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ORIGINAL CONTRIBUTION

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Molecular cloning of the rat β-carotene 15,15′-monooxygenase gene and its regulation by retinoic acid

Summary *Background* β-Carotene exhibits biological activity as provitamin A. Key step in vitamin A formation is the cleavage of β-carotene to retinal by an enzyme designated as β-carotene 15,15′-monooxygenase (BCM). Recently, it is reported that expression of BCM gene in the intestine is under feedback regulation by retinoic acid (RA). However, the regulation of BCM gene expression in various other tissues is still unknown. *Aim of the study* In the present study, we identified the full-length cDNA encoding the rat BCM gene and investigated the regulation of its expression in several tissues by RA in the presence of vitamin A deficiency. *Methods* We cloned the full-length cDNA encoding BCM gene from a rat intestinal cDNA library by hybridization screening. The BCM gene expression was examined using Northern blotting and reverse transcription-PCR analysis. We also investigated whether BCM gene expression was regulated by retinoids in several tissues of vitamin A-deficient rats. *Results* Sequence analysis of this clone revealed an open reading frame of 1,701 bases encoding a protein of 566 amino acids. The predicted polypeptide showed 94%, 81%, and 66% identity with mouse, human, and chicken BCM, respectively. Rat BCM mRNA was highly expressed in the intestine and liver, while there was weak expression in the testes, kidneys, and lungs. Immunoblotting revealed that rat BCM is a 64-kDa protein. BCM gene expression was increased in the small intestine by vitamin A deficiency compared with that in rats on a control diet, while this upregulation was suppressed by all-*trans* RA (ATRA) or 9-*cis* RA (9-*cis* RA). BCM gene expression in the lungs and testes was also suppressed by ATRA or 9-*cis* RA in rats with vitamin A deficiency. However, hepatic BCM gene expression was only decreased by ATRA and renal expression was not affected by either retinoid. As the small intestine is the major site of β-carotene conversion, intestinal BCM gene expression may be more tightly regulated. *Conclusion* These data suggest that BCM gene expression in several tissues may be down-regulated by RA at the level of conversion of β-carotene to retinal. To prevent an excess of retinol, homeostasis may occur at the level of conversion of β-carotene to retinal in several tissues.

Key words β-carotene 15,15′-monooxygenase · retinoic acid · regulation

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Introduction

Retinoic acid (RA) is a metabolite of vitamin A that plays an important role in cell growth and differentiation, immunity, reproduction, and development [1]. The physiological actions of retinoids are mediated by specific nuclear receptors such as the RA receptor (RAR) and the retinoid X receptor (RXR) [2]. These receptors are members of the steroid/thyroid hormone nuclear receptor superfamily, which act as ligand-dependent transcriptional factors. RAR and RXR regulate the transcription of target genes by

EJN 601

binding to RA response elements (RARE) in their promoters. There are three subtypes (α,β, and γ) of RAR and RXR; the RAR subtypes bind with all-*trans* RA (ATRA) and 9-*cis* RA (9-*cis* RA), while the RXR subtypes only bind with 9-*cis* RA.

β-Carotene is the most plentiful carotenoid in fruits and vegetables and it exhibits biological activity as provitamin A and an antioxidant. Carotenoids are absorbed by the mucosal cells of the small intestine [3]. Within the mucosal cells, β-carotene is cleaved to form retinal through an enzymatic process. This cleavage of β-carotene is the key step in the formation of retinal, which is subsequently reduced to yield retinol or oxidized to create RA [4]. The enzyme involved in cleaving β-carotene was formerly known as β-carotene 15,15′-dioxygenase and it has been investigated by several groups over about 40 years. Recently, it was demonstrated that this enzyme acts as a monooxygenase, so this cleavage enzyme is now designated as β-carotene 15,15′-monooxygenase (BCM) [EC.1.14.99.36] [5]. Molecular cloning and characterization of BCM has been reported for the enzymes from various species, including *Drosophila melanogaster* [6], chickens [7], mice [8, 9], humans [10], and zebra fish [11]. Histochemical studies in human tissues have revealed that the BCM gene is expressed in the kidneys, lungs, skin, testes [12] and retinal pigment epithelium [13] besides the small intestine and liver.

It is well known that RA metabolism is regulated by both RA and vitamin A. Moreover, the conversion of β-carotene to retinal in the rat intestine is enhanced by vitamin A deficiency [14]. Recently, Bachmann and colleagues reported that expression of BCM mRNA in the chicken intestine is under feedback regulation by RA and that BCM activity in the rat intestine is down-regulated by RA [15]. However, the regulation of BCM gene expression in various other tissues is still unknown. In the present study, we identified the full-length cDNA encoding the rat BCM gene and investigated the regulation of its expression in several tissues by RA in the presence of vitamin A deficiency.

Materials and methods

cDNA library screening

In order to obtain the full-length rat BCM cDNA, a 2,100 base pair Pst I fragment of human BCM (accession number: AK001592, provided by Helix Research Institute, Japan) was radiolabeled with [α-^{32}P]dCTP using a random primer labeling kit (Takara, Japan) and was employed to screen a rat intestinal cDNA library (Stratagene, CA) by the plaque hybridization method. Several positive clones were subsequently subjected to tertiary screening under increasingly stringent hybridization/washing conditions and were subcloned into the pBluscript II KS vector according to the supplier's instructions. The DNA sequence of the clone thus obtained was determined by automated sequencing using a Genetic Analyzer ABI PRISM 310 (Applied Biosystems, CA).

Northern blotting

Prehybridization and hybridization of rat poly(A)$^+$ RNA for 12 major tissues (Origene Technologies, MD) was performed using ExpressHyb hybridization solution (Clontech, CA) according to the manufacturer's instructions. cDNA fragments isolated from pBluscript II-rat BCM were labeled with [α-^{32}P]dCTP using a random primer labeling kit (Takara, Japan). The membrane was exposed to X-ray film at −80°C for 7 days with intensifying screens.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated by the acid guanidium thiocyanate/phenol/chloroform(AGPC) method [16]. The rat BCM gene was detected by PCR. Using total RNA (2 μg) isolated from various tissues, RT reactions were carried out with Omniscript (Qiagen, CA). The following PCR primer pair was used to detect rat BCM (accession number: AB062912): 5′-CAAGTCCTCCT TAAAGTGGT-3′ (sense) and 5′-AATAAACCATGC AGGTCCA-3′ (antisense). After an initial hot start at 95°C for 10 min, 36 cycles (94°C for 30 s, 48°C for 30 s, and 72°C for 3 min) were run using Ampli Taq Gold and a Geneamp PCR System 9700 (Applied Biosystems, CA). As an internal control, rat β-actin (accession number: B063166) was analyzed using the following primers: 5′-CCTGTATGCCTCTGGTCGTA-3′ (sense) and 5′-CCATCTCTTGCTCGAAGTCT-3′ (antisense). After an initial hot start at 95°C for 10 min, 36 cycles (94°C for 30 s, 48°C for 30 s, and 72°C for 2 min) were run under the same conditions as above.

Antiserum for rat BCM and immunoblotting

An antigenic peptide with the sequence LETLEKVDYRKY, corresponding to amino acids 152–163 of rat BCM, was synthesized and used to immunize rabbits. Harvested serum was purified by affinity column chromatography. The antiserum thus obtained was used at a final dilution of 1:5,000 in Tris-buffered saline containing Tween-20 (TBS-T). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, CA), and the

target band was detected by using the ECL Western blotting Detection System (Amersham Biosciences, Sweden). Dissected liver tissue was homogenized in 3 volumes of buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4). The cytosolic fraction was prepared from the homogenate by ultracentrifugation at 100,000*g* for 60 min, while the supernatant was also saved and analyzed by immunoblotting under the conditions described above. Protein content was measured according to the method of Lowry [17].

Animal experiments

Male weanling Wistar rats were obtained from Charles River Japan Inc. (Kanagawa, Japan) and were used for this study. Care and handling of the experimental animals were done according to Osaka Medical College guidelines for the ethical treatment of laboratory animals. Male weanling rats (4 weeks old, 50–60 g, $n = 16$) were randomized to two groups. The vitamin A-replete group (control, $n = 4$) was fed a standard AIN-93G diet [18], while the vitamin A-deficient group ($n = 12$) was fed the same diet without vitamin A. Rats were fed the vitamin A-containing or -deficient diet for 40 days. Animals were judged to be vitamin A deficient when the retinol level was below 0.15 mmol/l according to high-performance liquid chromatography using authentic retinol as the standard (Wako Pure Chemical Industries, Japan) [19].

An excess of RA (1 mg of ATRA or 1 mg of 9-*cis* RA in 0.5 ml of soybean oil) was administered intragastrically to vitamin A-depleted rats ($n = 4$ for each retinoid) and the animals were sacrificed four hours later [19]. As control groups, vitamin A-replete rats ($n = 4$) and vitamin A-deficient rats (VAD) ($n = 4$) were administered intragastrically soybean oil (0.5 ml) and then killed 4 h later. ATRA and 9-*cis* RA were purchased from Sigma-Aldrich Corporation (MO, USA).

Real-time RT-PCR

Total RNA was isolated as described above. Then quantitative real-time RT-PCR was performed to determine the levels of BCM and β-actin in RNA samples. The RT reaction was carried out using Omniscript (Qiagen, CA). Subsequently, one-tenth (2 μl) of each RT reaction mixture was amplified by a LightCycler PCR (Roche Molecular Biochemicals, Switzerland), as described previously [20]. After initial denaturation at 94°C for 2 min, 40 PCR cycles were done using denaturation at 95°C for 15 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s.

The following oligonucleotide primers were used to detect rat BCM and rat β-actin: BCM, 5′-CAAGT CCTCCTTAAAGTGGT-3′ (sense), 5′-AATAAACCATG CAGGTCCA-3′ (antisense), 5′-CATCATCTCTACAGA TCCCCAAAAGC-3′-FITC (sense-probe), LC-5′-GCCC TTTTTACTTATTCTGGATGCG-3′ (antisense-probe), β-actin, 5′-CCTGTATGCCTCTGGTCGTA-3′ (sense), 5′-CCATCTCTTGCTCGAAGTCT-3′ (antisense), 5′-CGGGACCTGACAGACTACCTCATG-3′-FITC (sense-probe), LC-5′-AGATCCTGACCGAGCG TGGCTAC-3′ (antisense-probe). RT-PCR products of 225 bp (BCM) and 260 bp (β-actin) were verified by DNA sequencing and used as external PCR standards. Using the LightCycler software, the amplification curves of the experimental samples were plotted against these standard curves to obtain an estimate of the number of specific mRNA copies. To compensate for differences in RT efficiency among the samples, BCM values were then normalized by the copy number for β-actin.

Statistical analysis

All results are expressed as the mean ± SD. One-way analysis of variance (ANOVA) was used to compare means among groups. If the ANOVA was significant at a p-value of less than 0.05, Dunnett's test was used as a post hoc test, comparing all treatments with the VAD treatment (StatView version 5.0, SAS Institute, NC). Differences between groups were considered significant at a p-value of less than 0.05.

Results

Identification of a rat BCM cDNA clone

When approximately 7.5×10^5 plaques of the rat intestinal cDNA library were screened using human BCM cDNA as a probe, one cDNA clone was obtained. This clone contained a 2.2-kb insert (deposited in the DataBank under accession number AB062912) and it was predicted to be 566 amino acids in length with a predicted molecular weight of 63.6 kDa (Fig. 1). The predicted rat polypeptide showed 94%, 81%, and 66% identity with mouse, human, and chicken BCM, respectively, while there was 60% identity among all of these sequences. The primary peptide sequences of mouse (accession number: AW044715), human (accession number: AK001592), and chicken (accession number: AJ271386) BCM are aligned in Fig. 1. Based on the findings described above, we designated this clone as the cDNA for rat BCM.

Expression of rat BCM in various tissues

Northern blotting showed that rat BCM was expressed in the liver and small intestine (Fig. 2A). The size of

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rat     MEIIFGRNKKEQLEPLRATVTGSIPAWLQGTLLRNGPGMHTVGDSKYNHWFDGLALLHSF   60
mouse   ·····Q·····VQ·K·····························E·················   60
human   ·D·····R·····V··K···K·····················E·R·············   60
chicken ··T··N···E·HP··IK·E·Q·QL·T····V··········I··T·············   60

rat     SIRDGEVFYRSKYLQSDTYNANIEANRIVVSEFGTMAYPDPCKNIFSKAFSYLSHTIPDF   120
mouse   ···················T·····································   120
human   T·····Y·····R·····T·····································   120
chicken TFKN···Y·····R·····C·····························A··········E·   120

rat     TDNCLINIMKCGEDFYATTETNYIRKIDPQTLETLEKVDYRKYVAVNLATSHPHYDEAGN   180
mouse   ·····························································   180
human   ···················S·····N·····························   180
chicken ··········T·D·Y···S···F···········D····S·············S···   180

rat     VLNMGTSIADKGGTKYVMFKIPATAPGSKKKGKNPLKHSEVFCSIPSRSLLSPSYYHSFG   240
mouse   ·····VV···R····I·····V·D·····S·V··A·····S·············   240
human   ·····VE··K····I·····V·EG··Q··S·W··T·····················   240
chicken I·····V···R····L····SSV·EKE····SCF··L··V·········Q·····   239

rat     VTENYVVFLEQPFKLDILKMATAYMRGVSWASCMTFCKEDKTYIHIIDQKTRKPVPTKFY   300
mouse   ·····································S·DR···········R·········   300
human   ·····I·····R··········I·RM·····LA·HR·E·········R··Q··Q····   300
chicken I····I··I·····V·L····I···N····LS·H·····WF·FV·R··K·E·S····   299

rat     TDPMVVFHHVNAYEEDGCVLFDVIAYEDNSLYQLFYLANLNKDFEEKSRLTSVPTLRRFA   360
mouse   ···························S·····························   360
human   ·GA·············IV·····················Q··K·N···········   360
chicken ··AL·LY··I·····H·V··IV··R·····DM···KK·D····VNNK···I··CK··V   359

rat     VPLHVDKDAEVGSNLVKVSSTTATALKEKDDHVYCQPEVLYEGLELPRINYAHNGKPYRY   420
mouse   ····························G·····················Y·····   420
human   ·····N····T··I··A·········E·GQ·····F·········V·····Q····   420
chicken ···QY···········LP··S···V····GSI·····I·C··I····V··DY···K·K·   418

rat     IFAAEVQWSPVPTKILKYDVLTKSSLKWSEESCWPAEPLFVPTPGAKDEDDGVILSAIIS   480
mouse   ·····················I·····································V·   480
human   V··TG·····I····I···I·········R·DD··········A·············V·   480
chicken VY·T··········A·LN·Q··EV·H·G·DH···S··I···S·D·RE··E··V·TCVVV   478

rat     TDPQKLPFLLILDAKSFTELARASVDVDMHLDLHGLFIPDAGWNAVKQTPAKTQEDENSD   540
mouse   ····························A·············D·········E···V····   540
human   ····························M·····T·MD·DTK··AASEE·R·RA··   540
chicken SE·N·A·········T·K··G··T·N·E·····M···QNDLG·ETE-----------   456

rat     HPTGLTAPGLGHGENDFTAGHGGKSL   566
mouse   ···DP···E·S·S··········S··   566
human   CHGAPLT-----------------   547
chicken -------------------------
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Fig. 1 Comparison of the deduced amino acid sequences. Alignment of the deduced amino acid sequences for rat, mouse, human and chicken BCM is shown. The rat cDNA is deposited in the Gene Bank (accession number: AB062912)

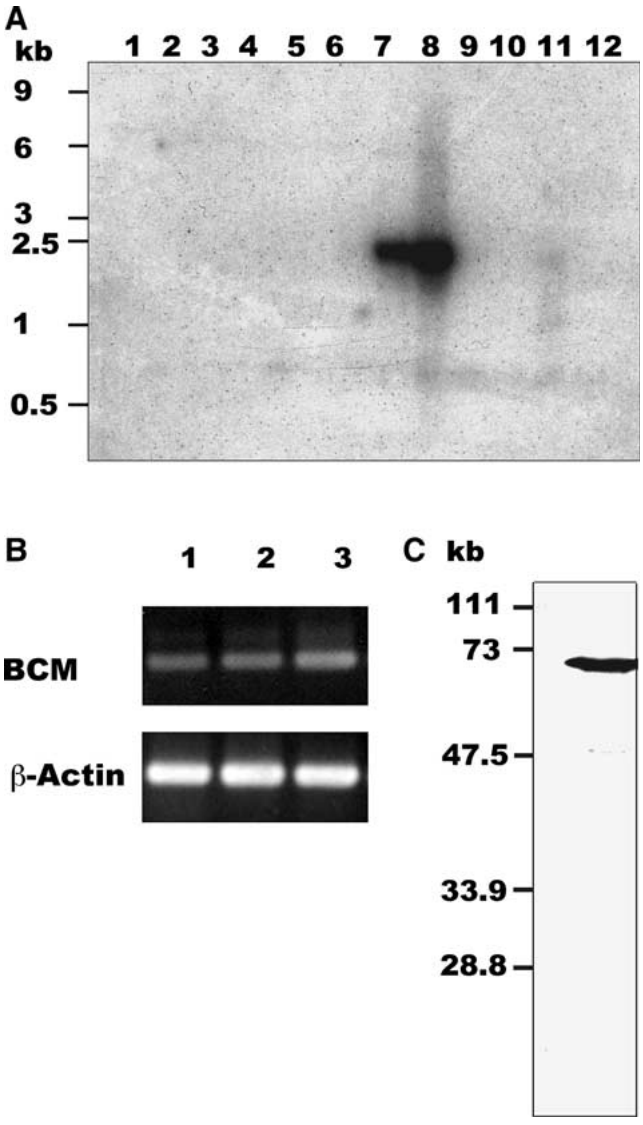

Fig. 2 A. Northern blot analysis of rat BCM in various tissues of adult rats. Northern blots with poly(A)$^+$ RNA from the brain (lane 1), thymus (lane 2), lung (lane 3), heart (lane 4), muscle (lane5), stomach (lane 6), small intestine (lane 7), liver (lane 8), kidney (lane 9), spleen (lane 10), testis (lane 11) and skin (lane 12) were hybridized with a [α-^{32}P]dCTP-labeled DNA probe for rat BCM cDNA. Positions of the size standards are indicated on the left. **B.** Expression of rat BCM gene analyzed by RT-PCR. The rat BCM primers yielded a 225-bp fragment and the rat β-actin primers yielded a 260-bp fragment. The PCR products were electrophoresed on 1.0% agarose gel and stained with ethidium bromide. Lane numbers: (1) lung, (2) kidney, and (3) testis. **C.** Immunoblotting of rat BCM expression. Twenty μg of liver protein was electrophoresed, transferred to a nylon membrane, and immunoblotted with the anti-rat BCM IgG as described in Materials and methods. Positions of the size standards are indicated on the left

the mRNA was estimated to be approximately 2.4 kb. To further analyze the expression of BCM in rats, RT-PCR was carried out with total RNA from the kidneys, lungs, and testes, organs in which β-carotene cleavage activity is exhibited [21]. As a result, the BCM gene was found to be expressed in the lungs, kidneys, and testes of rats (Fig. 2B).

Immunoblotting of rat BCM

We generated a polyclonal antibody against rat BCM to analyze the expression of its protein. On immunoblotting of liver samples, a band of the expected size (64 kDa) was detected (Fig. 2C), indicating that the size of rat BCM protein is similar to that of human [10] and mouse [9] BCM protein.

Regulation of the rat BCM gene by retinoids

In the small intestine of VAD rats, the BCM expression was increased compared with that in control rats, as shown by real-time RT-PCR (Fig. 3A). Intestinal expression of BCM mRNA was suppressed at 4 h after the oral administration of ATRA or 9-*cis* RA. Similarly, the expression of BCM was decreased in the testis and lung of VAD rats, after administration of ATRA or 9-*cis* RA (Fig. 3C, D). However, these tissues showed no

European Journal of Nutrition (2006) Vol. 45, Number 6

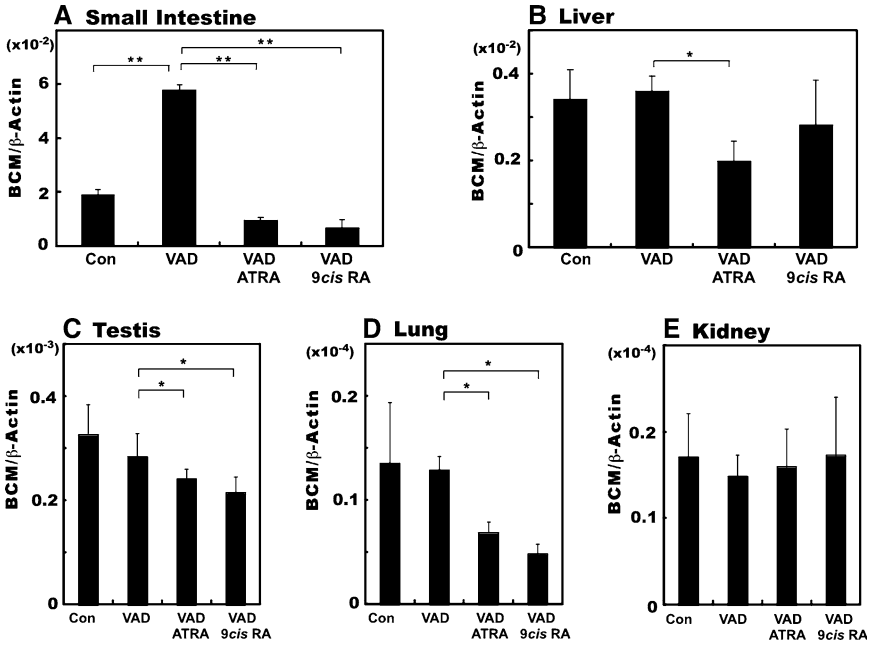

Fig. 3 Tissue-specific response of rat BCM to RA. Control and VAD rats were fed and treated as described in Materials and methods. The rats in each group ($n = 4$ each) were sacrificed, total RNA was isolated, and RT-PCR was performed. The BCM/β-actin ratio was determined using real-time RT-PCR. Bars indicate the standard deviation and asterisks indicate significant differences (*$p < 0.05$ and **$p < 0.01$)

difference of BCM expression between controls and VAD rats. Hepatic expression of BCM was decreased by administration of ATRA to VAD rats, but was not affected by 9-*cis* RA (Fig. 3B), while renal BCM expression in VAD rats was not influenced by either retinoid (Fig. 3E). Therefore, BCM gene expression was down-regulated by retinoids in the testes, lungs, and liver of VAD rats, as well as in the small intestine.

Discussion

In the present study, we cloned the rat BCM gene. The polypeptide sequence of BCM is well conserved among various mammals and the N-terminal domain of the protein is conserved in different species, as described previously [8–10, 12]. The mRNA size and peptide sequence of the rat BCM gene are especially similar to those of the mouse. The predicted protein has no transmembrane domains or potential sites for *N*-linked glycosylation, as is the case for the other homologues [9, 10, 12].

In several previous studies, the expression and distribution of BCM in various species have been analyzed by Northern blotting, RT-PCR, in situ hybridization and histochemistry [22]. In the chicken, BCM is most strongly expressed in the duodenum, followed by the liver, testes, lungs and ileum [12], whereas BCM mRNA is present at high levels in the testes, liver, and kidneys of mice, as well as at lower levels in the skin and intestine [9]. Northern blotting has revealed that BCM is most strongly expressed in the jejunum and ileum in humans followed by the kidneys, liver, testes, ovaries, colon, prostate, and skeletal muscles [23]. In the present study, the BCM gene was found to be expressed in the liver and small intestine of rats using Northern blotting, while it was detected in the testes, lungs, and kidneys using RT-PCR. In rats, β-carotene cleavage activity has been reported to exist in the intestinal mucosa, liver, kidneys, lungs, fatty tissues and brain [21]. There are marked inter-species differences of carotenoid absorption and metabolism [24], and the variations of BCM expression among species may be related to such differences in carotenoid metabolism.

The host nutritional status affects intestinal β-carotene cleavage enzyme activity, since the activity of this enzyme is enhanced by a high polyunsaturated fatty acid diet (rat) [25] and Cu depletion (rat) [26], and is inhibited by a protein-deficient diet (rat) [27] and flavonoid supplemention (pig) [28]. The absorption and metabolism of β-carotene are also affected by retinol and the β-carotene level in the diet. Intestinal β-carotene cleavage activity is enhanced by retinol deficiency and is reduced by a high vitamin A diet [14], while β-carotene supplemention also decreased intestinal β-carotene cleavage activity. To prevent an excess of retinol, homeostasis may occur at the level of conversion of β-carotene to retinal.

Our data on the regulation of BCM gene expression in the rat small intestine are in agreement with previous findings that β-carotene cleavage enzyme activity is enhanced in vitamin A deficiency [14]. Four hours after administration of RA, intestinal BCM gene expression was markedly decreased, so the expression of this gene might be regulated transcriptionally.

Analysis of the human BCM gene promoter has demonstrated that this gene contains a peroxisome proliferator response element (PPRE) and that peroxisome proliferator activated factor (PPAR) γ binds to this element [29]. In addition, BCM gene expression in the adipose tissue of humans is transcriptionally upregulated by PPAR γ agonists. However, analysis of the promoter has not been performed for rat BCM, and further investigations are needed to determine whether transcriptional regulation is mediated by RAR or RXR.

We found that hepatic BCM gene expression was suppressed by ATRA, but not 9-*cis* RA, in rats. Bachmann et al. reported that BCM activity in the liver was not affected by RA and speculated that transcription factors are involved in a tissue-specific manner [15]. The significance of the down-regulated hepatic BCM gene has been unclear. This discrepancy might be due to translational regulation of the BCM gene, which may occur in concert with transcriptional factors.

It is well known that VAD male animals show sterility and that the testis is a major target tissue for RA [30]. RAR- and RXR-mediated signaling is essential for spermatogenesis because the RARα or RXRβ genes disruption in mice lead to abnormalities of spermatogenesis. Therefore, metabolism of vitamin A in the testis may be controlled at the level of conversion of β-carotene to retinal.

Retinoids also play important roles in lung development and function [31]. Thus, it may be very important to regulate retinol or RA levels in the lungs. We demonstrated BCM gene expression in the rat lung using RT-PCR and pulmonary BCM expression is also detected in chicken [12], but not in mice or humans [8–10]. A previous study of cleavage enzyme activity showed that the K_m value in rat lung tissue was lower than in the liver or small intestine [21], and such data support the existence of BCM in the rat lung. The reason for this species difference of pulmonary BCM gene expression is not clear. In the rat lung, RA regulates the expression of the enzyme metabolizing provitamin A, but the role of BCM has not been determined. Therefore, further investigation is needed to better understand the physiological role of BCM in the lungs and the species differences of BCM gene expression.

In this study, the regulation of BCM gene expression by RA showed differences between several tissues. Since the intestine is the major site of β-carotene conversion, intestinal BCM gene expression may be more tightly regulated. In other tissues, except the kidneys, BCM gene expression is controlled by the retinoid level. An excess of β-carotene does not induce vitamin A toxicity [15]. This may be due to negative regulation of BCM gene expression in the intestine, liver, lungs, and testes, which are all target organs for RAR and RXR. Further investigations of BCM regulation will be needed to clarify the mechanisms of vitamin A metabolism from β-carotene in the peripheral tissues.

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