

結　論

1. 可食部 100 g あたりの B_{12} 含量は，普通肉の背部（9.9 ± 0.6 μg）と腹部（8.4 ± 0.6 μg）でほぼ同レベルとなり，五訂日本食品標準成分表の値とよく一致していた．
2. 血合肉には普通肉に比べ多量（158.5 ± 16.3 μg）の B_{12} が含まれており，カツオ魚肉全体に含まれる B_{12} の約 74% が血合肉に含まれていた．
3. カツオ血合肉抽出液から各種クロマトグラフィーを用

表 2. R_f values and retention times of the purified compound and authentic B_{12} on silica gel 60 TLC plate and reversed-phase HPLC. Solvent I, 1-butanol/2-propanol/water (10: 7: 10); Solvent II, 2-propanol/25% NH_4OH /water (7: 1: 2) Mobile phase for HPLC, 20% (v/v) methanol solution containing 1% (v/v) acetic acid

	TLC (R_f)		HPLC (min)
	Solvent I	Solvent II	
Purified compound	0.25	0.63	9.5
Authentic B_{12}	0.25	0.63	9.5

表 3. Loss of vitamin B_{12} from the skipjack meat putty treated under various cooking conditions.

Cooking conditions	Relative contents (%) of remaining vitamin B_{12}*
None	100
Boil	91.6 ± 3.5
Steam	94.5 ± 3.9
Sauté	97.7 ± 0.9
Fly	95.8 ± 2.4
Microwave	85.2 ± 7.1

*Percent against vitamin B_{12} content of the meat without cooking

いてコリノイド化合物を精製・同定した結果，カツオ血合肉に含まれるコリノイド化合物は真の B_{12} であった．

4. ゆでる・焼く・蒸す・揚げる・電子レンジ加熱によるカツオ魚肉の B_{12} 残存率は 85.2 ～ 97.7% であった．

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(平成18.1.6受付)

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市販ふりかけおよび茶漬けの素のビタミンB_{12}含量

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Vitamin B_{12} contents of commercially available seasoned powders for sprinkling over boiled rice and for boiled rice with tea.

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要　旨

市販されているふりかけおよび茶漬けの素に含まれるビタミンB_{12}含量を五訂日本食品標準成分表に準じた*Lactobacillus delbrueckii* subsp. *lactis*　ATCC7830を用いた微生物学的定量法で検討した。かつお，小魚，のり，たまごを原料に含むふりかけのB_{12}含量は，比較的高値を示したが，その他のふりかけのB_{12}含量は，低値であった（平均3.84　μg/100g)。一方，茶漬けの素のB_{12}含量は，平均2.25　μg/100gであった。

Abstract

Vitamin B_{12} contents of commercially available seasoned powders for sprinkling over boiled rice and for boiled rice with tea were assayed by the microbiological method authorized in the standard tables of food composition in Japan (5th Edition). These seasoned powders (100 g) for sprinkling over boiled rice and for boiled rice with tea contained 3.84 μg and 2.25 μg of vitamin B_{12} (mean values), respectively.

キーワード：ふりかけ，茶漬け，調味料，ビタミンB_{12}

Key words : seasoned powder for sprinkling over boiled rice, seasoned powder for boiled rice with tea, seasonings, vitamin B_{12}

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1. 緒　言

日本は世界有数の水産食品の消費国であり，魚介類や藻類は栄養素（タンパク質，脂質，ビタミン，ミネラル）のよい供給源ともなっており[1]，日本人にとって重要な食品である。

これらの栄養素のうち，ビタミンB_{12}（B_{12}）は一部の藻類（あおのりやあまのり）[2~5]を除き，植物性食品には含まれておらず，主に動物性食品から摂取している。米国では，畜肉や牛乳がB_{12}のよい供給源であるが[6]，我が国では魚介類や藻類が主要な供給源となっており[7]，栄養学的に重要である。

しかし，B_{12}のよい供給源である魚介類や藻類を用いた加工食品の中で，日本人が日常的に飯と共に食するふりかけと茶漬けの素については，五訂日本食品標準成分表に記載されていない。そこで，これらふりかけおよび茶漬けの素に着目し市販品中のB_{12}含量を測定し，数値を定めると共に，B_{12}のよい供給源になりえるかどうかを検討した。

2. 実験方法

(1) 試　料

市販のふりかけおよび茶漬けの素は，高知市内の市場で購入して実験に用いた。

(2) ビタミンB_{12}の抽出・定量法

B_{12}の定量は，五訂日本食品標準成分表で採用されている分析マニュアル[8]に準じて*Lactobacillus delbrueckii* subsp. *lactis*（旧名*L. leichimannii*）ATCC7830を用いた微生物学的定量法で行った。なお，定量に用いる培地は，日水製薬株式会社製のライヒマニ保存用培地，ライヒマニ接種用培地，ライヒマニ用B_{12}定量基礎培地を用いた。

ふりかけおよび茶漬けの素５～10ｇ程度をフードプロセッサーで粉砕後，分析試料2ｇを正確に秤量し，常法によりシアン化カリウムを含む酢酸緩衝液（pH 4.5）中でB_{12}を加熱抽出した。

また，本定量菌は，B_{12}以外にデオキシリボースやデオキシリボヌクレオチドにもB_{12}活性を示すため，分析マニュアル[8]に記載された以下の方法で補正した。

上記B_{12}抽出液の一部に１mol/L水酸化ナトリウム溶液を加え，pHを11～12に調整後，オートクレーブ（121℃，30分）処理を行い，B_{12}の分解を行った。その後，１mol/L塩酸溶液でpHを６に調整・定容し，この溶液をアルカリ耐性因子画分としてB_{12}抽出液と同様に微生物学的定量法でB_{12}含量を測定した。

B_{12}抽出液画分に検出されたB_{12}含量からアルカリ耐性因子画分に検出されたB_{12}含量を差し引き，試料中の正味のB_{12}含量を算出した。

3. 結果および考察

五訂日本食品標準成分表で採用されている*L. delbrueckii* subsp. *lactis* ATCC7830を用いたB_{12}定量法で市販ふりかけ中のB_{12}含量を測定した（Table 1）。その結果，かつおや小魚ふりかけ中のB_{12}含量は，2.39～10.88 μg/100gと比較的高く，次いでのりやたまごのふりかけ（1.32～6.50 μg/100g）であった。一方，さけ，たらこ，その他のふりかけのB_{12}含量は，低値を示した。使いきりタイプの小包装では，内容物の重量が２ｇ程度であったので，一食あたりの摂取含量の目安として併記した。今回測定した26種の市販ふりかけ中のB_{12}含量の平均値は3.84 μg/100gであり，一食あたり約0.1 μgが摂取できると考えられる。

一方，市販茶漬けの素中のB_{12}含量において，主要な素材の違いがB_{12}含量へ及ぼす影響は，ふりかけほど大きくなかった（Table 2）。内容物の重量が８ｇ程度のものが多かったので，一食あたりの摂取含量の目安として併記した。茶漬けの素中のB_{12}含量の平均値は，2.25 μg/100gとなり，一食あたり約0.18 μgのB_{12}が摂取できると推定される。

以上の結果から飯にかけて食するふりかけや茶漬けの素の中には比較的高いB_{12}含量を示すものもあったが，推定される一食あたりのB_{12}摂取量は日本人の食事摂取基準（2005年版）[9]の成人男女の推奨量（2.4 μg）の1/10程度であった。

Table 1 市販ふりかけに含まれるビタミンB_{12}含量

種類	ビタミンB_{12}含量 (μg/100g)	(μg/2g)
かつお，小魚など		
A	9.14	0.18
B	2.39	0.05
C	9.02	0.18
D	7.04	0.14
E	10.88	0.22
F	8.59	0.17
G	8.95	0.18
H	7.67	0.15
のり，たまご		
I	3.55	0.07
J	3.00	0.06
K	2.40	0.05
L	1.32	0.03
M	6.50	0.13
たらこ（めんたいこを含む）		
N	4.59	0.09
O	0.44	0.01
P	1.15	0.02
さけ		
Q	0.23	Trace*
R	0.48	0.01
S	2.26	0.05
その他		
T	3.14	0.06
U	0.20	Trace
V	2.87	0.06
W	1.30	0.03
X	0.35	Trace
Y	1.60	0.03
Z	0.80	0.02
平均**	3.84±0.67	0.09±0.01

*0.01μg以下の含量は，Traceとして示した。
**平均値±SEM

4．結　論

1．ふりかけのB_{12}含量は，平均3.84μg/100gであり，一食あたり約0.1μgが摂取できると評価された。

2．茶漬けの素のB_{12}含量は，平均2.25μg/100gとなり，一食あたり約0.18μgのB_{12}が摂取できると評価された。

Table 2 市販茶漬けの素に含まれるビタミンB_{12}含量

種類	ビタミンB_{12}含量 (μg/100g)	(μg/2g)
A	3.05	0.24
B	1.80	0.14
C	2.25	0.18
D	4.60	0.37
E	5.25	0.42
F	1.15	0.09
G	1.50	0.12
H	2.00	0.16
I	0.90	0.07
J	0.25	0.02
K	1.95	0.16
平均*	2.25±0.46	0.18±0.04

*平均値±SEM

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Low folate status increases chromosomal damage by X-ray irradiation

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Abstract

Purpose: To examine how folate status influences chromosomal damage following X-ray irradiation.

Material and methods: In an animal study, mice were fed either a low, basal, or high folic acid diet (0, 2, or 40 mg/kg diet, respectively) for 4 weeks, and then given total body irradiation (TBI) at 0.5 Gy. In a human study, subjects were supplemented with folic acid (800 μg/day) for 2 weeks and their peripheral blood was irradiated at 0.5 Gy *in vitro*. Chromosomal damage was determined by micronucleus assay.

Results: In an animal study, TBI-induced chromosomal damage was higher and folate concentration was lower in the bone marrow of the low folic acid group compared to the other two diet groups. The chromosomal damage and folate concentration were comparable between the basal and high folic acid groups. TBI administered to mice decreased folate in the plasma, erythrocyte and bone marrow. In a human study, supplementation with folic acid increased plasma folate, but did not influence either plasma homocysteine or X-ray-induced chromosomal damage in lymphocytes.

Conclusion: Low folate status increases susceptibility to X-ray-induced chromosomal damage, but excessive folic acid supplementation under normal conditions yields no further protection due to folate saturation in the target tissue.

Keywords: *Folate, chromosomal damage, irradiation, bone marrow, diet*

Introduction

Irradiation produces oxygen radicals and thereby induces oxidative damage to biomolecules such as lipids, proteins, and DNA (Riley 1994). Oxidative damage can be controlled by antioxidant defence systems such as antioxidants and antioxidant enzymes. As endogenous antioxidants do not have the capacity to prevent oxidative damage completely (Halliwell 1994), exogenous antioxidants from food have been gaining much attention for the prevention of oxidative damage. We have examined the effects of antioxidant vitamins and oxidative damage in DNA and lipids, especially in the bone marrow of mice and rats given total body irradiation (TBI) (Umegaki et al. 1995, 1999a, 2001, Umegaki & Ichikawa 1994, Yoshimura et al. 2002). In those studies, we showed that bone marrow is particularly susceptible to radiation-induced oxidative damage, which was accompanied by a marked decrease in antioxidants in the bone marrow. We have also examined the preventive effect of dietary antioxidants such as vitamin C, vitamin E and beta-carotene against radiation-induced DNA damage in bone marrow, and found that few antioxidant vitamins prevent radiation-induced DNA damage (Sugisawa et al. 2002, Umegaki et al. 1994a, 1994b, 1997).

Various epidemiological studies strongly suggest that a low folate status increases the risk of cancer (Choi & Mason 2000, Duthie et al. 2002, Kim 1999). In mouse studies, it has been reported that mice with a low folate status showed an increase in DNA damage in the bone marrow after treatment with caffeine and sodium arsenate (MacGregor et al. 1990, McDorman et al. 2002). The mechanism of enhanced DNA damage with a low folate status could be related to DNA synthesis and/or repair based on one-carbon metabolism by folate (Choi &

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Mason 2000). On the other hand, it has been reported that the radical scavenging capacity of folate is equivalent to that of vitamin C (Stocker et al. 2003). If folate acts as a radical scavenger in the body, folate could prevent radiation-induced DNA damage in two ways: by the modification of DNA synthesis and/or repair through one-carbon metabolism, and as a radical scavenger that traps oxygen radicals produced by irradiation. Several *in vitro* studies have shown that folate affects chromosomal stability (Duthie et al. 2002, Fenech 2001). Low folate status and gamma-ray irradiation increase chromosomal damage *in vitro* (Beetstra et al. 2005, Courtemanche et al. 2004). To our knowledge, however, only a few reports have been published that have examined these topics *in vivo*.

In this study, we fed mice various folic acid diets, and then gave them TBI to examine changes in folate concentration and the modification of chromosomal damage in bone marrow. We also examined radiation-induced chromosomal damage in a human study, where folic acid was given as a dietary supplement to healthy volunteers for 2 weeks and their peripheral blood was sampled before and after the study to determine radiation-induced chromosomal damage in lymphocytes *in vitro*. Micronucleus (MN) assays in mice (Hayashi et al. 1994) and human lymphocytes (Fenech 2000) are able to detect DNA damage better than the measurement of 8-hydroxydeoxyguanosine (Kobus et al. 1993), a well-known biomarker of oxidative DNA damage, and X-ray-induced damage is mainly related to oxidative damage (Riley 1994). Therefore, in this study we used the MN assay to evaluate X-ray-induced DNA damage.

Materials and methods

Materials

Fetal bovine serum, Roswell Park Memorial Institute (RPMI) 1640 medium, antibiotic solution, phytohemagglutinin, Hanks' Balanced Salt Solution, and L-glutamine were purchased from GIBCO (Grand Island, NY, USA). Cytochalasin B and trypsin were obtained from Sigma (St Louis, MO, USA). Giemsa's Solution and 2-thiobarbituric acid were from Merck (Darmstadt, Germany). Ficoll-Paque was from Amersham Bioscience (Uppsala, Sweden). Folinic acid calcium salt pentahydrate was from Fluka (Buchs, Switzerland). *Lactobacillus rhamnosus* (American Type Culture Collection (ATCC) number 27773) was from the ATCC (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).

Animals and diets

Four-week-old male ICR mice (Japan Clea, Tokyo, Japan) were individually housed in polypropylene cages in a room with a constant temperature of 23 ± 1°C and a 12-h light-dark cycle. The mice were fed either low, basal or high folic acid diets (folic acid; 0, 2, 40 mg/kg diet, respectively), which were prepared based on the AIN-93G formula (Reeves 1997). The composition was as follows: cornstarch 397.486 g/kg diet, vitamin-free casein (>85% protein) 200 g/kg diet, dextrinized cornstarch (90–94% tetrasaccharides) 132 g/kg diet, sucrose 100 g/kg diet, soybean oil (no additives) 70 g/kg diet, fiber 50 g/kg diet, mineral mix (AIN-93G-MX) 35 g/kg diet, folic acid-free vitamin mix (AIN-93-VX) 10 g/kg diet, L-cystine 3 g/kg diet, choline bitartrate (41.1% choline) 2.5 g/kg diet, *tert*-butylhydroquinone 14 mg/kg diet. Mice were divided into six groups (six mice per group), given *ad libitum* access to food and tap water, and weighed once every 3 days throughout the study. After a 4-week feeding period, half of the mice in each diet group received TBI with X-rays by a soft X-ray unit (OM-150-RS; Ohmic, Tokyo, Japan) at a dose rate of 0.5 Gy/min (140kV, 9mA). The beam was filtered through Cu (0.1 mm) and Al (0.2 mm). For the analysis of chromosomal damage in bone marrow, peripheral blood was taken from the tail after an overnight fast at 44 h post TBI. The mice were anesthetized with sodium pentobarbital and blood was taken from the large abdominal vein with a heparinized syringe. Part of the blood was collected in a capillary tube and centrifuged at 10,000g for 10 min for hematocrit measurement and the rest of the blood was immediately centrifuged (RS-20IV; TOMY SEIKO Co., LTD, Tokyo, Japan) at 1,500g for 15 min at 4°C to prepare plasma. The liver was immediately removed, frozen, and stored at −80°C until analyses. The bone marrow cells were prepared from the femurs and tibiae according to the method reported previously (Umegaki & Ichikawa 1994). For the analysis of folate, the blood was immediately mixed with 0.5% ascorbic acid and the bone marrow cell samples were similarly mixed with 0.5% ascorbic acid sodium salt for the analysis of folate. The plasma was immediately mixed with 5 volumes of 6% metaphosphoric acid for the analysis of vitamin C. In an *in vitro* study, mice plasma was prepared and irradiated with X-ray at a dose of 3Gy to examine changes in the concentration of folate and vitamin C 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.

Human study

The subjects were 22 male healthy volunteers in their 20s (22.7 ± 2.0 years old). They were randomly divided into a placebo ($n = 10$) or folic acid group ($n = 12$) and their diets were supplemented with folic acid 800 μg/day (Nature Made Folic acid, Otsuka Pharmaceuticals, Tokyo, Japan) or a placebo (Lactose tablets) for 2 weeks. They consumed folic acid supplements three times a day just after each meal. During the study, the consumption of the supplement was recorded and the subjects were instructed to maintain their habitual diet and physical activity. Before and after the supplementation period, their peripheral blood was collected after an overnight fast. Chromosomal damage in the lymphocytes and the plasma concentration of folate were determined. The Japan Women's University Human Ethics Committee approved this study, and written informed consent was obtained from the subjects.

Analytical methods

Folate was analysed as follows: The liver and bone marrow cells samples were homogenized (Polytron PT3100; Kinematica, Lucerne, Switzerland) or sonicated (Ultra Sonic processor; Seiko Sonics & Materials, Tokyo, Japan) with 9 volumes of folate extraction buffer (50 mM phosphate buffer, 0.5% ascorbic acid, final pH 6.1). These samples were autoclaved (KS-323; Tomy Seiko Co., LTD, Tokyo, Japan) 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 whole blood samples were incubated at 37°C for 30 min to hydrolyze pteroylpolyglutamates with endogenous conjugase. The plasma and conjugase-treated liver, bone marrow, and whole blood samples were diluted with 0.5% ascorbic acid sodium salt, if necessary (O'Leary & Sheehy 2001). The folate concentration in the samples was analysed by microbiological assay using 96-well microplate and glycerol-cryoprotected *Lactobacillus rhamnosus* methods (Horne 1997). Erythrocyte folate concentration was calculated using the following formula: [whole blood folate − {plasma folate × (1-hematocrit)}]/hematocrit (Bills et al. 1992).

In the mouse study, chromosomal damage in the bone marrow cells was evaluated with the micronucleus assay using peripheral blood according to the method reported previously (Umegaki et al. 1994c). Briefly, reticulocytes with and without micronuclei (MN) among over 1000 cells were counted, and chromosomal damage was expressed in terms of the number of cells containing MN per 1000 cells by fluorescence microscope (Olympas BHS, Tokyo, Japan). In the human study, chromosomal damage to lymphocytes was assessed by the cytokinesis-block micronucleus (CBMN) assay according to the method of Fenech (2000). Briefly, peripheral whole blood from each human subject was divided into two parts (0.5 ml each) in culture tubes. One sample was not irradiated and the other was irradiated with X-rays at 0.5 Gy. The two samples from each subject were each added to 4.5 ml of RPMI-1640 culture medium supplemented with 1% L-glutamine, 10% fetal bovine serum and 1% antibiotics. Culture was immediately initiated by adding phytohemagglutinin. Cytochalasin B (final concentration 6.6 μg/ml) was added at 44 h to induce binucleated cells. The cells were harvested at 72 h and slides were prepared using a cytocentrifuge (Cytospin-3; Shandon Southern Products, Cheshire, UK). At least 500 binucleated cells with preserved cytoplasma were scored for each culture using a microscope. Percentages of lymphocytes with MN in the binucleated cells were calculated from each culture.

Vitamin C was analysed by high pressure liquid chromatography (HPLC) with an electrochemical detector (Shiseido, Tokyo, Japan) according to the method reported previously (Umegaki et al. 1999b). Plasma homocysteine concentration was determined by HPLC with a fluorescence detector (Shimadzu, Kyoto, Japan) by the method of Frick et al. (2003). 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. Correlation analysis between TBI-induced chromosomal damage and folate concentration in various tissues was performed with Pearson's correlation coefficient. 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

Animal study

The final body weight and relative liver weight did not differ among the groups (Table I). Relative spleen weight was lower in the TBI groups, but no difference was found among the three diet groups. The proportion of micronucleated (MNed) reticulocytes in the peripheral blood, an indicator of

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

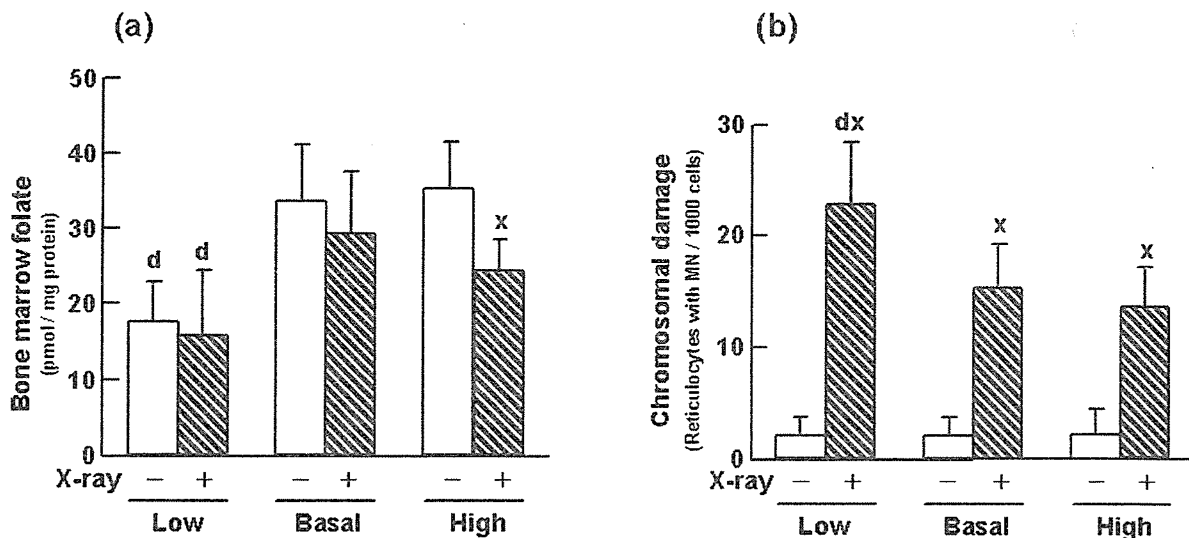

Figure 1. Folate status (a) and TBI-induced chromosomal damage (b) in the bone marrow of mice fed various folic acid diets for 4 weeks (animal study). Male ICR mice (4 weeks old) were fed a low folic acid diet (0 mg/kg), basal diet (2 mg/kg) or high folic acid diet (40 mg/kg) for 4 weeks, then given 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 ± 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; $p < 0.05$).

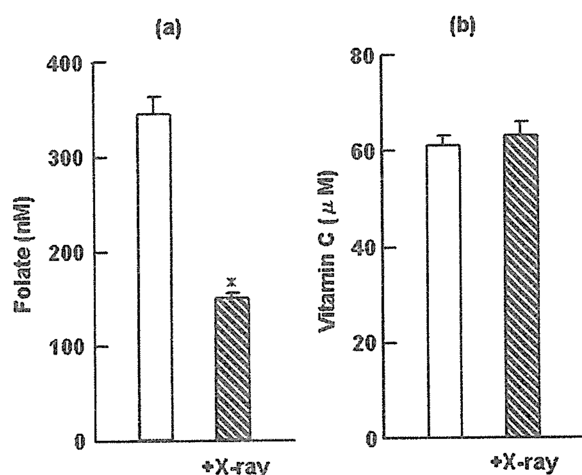

Figure 2. Concentration of folate (a) and vitamin C (b) in mice plasma with and without X-ray irradiation *in vitro*. Fresh mice plasma was irradiated with X-ray (3 Gy) and changes in the concentrations of folate and vitamin C were measured; each column and vertical bar indicates the mean and SD, n =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

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 ± 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 ± SD ($n = 10$ in placebo group, and $n = 12$ in supplemented group).

Discussion

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

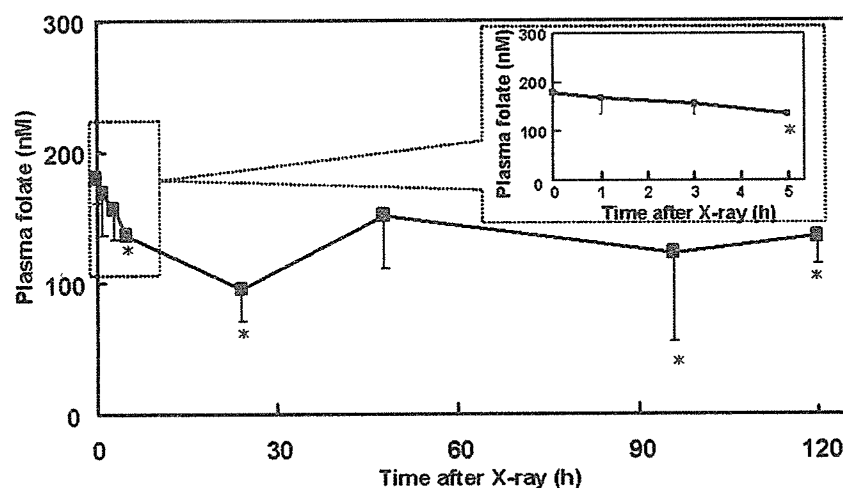

Figure 1. Time-dependent changes in folate in the plasma of mice after TBI at 3 Gy. Male ICR mice (4 weeks old) were subjected to TBI via X-rays at a dose of 3 Gy, and then sacrificed at 1, 3, 5, 24, 48, 96, 120 h for the analysis of antioxidant vitamins. Each point and vertical bar indicates the mean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