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—Review—

シトクロム P450 と転写因子の microRNA による発現制御

中島美紀

Role of MicroRNAs in the Regulation of Cytochrome P450s and Transcriptional Factors

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MicroRNAs (miRNAs) are endogenous ~22-nucleotide non-coding RNAs that regulate gene expression through the translational repression or degradation of target mRNAs. The human genome contains over 1400 miRNAs and over 60% of human mRNAs are predicted to be targets of miRNAs. The miRNAs have roles in fine-tuning the expression of their target genes forming intricate networks. Research on miRNA is growing exponentially, and it is now clear that miRNAs can potentially regulate every aspect of cellular processes such as differentiation, proliferation and apoptosis as well as a large range of physiological processes such as development, immune response, metabolism, tumor formation, and disease development. The roles of miRNAs in the metabolism of xenobiotics and endobiotics have only recently been revealed. This review describes the current knowledge on the regulation of cytochrome P450s and transcriptional factors by miRNAs, and its physiological and clinical significance, which were disclosed in our studies. The miRNA expression is readily altered by chemicals, carcinogens, drugs, hormones, stress, or diseases, and the dysregulation of specific miRNAs might lead to changes in the drug metabolism potency or pharmacokinetics as well as pathophysiological changes. Utilizing miRNAs opens a new era in the fields of drug metabolism and pharmacokinetics as well as toxicology.

Key words——microRNA; cytochrome P450; nuclear receptor; post-transcriptional regulation

1. はじめに

薬の効果や副作用に認められる個人差は，薬の体内動態並びに薬物代謝酵素活性の個人差に起因することが多い．その個人差の理解のため，薬物代謝酵素の遺伝子多型や転写調節の研究が行われてきた．しかし，それでもなお薬物代謝能の個人差を説明できない事象が存在する．薬物代謝能を制御する可能性のある新たな因子として microRNA（miRNA）が考えられた．miRNA はタンパク質をコードしない 22 塩基程度の小さな RNA で，標的となる mRNA に結合して翻訳を抑制あるいは mRNA を分解することにより，タンパク質の発現を負に制御する機能を有している．1993 年線虫で最初に発見されたのち，2001 年にヒトにも存在することが明らかになり，以降その存在意義が解明され，発生，分化，増殖，アポトーシスなど，重要な生命現象に係わっていることがわかってきた．これまでにヒトでは 1400 種以上の miRNA が同定され，ヒト遺伝子産物の 60%以上が，また二次的な影響を含めるとほぼすべての遺伝子産物が miRNA によって調節されていると考えられている．本稿では，miRNA の生合成と発現抑制機構について簡単に解説し，ある遺伝子の発現制御に係わる miRNA を同定するために用いられる一般的な方法について述べた後，筆者らの研究によって明らかになったヒトシトクロム P450 と転写調節因子の miRNA による発現制御とその意義について概説する．

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本総説は，平成 23 年度日本薬学会学術振興賞の受賞を記念して記述したものである．

2. miRNA の生合成と発現抑制機構

miRNA は，通常 RNA ポリメラーゼ II によってヘアピン構造を有する 200–5000 塩基ほどの転写産物 primary microRNA（pri-miRNA）として転写され，核内で Drosha などによりプロセッシングを受けて，ステムループ構造を持つ約 70 塩基の precur-

sor microRNA（pre-miRNA）となる．その後，pre-miRNA は Exportin-5 を介して細胞質に輸送され，Dicer によってプロセッシングを受けて約 22 塩基の二本鎖 miRNA:miRNA*となり，Dicer, Agonaute 2 (Ago2), tar-RNA-binding proteins (TRBP) などで構成される RNA-induced silencing complex (RISC) と複合体を形成する．そして，一本鎖化された mature miRNA がガイド鎖となり，標的 mRNA の主に 3′-非翻訳領域（untranslated region, UTR）に存在する認識配列(miRNA recognition element, MRE) に結合する．miRNA の標的 mRNA への結合は部分相補的であり，miRNA の 5′末端 2–7 塩基である seed 配列が相補的であることが重要と言われている．RISC はリボソーム・サブユニット会合の阻害，リボソームの脱落，キャップ構造の脱離，脱アデニル化など，種々のメカニズムを介して翻訳を抑制又は mRNA を分解し，遺伝子サイレンシングを起こす．

3. 発現制御に係わる miRNA の同定

1 つの miRNA は数百種類の mRNA を標的とする可能性があり，1 つの mRNA は複数の miRNA によって認識されることもあるため，遺伝子の発現調節に係わっている miRNA を予測することは容易ではない．いくつかの予測プログラムが利用可能であるが，それぞれのプログラムで用いるアルゴリズムが異なっており，予測されてくる miRNA が異なることも多い．偽陽性の確率も高く，実際に標的となるかどうかは実験により確かめなければわからない．また，予測プログラムでは miRNA の発現量は考慮されていないため，当該遺伝子が発現している組織に予測された miRNA がどの程度発現しているかは別途考慮する必要がある．

遺伝子の発現制御に miRNA が係わっているか調べる一般的な方法は以下の通りである．まず，細胞に miRNA を過剰発現させ，当該タンパク質又は mRNA の発現量が低下するか調べる．miRNA の過剰発現による人為的な影響の結果である可能性を否定するためには，miRNA に対するアンチセンスオリゴヌクレオチド（AsO）を導入し，内因性の miRNA を抑制した際に，当該タンパク質又は mRNA の発現量が増加するか調べることが肝要である．しかし，このような実験では，miRNA の作用が直接的なものか，別の標的遺伝子に作用した二次的な結果なのか判断できない．そこで有用なのが 3′-UTR や MRE をルシフェラーゼ遺伝子の下流に組み込んだプラスミドを用いたルシフェラーゼアッセイである．miRNA の過剰発現又は AsO の導入によってルシフェラーゼ活性に変動が認められれば，直接的な発現制御を証明することができる．MRE への変異の導入や欠失，複数連結などによるルシフェラーゼ活性の変動を調べることで，MRE の機能性を確認することも重要である．細胞内の miRNA の発現量を人為的に変動させることなく，常在的な状態で miRNA の関与を提唱するには，当該タンパク質発現量と miRNA の発現量に負の相関関係が認められるか調べることも有用である．正常細胞とがん細胞の比較，複数の細胞株間での比較，複数の個人サンプル間の比較など，様々なパターンで適用できる．

4. miR-27b によるヒト CYP1B1 の発現制御[1)]

CYP1B1 は多環芳香族炭化水素や芳香族アミンの代謝的活性化を触媒し，またエストロゲンを DNA 損傷性の代謝物に変換することから，発がんに関与している分子種である．CYP1B1 は卵巣，子宮，乳腺などの組織において mRNA レベルでは高く発現しているものの，タンパク質レベルではほとんど検出できないことから，転写後調節の寄与が示唆され，miRNA による発現制御の可能性を検討した．CYP1B1 mRNA の長さは約 5.2 kb であり，そのうち 3′-UTR は約 3.1 kb と半分以上を占める．CYP1B1 mRNA の配列をヒト，マウス，ラットで比較すると翻訳領域の相同性は 80％以上と高いのに対し，3′-UTR 全体の相同性は 30％ほどしかない（Fig. 1）．ところが，ポリ A に近い領域に 86％と高い相同性を示す領域が 44 bp ほど存在し，その中に miR-27b との結合が予想される配列が存在していた（Fig. 1）．miRNA の配列は種を超えて保存されていることが多く，MRE の配列も種で保存されているほど，その miRNA によって制御されることに意義があるものと推定される．ルシフェラーゼ遺伝子の下流に MRE や 3′-UTR を組み込んだプラスミドを Jurkat 細胞に pre-miR-27b とともに導入するとルシフェラーゼ活性の低下が認められた（Fig. 2）．一方，miR-27b の発現量の高い MCF-7 細胞に導入した際，MRE や 3′-UTR を組み込んだプラスミドでコントロールプラスミドと比べてルシフェ

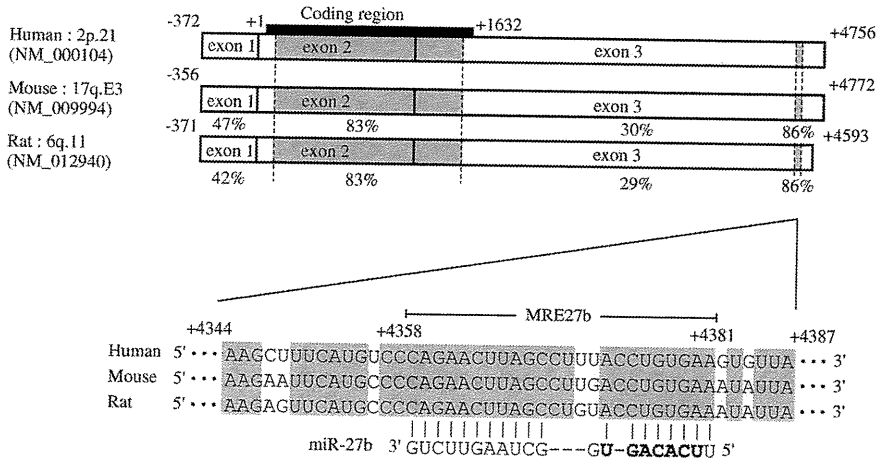

Fig. 1. Homology between Human, Mouse, and Rat CYP1B1 mRNAs and the Predicted Target Sequences of miR-27b

CYP1B1 mRNAs in human, mouse, and rat are ~5 kb in length and consist of three exons. The numbering refers to the translation start site as 1. The sequence of MRE27b is located on +4358 to +4381 in the 3′-UTR of human CYP1B1. Highly conserved regions are shown in gray color. Bold letters: seed sequence.

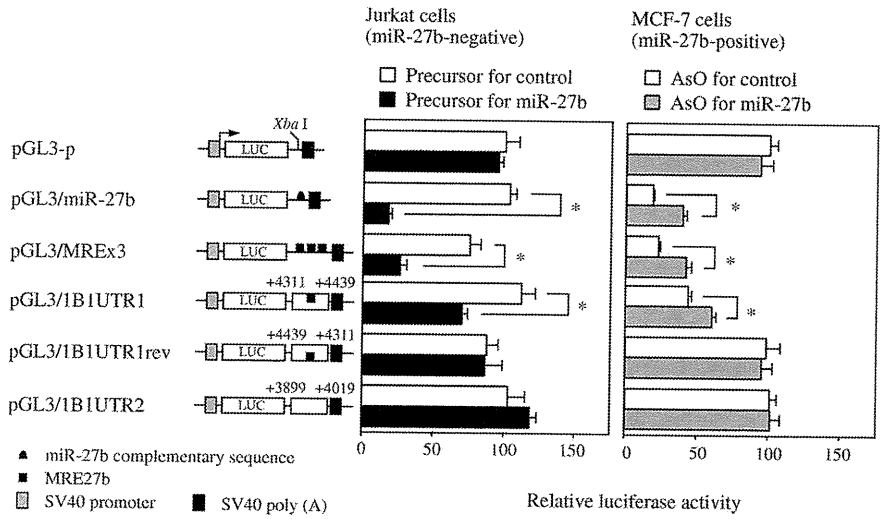

Fig. 2. Luciferase Assay with Reporter Constructs Containing MRE27b or 3′-UTR of Human CYP1B1 in Jurkat or MCF-7 Cells

A series of reporter constructs was transfected into Jurkat cells with precursor for miR-27b or control, or into MCF-7 cells with AsO for miR-27b or control. Values are expressed as percentages of the relative luciferase activity of pGL3 promoter plasmid. Each column represents the mean±S.D. of three independent experiments. *$p<0.05$.

ラーゼ活性は低値を示したが，miR-27b に対する AsO の導入により活性の回復が認められたことから，予測された MRE に miR-27b が結合し，発現を抑制していることが示された．また，miR-27b に対する AsO の導入により，MCF-7 細胞における内因性 CYP1B1 の発現量の増大が認められ，CYP1B1 が miR-27b によって制御されていることが明らかになった．この発現制御機構の生体内における意義を解明するにあたり，CYP1B1 タンパク質発現量は正常組織よりもがん組織で多いことに注目し，その現象に miR-27b が係わっている可能性を考慮した．乳がん組織とその周辺の非がん部における miR-27b の発現量を調べたところ，がん部では発現量が少ないことが明らかになり [Fig. 3(A)]，がん部における miR-27b の発現量と CYP1B1 タンパク質発現量との間に負の相関関係が認められた [Fig. 3(B)]．したがって，正常組織中では miR-27b が CYP1B1 の発現を抑制的に制御しており，がんで CYP1B1 が高発現している理由の 1 つとして miR-27b の低下が挙げられることを明らかにした．これ

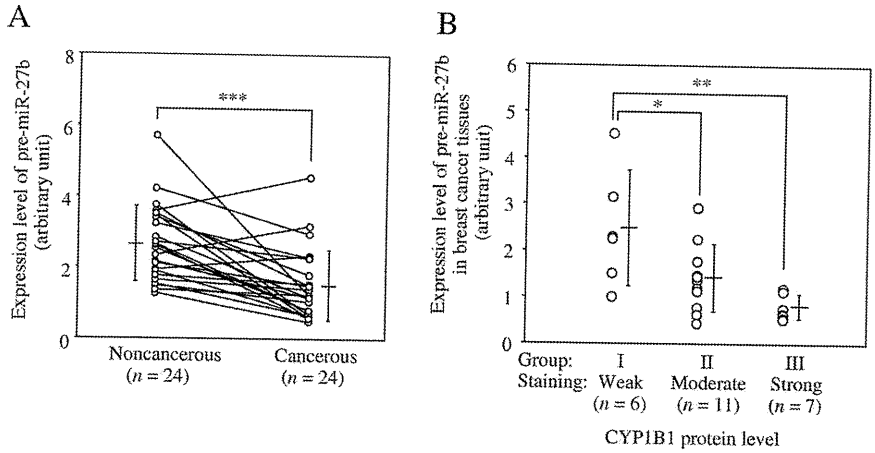

Fig. 3. Expression of miR-27b in Human Breast Cancerous and Adjacent Noncancerous Tissues (A) and the Relationship between the Expression Levels of miR-27b and CYP1B1 Protein Level in Human Breast Cancer (B)

The expression levels of pre-miR-27b and CYP1B1 protein were determined by real-time RT-PCR and immunostaining, respectively. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

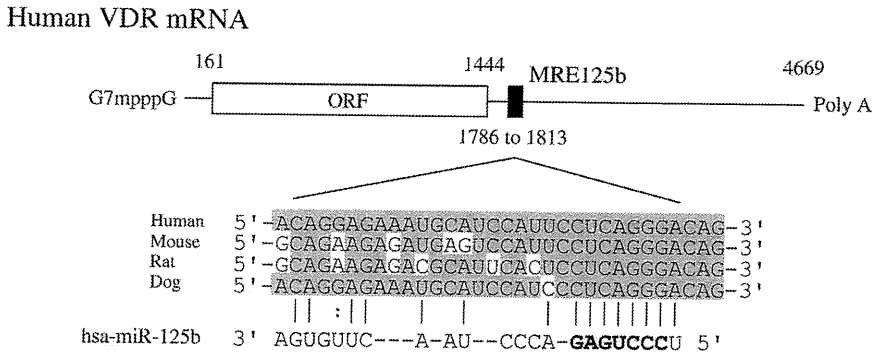

Fig. 4. Schematic Representation of Human VDR mRNA and the Predicted Target Sequence of miR-125b

The numbering refers to the 5′-end of mRNA as 1. The sequence of MRE125b is located on +1786 to +1813 in the 3′-UTR of human VDR. Highly conserved sequence is shown in gray color. Bold letters: seed sequence.

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はmiRNAが薬物代謝酵素の発現制御に係わっていることを示した世界初の研究成果である.[1]

5. miR-125bによるビタミンD受容体（VDR）[2]とCYP24[3]の発現制御

ビタミンD_3は血中カルシウム濃度の恒常性維持や骨代謝に重要な役割を担う一方，細胞増殖抑制作用や分化誘導及びアポトーシス誘導作用を有しており，抗がん薬としての可能性が期待されている．ビタミンD_3の作用はVDRを介して発揮される．VDRは，mRNA発現量としてはがん部位と正常部位で差が認められないものの，タンパク質発現量としては正常部位に比べがん部位で高いことが報告されており，転写後調節の関与が示唆された．VDR mRNAに結合する可能性のあるmiRNAを探索したところ，いくつかのmiRNAが予測されたが，中でもmiR-125bの認識部位MRE125bの配列は種を超えて高く保存されていた（Fig. 4）ことから，miR-125bがVDRの発現を制御している可能性を検討した．ルシフェラーゼアッセイによりMRE125bが機能的に働いていることが示された．miR-125bがVDRのタンパク質発現量を抑制しているか，ヒトがん由来細胞株におけるVDR発現量をウェスタンブロットで解析したが，市販の抗体では非特異的なバンドが多く，検出が困難であった．そこで，ゲルシフトアッセイを利用した検出を試みた．VDRは活性化されるとレチノイドX受容体α（RXRα）とヘテロダイマーを形成して，標的遺伝子の応答配列に結合して転写を活性化する．MCF-7細胞に

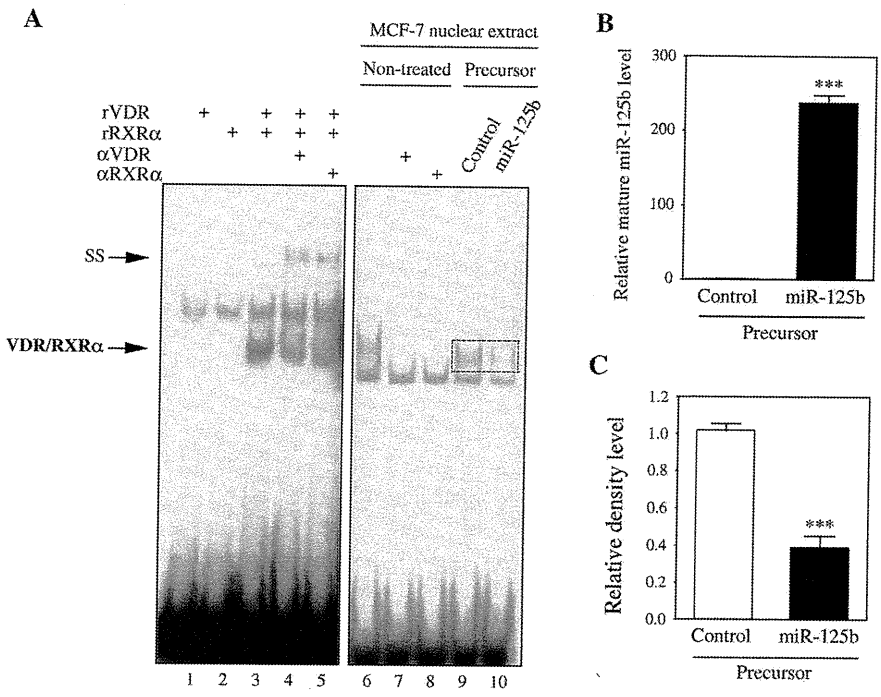

Fig. 5. Electrophretic Mobility Shift Assay to Evaluate the Endogenous VDR Protein Level

The ^{32}P labeled probe containing the VDRE in human CYP24 promoter was incubated with *in vitro*-synthesized VDR (rVDR) and RXRα (rRXRα) or the nuclear extract prepared from the precursors for miR-125b or control-transfected MCF-7 cells (A). The mature miR-125b level was determined by real-time RT-PCR analysis (B). The relative density of the shifted band including VDR/RXRα complex was shown as the mean±S.D. of three independent experiments (C). $^{***}p<0.001$.

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pre-miR-125b を導入して核抽出液を調製し，VDR の標的遺伝子の 1 つである CYP24 の応答配列をプローブとしてゲルシフトアッセイを行ったところ，VDR/RXRα ヘテロダイマーの結合量が低下し，VDR 発現量の低下が示された（Fig. 5）．また，VDR のリガンドである 1α,25-ジヒドロキシビタミン D_3 の処置により，標的遺伝子である CYP24 mRNA は顕著に誘導されるが，その誘導能は miR-125b により有意に抑制された (Fig. 6)．以上より，ヒト VDR が miR-125b によって発現制御されていることが明らかになった．[2)]

また興味深いことに，CYP24 の発現も miR-125b で制御されていることを，過剰発現又は阻害実験及びルシフェラーゼアッセイなどの手法を用いて明らかにした（Fig. 7）．[3)] つまり，CYP24 は miR-125b によって直接的に，及び VDR の発現抑制を介して間接的に発現抑制されていることになる．CYP24 も正常組織に比べてがん組織で高発現しており，がん組織における miR-125b の発現低下が原因であることも示された．様々ながん組織において多くの

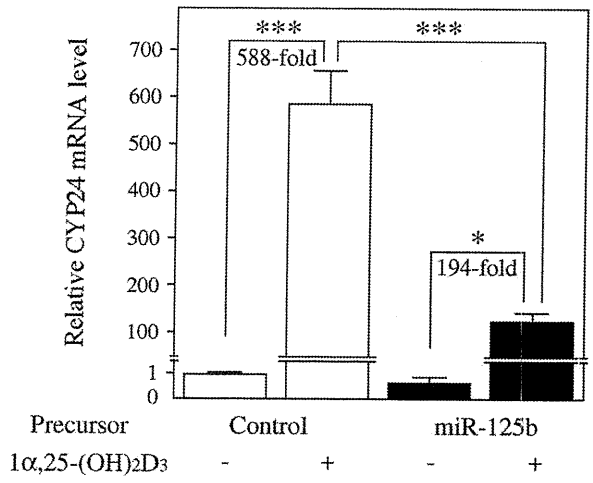

Fig. 6. Induction of CYP24 mRNA in MCF-7 cells by 1α,25-dihydroxyvitamin D_3

The precursors for miR-125b or control (50 nM) were transfected into MCF-7 cells. After 72 h, the cells were treated with 100 nM 1α,25-dihydroxyvitamin D_3 or 0.1% ethanol (vehicle) for 24 h and then CYP24 mRNA levels were determined by real-time RT-PCR and normalized with the GAPDH mRNA level. Each column represents the mean±S.D. of three independent experiments. $^{*}p<0.05$, $^{***}p<0.001$.

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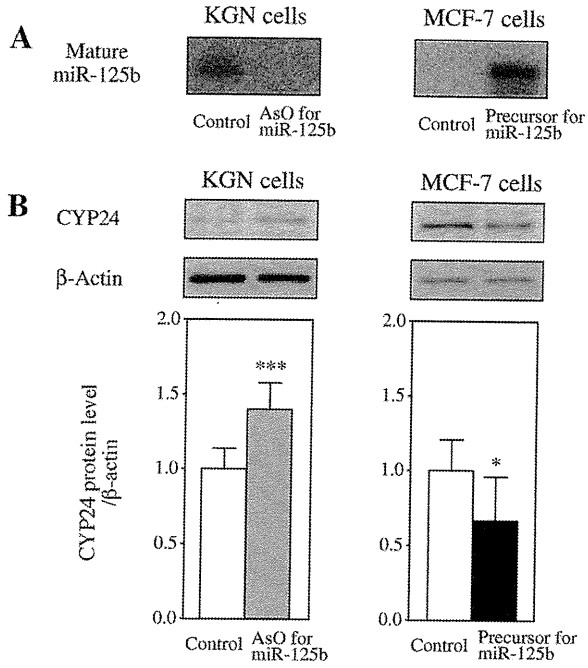

Fig. 7. Effects of miR-125b on the Endogenous CYP24 Protein Level in KGN or MCF-7 Cells

AsO for miR-125b or control (2.5 pmol/4×10^5 cells) were transfected into KGN cells and precursors for miR-125b or control (84 pmol/1.68×10^5 cells) were transfected into MCF-7 cells. After 72 h, total RNA and whole cell lysate were prepared. The expression levels of mature miR-125b were determined by Northern blot analysis (A). The expression levels of CYP24 protein were determined by Western blot analysis and normalized with β-actin protein level (B). Each column represents the mean±S.D. of three independent experiments. $^*p<0.05$, $^{***}p<0.005$

This figure is from our previous paper[3] with permission of The American Society for Pharmacology and Experimental Therapeutics.

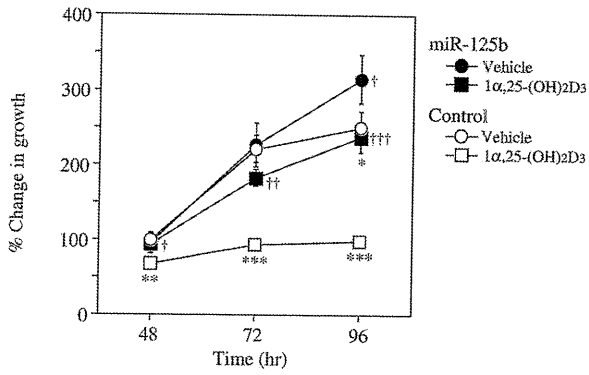

Fig. 8. Antiproliferative Effects of 1α,25-dihydroxyvitamin D_3 in MCF-7 Cells

The precursors for miR-125b or control (20 nM) were transfected into MCF-7 cells. After 24 h, the cells were treated with 1 μM 1α,25-dihydroxyvitamin D_3 or 0.1% ethanol (vehicle) for 48–96 h and then crystal violet assays were performed. Values are expressed as percentages change in growth relative to the cell viability in the precursor for control-transfected cells in the absence of 1α,25-dihydroxyvitamin D_3 after 48 h incubation. Each point represents the mean±S.D. of three independent experiments. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, compared with the vehicle. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$, compared with the precursor for control.

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miRNA の発現が大きく変動していることが示されている.[4] その原因として，転写活性の変化，エピジェネティック発現調節の変動，遺伝子変異，DNA コピー数の異常など，いくつかの理由が挙げられるが，miRNA をコードする遺伝子の半数以上はがんに関連する fragile site 上に存在することから，DNA コピー数の異常が主な原因と考えられている.[5] miR-125b は pre-miR-125b-1 と pre-miR-125b-2 の 2 つの前駆体から生成されるが，それらをコードする遺伝子はそれぞれ 11q24.1 と 21q11.2 にある. 11q23-24 は乳がん，卵巣がん，肺がんなどで欠失し易く,[6,7] 21q11-21 は乳がん，食道がん，胃がん，卵巣がん，肺がんなどで欠失し易い領域である.[8] そのために miR-125b の発現量ががん組織で低下しているものと考えられる.

CYP24 は活性型ビタミン D_3 の 24 位水酸化反応を触媒し，不活性化する酵素である．活性型ビタミン D_3 の効果発揮に必要な VDR と不活性化に係わる CYP24 がともに miR-125b により制御されていることが明らかになり，それがビタミン D_3 による細胞増殖抑制作用にどのような結果をもたらすかが疑問に残った．そこで，MCF-7 細胞の増殖能を評価することで検討したところ，細胞増殖能は 1α,25-ジヒドロキシビタミン D_3 により有意に抑制され，その抑制効果は pre-miR-125b の導入により低下した（Fig. 8）．したがって，この実験条件下では miR-125b は VDR に対する抑制効果を優先的に示すことが明らかになった．すなわちがん組織では miR-125b の発現が低下しており，VDR を介した抗腫瘍作用を増大させる生体防御機構が働いている可能性が考えられた.

6. miR-148a によるプレグナン X 受容体（PXR）の発現制御と CYP3A4 発現量への影響[9]

ヒト CYP3A4 は肝臓及び小腸に高く発現し，医薬品代謝の約 50%に関与する最も重要な薬物代謝酵素である．CYP3A4 の発現量や酵素活性には 100 倍ほどの大きな個人差が認められるが，遺伝子多型でも個人差を説明できない．筆者らは CYP3A4 とその発現に重要な役割を果たすプレグナン X 受容体（pregnane X receptor, PXR）の 3′-UTR に共通して miR-148a 認識配列が存在することを見い出し

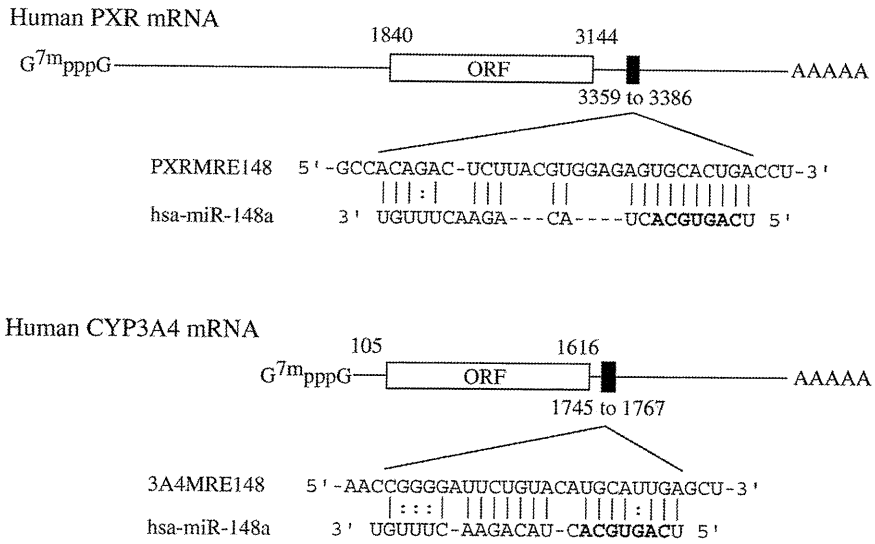

Fig. 9. Schematic Representation of Human PXR and CYP3A4 mRNAs and the Predicted Target Sequence of miR-148a

The numbering refers to the 5′-end of mRNA as 1. The sequence of MRE148a is located on +3359 to +3386 in the 3′-UTR of human PXR mRNA and +1745 to +1767 in the 3′-UTR of human CYP3A4 mRNA. Bold letters: seed sequence.

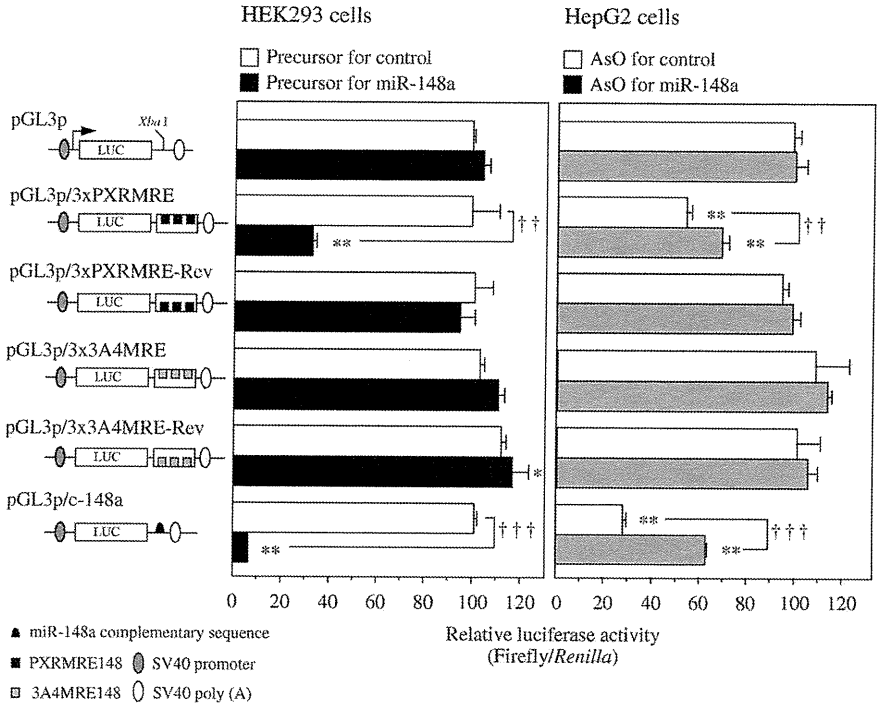

Fig. 10. Luciferase Assay with Reporter Constructs Containing MRE148a in the 3′-UTR of PXR or CYP3A4 in HEK293 or HepG2 Cells

A series of reporter constructs was transfected into HEK293 cells with precursor for miR-148a or control, or into HepG2 cells with AsO for miR-148a or control. Values are expressed as percentages of the relative luciferase activity of pGL-3 promoter plasmid. Each column represents the mean±S.D. of thee independent experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared with pGL3, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ compared with precursor or AsO for control.

(Fig. 9)，解析したところ，miR-148a は CYP3A4 には直接作用しないが，PXR の発現を抑制的に制御し（Fig. 10），CYP3A4 の発現量に影響を与えていることを明らかにした（Fig. 11）．タンパク質は DNA から転写された mRNA に基づいて合成されるため，かならず転写レベルでの調節を受けている．

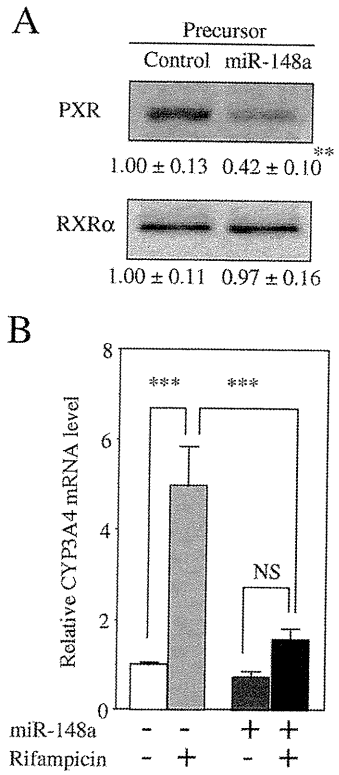

Fig. 11. Effects of Overexpression of miR-148a on the Endogenous PXR Level and the Induction of CYP3A4 mRNA in LS180 Cells

The precursors for miR-148a or control (50 nM) were transfected into LS180 cells. After 72 h, the cells were harvested and nuclear extracts were isolated. The PXR and RXRα protein levels were determined by Western blot analysis (A). The precursor-transfected LS180 cells were treated with 50 μM rifampicin or 0.1% DMSO for 24 h and the CYP3A4 mRNA levels were determined by real-time RT-PCR and normalized with the GAPDH mRNA level. Data are the mean±S.D. of three independent experiments. $^{**}p<0.01$, $^{***}p<0.001$; NS: Not significant.

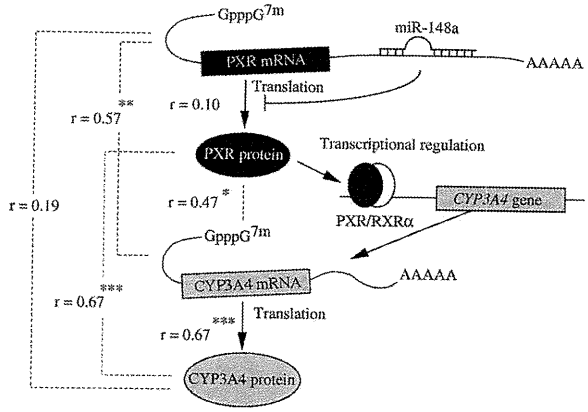

Fig. 12. Schematic Representation of miR-148a-dependent Post-transcriptional Regulation of Human PXR Affecting the Expression Level of CYP3A4 in Human Livers

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

25検体のヒト肝試料を用いた検討において，PXRではmRNA発現量とタンパク質発現量との間に正の相関関係が認められず（r＝0.1），転写後調節が大きく寄与していることが示唆された（Fig. 12）．一方，CYP3A4ではmRNA発現量とタンパク質発現量との間に有意な正の相関関係が認められた（r＝0.67, $p<0.001$）ことから，転写調節が主要であり，miRNAによる転写後調節の寄与は大きくないことが示された．

7. miR-24とmiR-34aによるhepatocyte nuclear factor 4α（HNF4α）の発現制御と胆汁酸合成への影響[10]

肝臓や腎臓，腸管などに発現しており，非常に多くの遺伝子発現を制御することからマスターレギュレーターとよばれる肝細胞核因子4α（hepatocyte nuclear factor 4α, HNF4α）がmiRNAで制御されている可能性を検討した．興味深いことに，miR-34aは3′-UTRに結合して翻訳を抑制し，miR-24は翻訳領域に結合してmRNAの分解を介して発現を抑制することを明らかにした［Fig. 13(A)］．この発現制御は肝臓中においてHNF4αの下流遺伝子であるCYP7AやCYP8Bなどの胆汁酸合成酵素の発現低下を招くことが示された［Fig. 13(B)］．胆汁酸はHNF4αの発現を低下させ，胆汁酸合成を抑制する，というネガティブフィードバック機構が存在することが報告されていたが，そのメカニズムは不明であった．本研究では，胆汁酸によるプロテインキナーゼCの活性化や活性酸素種の産生を介したシグナル伝達経路の活性化がmiR-24及びmiR-34aの発現を増加させ，それがHNF4αの発現低下をもたらしていることを示し，メカニズムの一因にmiRNAが係わっていることを明らかにした(Fig. 14)．

8. おわりに

上述の研究に加え筆者らは，ヒトCYP2E1がmiR-378で制御されていること，[11] ヒトPPARαがmiR-21及びmiR-27bで制御されていること[12]も最近明らかにしており，薬物・異物代謝におけるmicroRNAの役割についてかなり情報が蓄積されてきた．[13] 興味深いことに，ジヒドロ葉酸還元酵素(dihydrofolate reductase, DHFR)[14]や硫酸転移酵素(sulfotransferase, SULT) 1A1[15]の3′-UTRに存在する一塩基多型（single nucleotide polymorphism, SNP）が，miRNAによる結合・制御能に影響を及

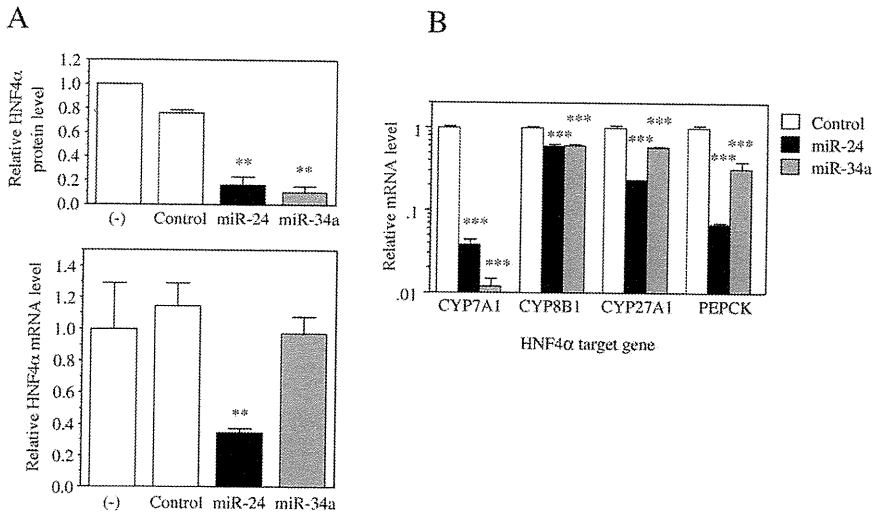

Fig. 13. Effects of miR-24 and miR-34a on the Human HNF4α Protein or mRNA Levels (A) and Its Downstream Genes (B)

The precursors for miR-24, miR-34a or control (50 nM) were transfected into HepG2 cells. After 48 h, total RNA and whole cell lysates were prepared. The HNF4α protein levels were determined by Western blot analysis and normalized with GAPDH protein level (A). The HNF4α mRNAs levels (A) and the CYP7A1, CYP8B1, CYP27Al, and PEPCK mRNA levels (B) were determined by real-time RT-PCR analysis and normalized with GAPDH mRNA level. Each column represents the mean±S.D. of three independent experiments. $^{**}p<0.01$, $^{***}p<0.001$.

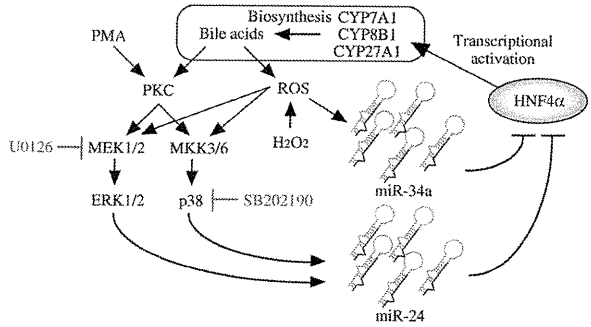

Fig. 14. The Regulatory Loop of miR-24, miR-34a and HNF4α in Bile Acid Synthesis

Bile acids are known to activate protein kinase C (PKC) and reactive oxygen species (ROS) generation, resulting in the activation of mitogen-activated protein kinase (MAPK) pathway. The expression of miR-24 and miR-34a is induced by MAPK-dependent and -independent pathways, respectively. In turn, miR-24 and miR-34a negatively regulate the HNF4α. The down-regulation of HNF4α decreases the expression of bile acid-synthesizing enzymes CYP7A1 and CYP8B1 resulting in the decrease of bile acids. ERK; extracellular signal-regulated kinase, MEK; MAPK/ERK kinase, MKK; mitogen-activated protein kinase kinase, PMA; phorbol 12-myristate 13-acetate.

ぼし，酵素の発現量に個人差をもたらす原因となっていることが報告された．mature miRNA 上に遺伝子多型がある場合も同様に発現制御機能に影響を及ぼす可能性もあり，pri-miRNA や pre-miRNA 上に遺伝子多型がある場合は mature miRNA の発現量の変動をもたらし，それが標的遺伝子の発現変動をもたらすこともある.[13] ファーマコジェネティクスの研究領域に miRNA を取り込むことで，これまで解明できなかった薬効・副作用の個人差が解明できる可能性があり，今後の研究の発展が望まれる．

miRNA の発現は様々な疾患において変動する.[13] また，薬物，毒物，発がん物質などの曝露や，ストレスに応答して miRNA の発現が変動することも示されている.[13] このような miRNA 発現の変動が，薬物の体内動態にどの程度影響を及ぼしているか解明することは今後の課題である．

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MOLECULAR TOXICOLOGY

Stimulation of pro-inflammatory responses by mebendazole in human monocytic THP-1 cells through an ERK signaling pathway

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Abstract Oral helminthic mebendazole (MBZ) has been reported to cause liver injury with inflammatory responses. However, the underlying mechanism remains unknown. To examine the inflammatory reactions, we investigated whether MBZ and other helminthic drugs increase the release of pro-inflammatory cytokines and chemokines using human monocytic cells. The release of interleukin (IL)-8 and tumor necrosis factor (TNF) α from human monocytic THP-1 cells was significantly increased by treatment with MBZ, albendazole (ABZ), fenbendazole (FBZ), or oxibendazole (OBZ), but not by albendazole sulfoxide or praziquantel, suggesting that MBZ and structurally similar drugs can stimulate monocytes and increase the release of pro-inflammatory cytokines. MBZ also significantly increased the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and c-Jun N-terminal kinase (JNK) 1/2 in THP-1 cells. Pretreatment with the MAP kinase/ERK kinase 1/2 inhibitor U0126 significantly suppressed the increase of IL-8 and TNFα levels by MBZ, ABZ, FBZ, or OBZ treatment in THP-1 cells, but the p38 mitogen-activated protein kinase inhibitor SB203580 or JNK1/2 inhibitor SP600125 did not. These results suggested that an ERK1/2 pathway plays an important role in the release of IL-8 and TNFα in THP-1 cells treated with MBZ and structurally similar drugs. In conclusion, the release of inflammatory mediators by MBZ might be one of the mechanisms underlying immune-mediated liver injury. This in vitro method may be useful to predict adverse inflammatory reactions that lead to hepatotoxicity.

Keywords Mebendazole · THP-1 cell · Hepatotoxicity · IL-8 · TNFα

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Introduction

Drug-induced liver injury is the most frequent reason for the withdrawal of an approved drug from the market and for failures in drug development in pharmaceutical companies. Because of significant adverse drug reactions associated with hepatotoxicity, several drugs have been removed from the pharmaceutical market (Holt and Ju, 2006). Inflammatory stress might be caused by xenobiotics or drugs leading to idiosyncratic adverse drug reactions. The sporadic occurrence of acute inflammatory episodes could explain the onset of some idiosyncratic reactions during clinical drug therapy (Ganey et al. 2004; Roth et al. 2003; Tafazoli et al. 2005). Inflammatory reactions in liver are induced by the activation of immune cells, such as monocytes, macrophages and Kupffer cells. Activated monocytes and macrophages release large amounts of pro-inflammatory cytokines and chemokines, including interleukin (IL)-1, tumor necrosis factor (TNF) α, and IL-8. TNFα triggers the release of a cascade of other cytokines and recruits activated immune cells, including lymphocytes and macrophages (Bradham et al. 1998). IL-8 exhibits multiple effects on neutrophils, including the induction of lysosomal enzyme release, the increase in the expression of adhesion molecules, and rapid infiltration (Leonard et al. 1991; Baggiolini et al. 1994). In several rodent models, it was shown that the production of TNFα and neurtophil infiltration in liver play a critical role in immune-mediated

liver injury by drugs such as acetaminophen, non-steroidal anti-inflammatory drugs, and antibiotics (Jaeschke 2005; Deng et al. 2009).

Recently, it has been reported that human monocytic cell lines were useful to examine the inflammatory responses mediated by drugs withdrawn from the market. In human monocytic THP-1 cells, the mRNA expression levels and/or the release of pro-inflammatory cytokines and chemokines were increased by the treatment with troglitazone or ximelagatran (Edling et al. 2008, 2009).

Many benzimidazoles have been launched on the market and used in clinical drug therapy. Mebendazole (MBZ) and other structurally related drugs are used for the therapy of various helminthic infections as well as for the treatment of hydatid disease and alveolar echinococcosis (Ammann and Eckert 1996). However, MBZ has been reported to cause hepatic injury. Bekhti and Pirotte (1987) described a case of acute hepatocellular injury in a patient treated with MBZ 600 mg/day for echinococcosis. Colle et al. (1999) reported a case of granulomatous hepatitis with eosinophilia after the administration of MBZ. Seitz et al. (1983) and Junge and Mohr (1983) reported MBZ-induced hepatic injury, and the liver biopsy of the patient revealed hepatocytic necrosis and portal inflammation with eosinophils during long-term (49–60 days) and high-dose (2–3.5 g/day) therapy with MBZ. Recently, MBZ has been carefully used in clinical drug therapy, thus case reports of severe hepatic injury are very rare (Bagheri et al. 2004). Chen et al. (2003) reported that MBZ is associated with Steven-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN), suggesting the involvement of immune-mediated factors. However, the mechanism underlying the hepatic injury by MBZ remains to be clarified.

Considering the case reports of hepatic injury by MBZ, we hypothesized that benzimidazoles stimulate inflammatory responses that may result in immune-mediated hepatic injury. The purpose of this study is to investigate whether benzimidazoles stimulate the release of pro-inflammatory cytokines and chemokines from human monocytic cells and to clarify the involvement of cell signaling in the release of pro-inflammatory cytokines and chemokines from THP-1 cells.

Materials and methods

Materials

ABZ, fenbendazole (FBZ), MBZ, oxibendazole (OBZ), and praziquantel (PZQ) were purchased from Sigma–Aldrich (St. Louis, MO). Albendazole sulfoxide (ABZSO) was purchased from Toronto Reserch Chemicals (Ontario, Canada). Lipopolysaccharide (LPS) was also from Sigma–Aldrich (St. Louis, MO). Primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). The monoclonal antibodies of anti-Thr202/Tyr204 phosphorylated extracellular signal-regulated kinase (ERK) 1/2, anti-Thr180/Tyr182 phosphorylated p38 mitogen-activated protein (MAP) kinase, and anti-Thr183/Tyr185 phosphorylated c-Jun N-terminal kinase (JNK) 1/2 were purchased from Cell Signaling Technology (Beverly, MA). The monoclonal antibodies against ERK1/2 and JNK1/2 and the polyclonal antibody against p38 MAP kinase were also from Cell Signaling Technology. All other reagents were of the highest grade commercially available.

Cell culture

The human monocytic leukemia cell line THP-1 was obtained from Riken Gene Bank (Tsukuba, Japan). HL-60 and KG-1 cells were obtained from American Type Culture Collection (Manassas, VA). THP-1 cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). HL-60 and KG-1 cells were cultured in RPMI 1640 medium supplemented with 20% FBS. These cells were maintained at 37°C under an atmosphere of 5% CO_2.

Drug treatment of human monocytic cell lines

THP-1, HL-60, and KG-1 cells were seeded at a density of 1×10^6 cells/well in 24-well plates with the medium containing the indicated concentration of helminthic drugs, and then incubated at 37°C. The final concentration of dimethyl sulfoxide (DMSO) in medium was 0.1%. In experiments using MAP kinase inhibitors, cells were pretreated with MAP kinase/ERK kinase (MEK) 1/2 inhibitor U0126 (Wako Pure Chemical Industries), p38 MAP kinase inhibitor SB203580 (Wako Pure Chemical Industries), or JNK1/2 inhibitor SP600125 (Calbiochem, Los Angeles, CA) for 1 h, and then treated with the helminthic drugs. Supernatants were separated from cell cultures by centrifugation and stored at −70°C until assayed. For immunoblot analysis, the cells were suspended in TGE buffer (10 mM Tris–HCl, 20% glycerol, 1 mM EDTA, pH 7.4) and disrupted by freeze-thawing three times.

Enzyme-linked immunosorbent assay (ELISA)

The pro-inflammatory cytokine TNFα and the chemokine IL-8 in cell supernatants were measured by Human TNFα and IL-8 ELISA Ready-SET-GO!™ (eBioscience, San Diego, CA) according to the manufacturer's instructions.

Real-time reverse transcription-polymerase chain reaction (RT–PCR)

Total RNA was extracted from THP-1 cells with RNAiso (Takara Bio, Shiga, Japan) according to the protocol supplied by the manufacturer. Reverse transcription was performed with ReverTra Ace (Toyobo, Tokyo, Japan) according to the manufacturer's protocol. For quantitative analysis, real-time RT–PCR was performed for inflammatory cytokine mRNA using an MX3000P real-time PCR system (Stratagene, La Jolla, CA). The primers used in this study were human IL-8 (forward: 5′-CAGCCTTCCTG ATTTCTCTGCAG-3′, reverse: 5′-AGACAGAGCTCTC TTCCATCAG-3′) and human TNFα (forward: 5′-CTT CTGCCTGCTGCACTTTGGAG-3′, reverse: 5′-GGCTAC AGGCTTGTCACTCGG-3′). An 1 μL portion of the reverse-transcribed mixture was added to a PCR mixture containing 10 pmol of each primer and 10 μL of SYBR Premix ExTaq solution in a final volume of 20 μL. After an initial denaturation at 95°C for 30 s, the amplification was performed by denaturation at 94°C for 20 s and annealing and extension at 64°C for 20 s for 45 cycles. The IL-8 and TNFα mRNA levels were normalized with human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (forward: 5′-CCATGAGAAGTATGACAACAGCC-3′, 5′-TG GGTGGCAGTGATGGCATGGA-3′).

Immunoblot analysis

SDS–polyacrylamide gel electrophoresis and immunoblot analysis were performed according to Laemmli (1970). Cell sources (25 μg) were separated on 10% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membrane, Immobilon-P (Millipore Corporation, Billerica, MA). The membranes were probed with the monoclonal antibodies of anti-Thr202/Tyr204 phosphorylated ERK1/2, anti-Thr180/Tyr182 phosphorylated p38 MAP kinase, and anti-Thr183/Tyr185 phosphorylated JNK1/2, and the corresponding fluorescent dye-conjugated second antibody and an Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE) were used for the detection. The relative expression level was quantified using ImageQuant TL Image Analysis software (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Cell viability assay

For the cell viability assay, THP-1 cells were seeded at a density of 1×10^5 cells/well in 96-well plates with the medium containing the indicated concentration of the halminthic drug, and then incubated at 37°C. The final concentration of DMSO in medium was 0.1%. After 6 or 24 h incubation, cell viability was evaluated by 3-(4,5-dimetylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) activities using a CellTiter-Blue Cell Viability Assay (Promega, Medison, WI) according to the manufacturer's protocol. The fluorescence of the generated resorufin was detected fluorometrically (excitation: 338 nm, emission: 458 nm) by using a luminometer (1420 ARVO MX, Wallac, Turku, Finland).

Statistical analysis

Data are expressed as mean ± SD of triplicate determinations. Comparison of 2 groups was made with an unpaired, two-tailed student's *t*-test. Comparison of multiple groups was made with ANOVA followed by Dunnett or Tukey test. A value of $P < 0.05$ was considered statistically significant.

Results

Comparative effect of helminthic drugs on human monocytic cell lines

To investigate whether the helminthic drugs increased the release of IL-8 and TNFα from human monocytic cells, cells were treated with 10 μM of the helminthic drugs for 6 h and then the release of IL-8 and TNFα in the cell supernatants was measured by ELISA. The helminthic drugs used in our study are shown in Fig. 1. ABZSO was the active metabolite of ABZ (Gottschall et al. 1990). FBZ and OBZ were used as drugs structurally similar to MBZ and ABZ, although they have never been administered in humans. PZQ was used because no case of symptomatic hepatic injury has ever been seen so far. The IL-8 and TNFα release from THP-1 cells was significantly increased by treatment with ABZ, FBZ, MBZ, or OBZ but not by ABZSO or PZQ compared with control (0.1% DMSO) (Fig. 2a, b). These results suggested that MBZ and structurally similar drugs have the ability to increase the release of pro-inflammatory cytokines and chemokines from monocytes that activate the inflammatory responses. In addition, MBZ and OBZ also significantly increased the IL-8 release from HL-60 and KG-1 cells and FBZ significantly increased the IL-8 release from KG-1 cells (Fig. 2c, e). In contrast, the TNFα release from HL-60 and KG-1 cells was not increased by these helminthic drugs (Fig. 2d, f). For the subsequent analyses, THP-1 cells were used because they showed the highest sensitivity for the release.

Time-dependent changes in the mRNA expression levels and the release of IL-8 and TNFα in THP-1 cells treated with MBZ

We next investigated the time-dependent changes of the IL-8 and TNFα levels in THP-1 cells. By the treatment

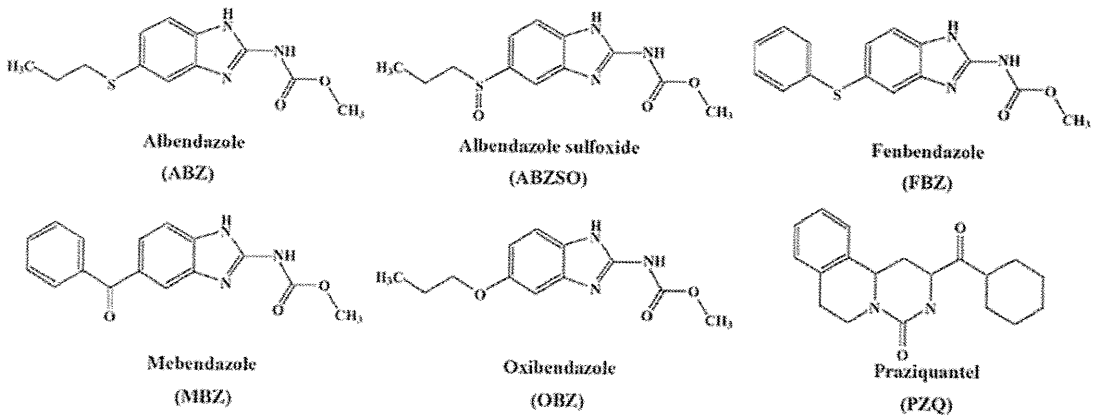

Fig. 1 Chemical structures of the helminthic drugs used in the present study

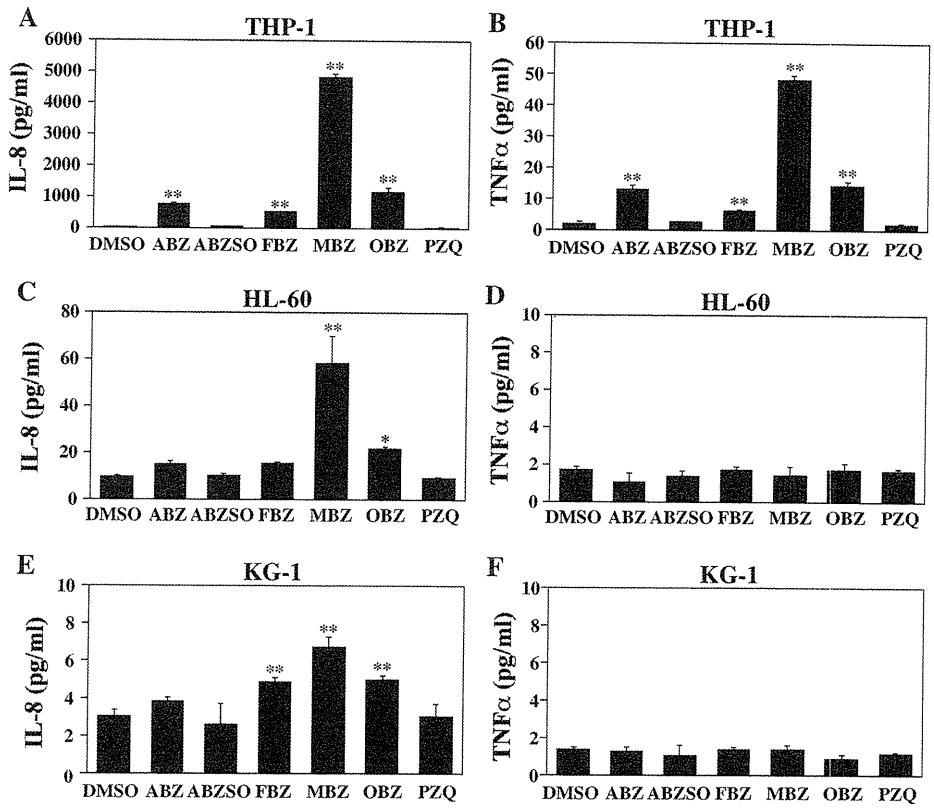

Fig. 2 Effects of helminthic drugs on the release of IL-8 and TNFα from human monocytic cell lines. Human monocytic cell lines including THP-1 (**a** and **b**), HL-60 (**c** and **d**), and KG-1 (**e** and **f**) were treated with 10 μM of the helminthic drugs for 6 h. The release of IL-8 (**a**, **c**, and **e**) and TNFα (**b**, **d**, and **f**) in the supernatant was measured by ELISA. Data represent the mean ± SD of triplicate determinations. $^{*}P < 0.05$; $^{**}P < 0.01$, compared with control (0.1% DMSO)

with 10 μM MBZ, the mRNA expression levels and the release of IL-8 and TNFα in THP-1 cells were significantly increased for 1.5–24 h compared with control (Fig. 3). The mRNA expression levels of IL-8 were mostly increased at 6 h incubation but the increase of IL-8 release was in a time-dependent manner (Fig. 3a, c). The highest increase of the mRNA expression levels and the release of TNFα appeared at 4.5 and 6 h-incubation, respectively (Fig. 3b, d). Therefore, an incubation time of 6 h was selected for further assay to measure the release of IL-8 and TNFα. To investigate whether there were cytotoxic effects on THP-1 cells caused by the leakage of intercellular cytokines and chemokines, a cell viability assay for THP-1 cells was performed. At 24 h-incubation, these helminthic drugs had no cytotoxic effects on THP-1 cells (data not shown).

Dose-dependent changes in the release of IL-8 and TNFα from THP-1 cells treated with helminthic drugs

To investigate whether the helminthic drugs at a lower concentration could also lead to IL-8 and TNFα release in

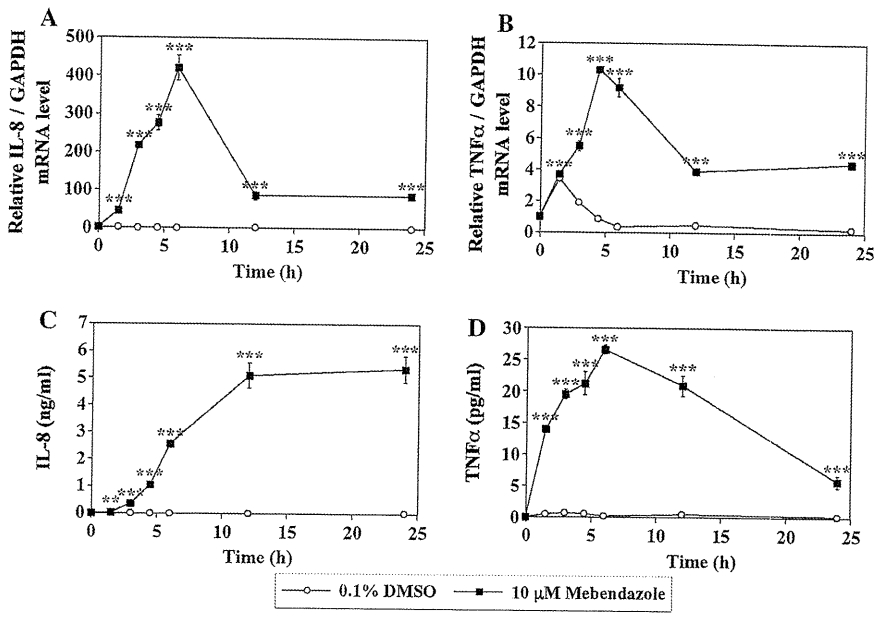

Fig. 3 Time-dependent changes in the mRNA expression levels and the release of IL-8 and TNFα in THP-1 cells treated with MBZ. THP-1 cells were treated with 10 μM MBZ for various durations. The mRNA expression levels of IL-8 (**a**) and TNFα (**b**) in THP-1 cells were measured by real-time RT–PCR analysis. The release of IL-8 (**c**) and TNFα (**d**) in the supernatant was measured by ELISA. Data represent the mean ± SD of triplicate determinations. $^{**}P < 0.01$; $^{***}P < 0.001$, compared with control (0.1% DMSO) of each time point

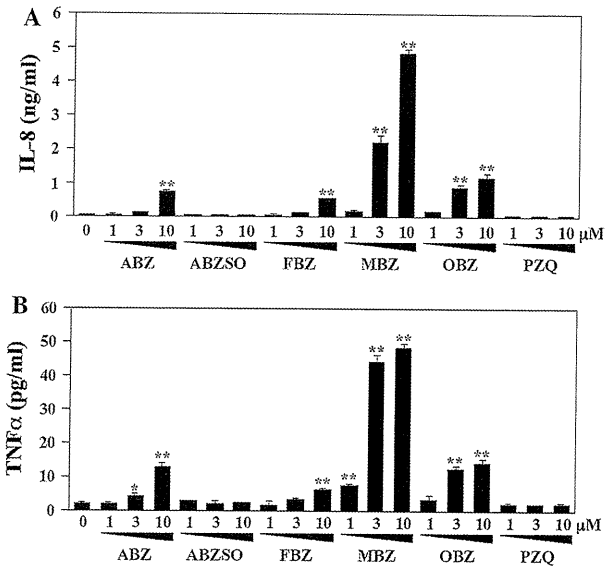

Fig. 4 Dose-dependent changes in the release of IL-8 and TNFα from THP-1 cells treated with helminthic drugs. THP-1 cells were treated with the indicated concentrations of the helminthic drugs. After incubation for 6 h, the release of IL-8 (**a**) and TNFα (**b**) in the supernatant was measured by ELISA. Data represent the mean ± SD of triplicate determinations. $^{*}P < 0.05$; $^{**}P < 0.01$, compared with control (0.1% DMSO)

THP-1 cells, THP-1 cells were treated with helminthic drugs at the indicated concentration for 6 h and then the release of IL-8 and TNFα was measured. As shown in Fig. 4, ABZ, FBZ, MBZ, and OBZ increased the IL-8 and TNFα levels in a dose-dependent manner. In addition, at least 3 μM MBZ was required to increase the release of IL-8 in THP-1 cells. In contrast, the TNFα release was significantly increased at 1 μM MBZ.

Activation of MAP kinase signaling pathway in THP-1 cells treated with MBZ

MAP kinases, including ERK1/2, p38 MAP kinase, and JNK1/2, are major components for many intracellular signaling pathways. The phosphorylation of MAP kinases, which is required for the enzyme activity, activates signaling cascades, the down stream effects of which have been linked to the regulation of the inflammatory response (DeFranco et al. 1998). To clarify the role of MAP kinase signaling pathway in the activation of THP-1 cells, the phosphorylation of ERK1/2 (44/42 kDa), p38 MAP kinase (43 kDa), and JNK1/2 (46/54 kDa) in cell lysates was assessed by immunoblot analysis. A sample treated with 2 μg/ml LPS was used as a positive control for the phosphorylation of MAP kinases. As shown in Fig. 5, MBZ treatment for 1 h significantly increased the phosphorylation of ERK1/2 and JNK1/2 but not p38 MAP kinase in THP-1 cells. These results suggested that MBZ activated ERK1/2 and JNK1/2 pathways in THP-1 cells. In addition, to confirm the effects of MAP kinase inhibitors on the phosphorylation of ERK1/2, p38 MAP kinase, and JNK1/2, THP-1 cells were pretreated for 1 h with various concentrations of MEK1/2 inhibitor U0126, p38 MAP kinase inhibitor SB203580, or JNK1/2 inhibitor SP600125 (English and Cobb, 2002) before the treatment with 10 μM MBZ. As a result, the phosphorylation of ERK1/2 was significantly suppressed by the pretreatment with the specific inhibitor U0126 but not that of JNK1/2 (Fig. 5).

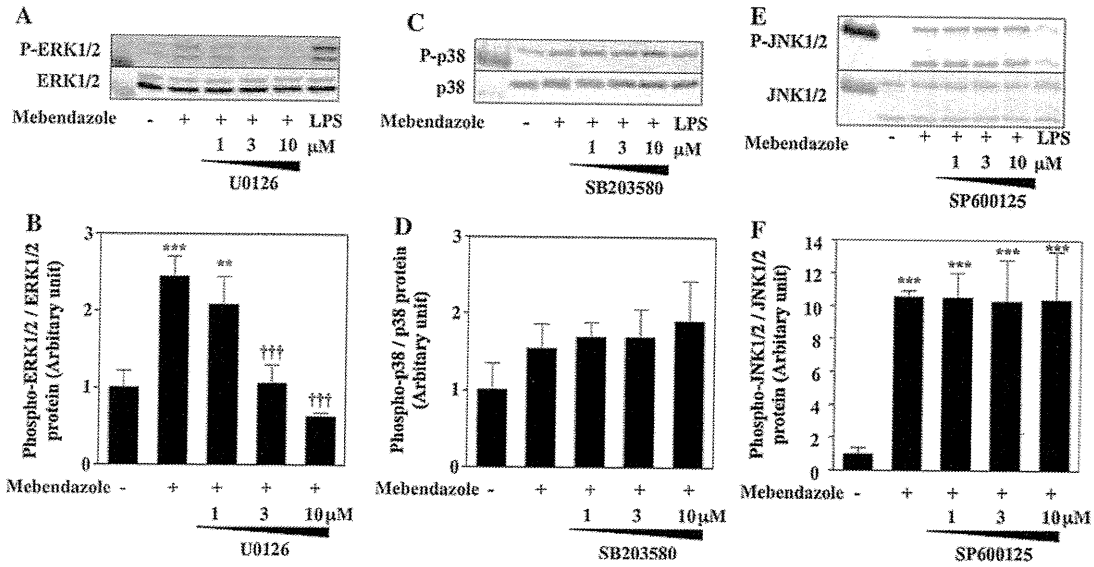

Fig. 5 Activation of MAP kinase signaling pathways in THP-1 cells treated with MBZ. Immunoblot analyses of MAP kinase proteins in THP-1 cells were performed (**a**, **c**, and **e**) and quantified (**b**, **d**, and **f**). Before the treatment with 10 μM MBZ, THP-1 cells were pretreated with the indicated concentrations of MAP kinase inhibitors for 1 h. U0126, SB203580, and, SP600125 were used as specific inhibitors of MEK1/2, p38 MAP kinase, and, JNK1/2, respectively. After 1 h-incubation with MBZ, cell lysates were subjected to immunoblot analyses using antibodies of anti-Thr202/Tyr204 phosphorylated ERK1/2 (**a** and **b**), anti-Thr180/Tyr182 phosphorylated p38 MAP kinase (**c** and **d**), and anti-Thr183/Tyr185 phosphorylated JNK1/2 (**e** and **f**). The same sample treated with 2 μg/ml LPS was used as a positive control. Data represent the mean ± SD of triplicate determinations. $^{**}P < 0.01$; $^{***}P < 0.001$, compared with control (0.1% DMSO). $\dagger\dagger P < 0.01$; $\dagger\dagger\dagger P < 0.001$, compared with MBZ only

The phosphorylation of JNK1/2 treated with LPS was suppressed by the pretreatment with the specific inhibitor SP600125 (data not shown).

Effects of MAP kinase inhibitors on the release of IL-8 and TNFα from THP-1 cells treated with helminthic drugs

To clarify which MAP kinase signaling pathway is mainly involved in the increase of IL-8 and TNFα release, the effects of MAP kinase inhibitors on the release of IL-8 and TNFα from THP-1 cells treated with MBZ were investigated. As shown in Fig. 6, the increased release of IL-8 and TNFα by MBZ treatment from THP-1 cells was significantly suppressed in a dose dependent manner by the pretreatment with U0126, suggesting that an ERK1/2 pathway plays an important role in the release of IL-8 and TNFα by MBZ treatment. A suppressing effect by SP600125 was not observed. In contrast, the increase of IL-8 and TNFα release was enhanced by the pretreatment with SB203580. Therefore, we investigated the effects of MAP kinase inhibitors on the release of IL-8 and TNFα from THP-1 cells treated with other helminthic drugs. As shown in Fig. 7, with ABZ, FBZ, or OBZ treatment, the pretreatment with U0126 remarkably suppressed the increase of IL-8 and TNFα in THP-1 cells and those of SB203580 and SP600125 had no suppressive effects. These results suggested that an ERK1/2 pathway also plays an important role in the increase of IL-8 and TNFα by ABZ, FBZ, or OBZ treatment as well as MBZ treatment. In contrast, even for the control, the basal IL-8 and TNFα levels in THP-1 cells were significantly suppressed by the pretreatment with U0126 and were increased by the pretreatment with SB203580, suggesting that the basal IL-8 and TNFα levels in THP-1 cells were affected by the MAP kinase inhibitors. In the case of ABZSO and PZQ, the effects of MAP kinase inhibitors on the IL-8 and TNFα levels in THP-1 cells were similar to those of the control (0.1% DMSO).

Discussion

For in vitro studies of the differentiation and activation of immune cells, human monocytic cell lines, THP-1, HL-60, and KG-1 cells are usually employed. In the present study, by the treatment with the helminthic drugs MBZ, IL-8 release from THP-1, HL-60, and KG-1 cells and TNFα release from THP-1 cells was significantly increased compared with the control (Fig. 2a–c, e). In the case of ABZ, FBZ, or OBZ, the release of IL-8 and TNFα from THP-1 cells was also significantly increased compared with the control (Fig. 2a, b). This suggested that MBZ and structurally similar drugs have the ability to stimulate the

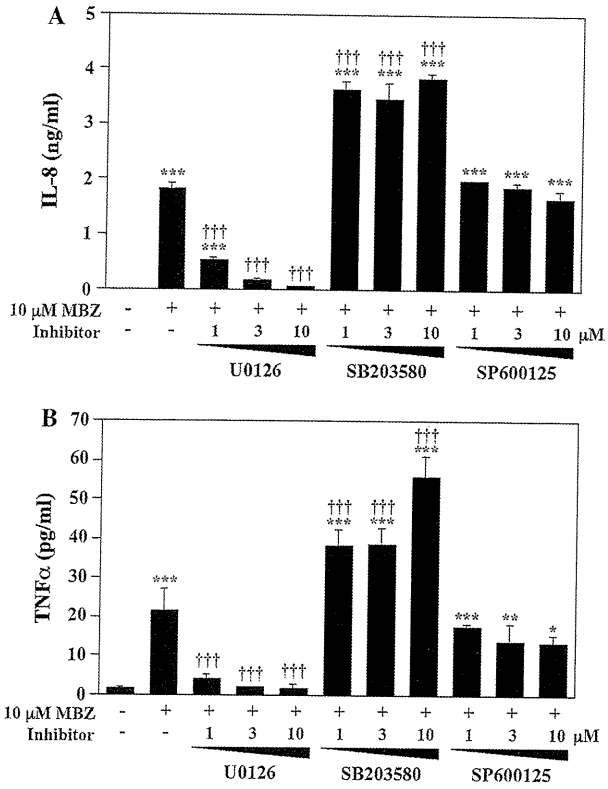

Fig. 6 Effects of MAP kinase inhibitors on the IL-8 and TNFα release from THP-1 cells treated with MBZ. Before the treatment with 10 μM MBZ, THP-1 cells were pretreated with the indicated concentrations of MAP kinase inhibitors for 1 h. After 6 h-incubation with MBZ, the release of IL-8 (**a**) and TNFα (**b**) in the supernatant was measured by ELISA. Data represent the mean ± SD of triplicate determinations. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, compared with control (0.1% DMSO). $\dagger P < 0.05$; $\dagger\dagger P < 0.01$; $\dagger\dagger\dagger P < 0.001$, compared with MBZ only

release of pro-inflammatory cytokines from monocytes, leading to the activation of the inflammatory reaction. In contrast, PZQ, used as a negative control for MBZ, had no effects on the cytokine release in human monocytic cells. This result was supported by the fact that no case of symptomatic hepatic injury has ever been seen so far.

We found that MBZ stimulates the release of pro-inflammatory cytokines and chemokines from human monocytic cells. The activation of inflammatory responses might be one of the mechanisms underlying the immune-mediated liver injury by MBZ. On the other hand, the activation of human monocytic cells by other drugs, such as ximelagatran and troglitazone, has been reported recently, although these drugs have already been withdrawn from the market due to idiosyncratic hepatic injury (Edling et al. 2008, 2009). Ximelagatran increased the release of chemokines from THP-1 cells but the types of released cytokines and the time dependent change of the cytokine release by the drug treatment were different from the case of MBZ (Edling et al. 2008). In addition, troglitazone increased the mRNA expression levels of pro-inflammatory cytokines and chemokines in THP-1 cells (Edling et al. 2009). Therefore, measurement of the release of pro-inflammatory cytokines and chemokines from human monocytic cells may be useful to predict the possibility of adverse reactions to drugs involving immune-mediated hepatic injury.

The activation of MAP kinases such as ERK1/2, p38 MAP kinase, and JNK1/2 is important in mediating many macrophage functions, including the activation of various transcription factors and the production of pro-inflammatory cytokines (Payne et al. 1991; DeFranco et al.

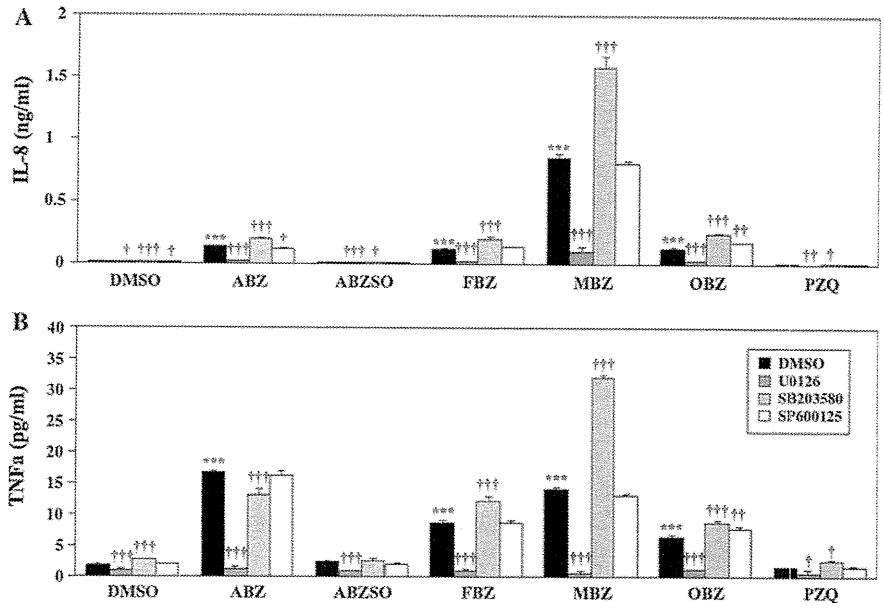

Fig. 7 Effects of MAP kinase inhibitors on the IL-8 and TNFα release from THP-1 cells treated with helminthic drugs. Before the incubation with 10 μM of the helminthic drugs, THP-1 cells were pretreated with 10 μM MAP kinase inhibitors for 1 h. After 6 h-incubation with the halminthic drugs, the release of IL-8 (**a**) and TNFα (**b**) in the supernatant was measured by ELISA. Data represent the mean ± SD of triplicate determinations. $***P < 0.001$, compared with control (0.1% DMSO). $\dagger P < 0.05$; $\dagger\dagger P < 0.01$; $\dagger\dagger\dagger P < 0.001$, compared with an helminthic drug only

1998). In this study, MBZ activated the ERK1/2 and JNK1/2 pathways in THP-1 cells (Fig. 6). Previous experiments with several MAP kinase inhibitors demonstrated that blocking MAP kinase prevents IL-8 and TNFα release from LPS-stimulated monocytes at the transcription and translation levels (Guha and Mackman 2001). To determine the involvement of MAP kinases in MBZ-induced IL-8 and TNFα release, blocking studies were performed using specific inhibitors of MAP kinases, including U0126, SB203580, and SP600125 (English and Cobb 2002). The release of IL-8 and TNFα increased by MBZ was significantly suppressed by the U0126 pretreatment (Figs. 5, 6). These results suggested that an ERK pathway is mainly involved in IL-8 and TNFα release from THP-1 cells. In addition, the increase of IL-8 and TNFα release from THP-1 cells was enhanced by the pretreatment with SB203580 (Fig. 5). At higher concentrations, the p38 MAP kinase inhibitors have been reported to increase the phosphorylation of ERK1/2 (Ishii et al. 2001; Hirosawa et al. 2009). THP-1 cells also have been reported to show enhanced ERK cascade by SB203580 (Numazawa et al. 2003). Considering these reports, the increase of IL-8 and TNFα release from THP-1 cells by SB203580 would be due to the activation of an ERK pathway.

Elevations of serum aminotransferases have been reported to occur in 9–13% of patients treated with 50–100 mg/kg/day; however, severe hepatic injury of MBZ is very rare. Seitz et al. (1983) and Junge and Mohr (1983) reported MBZ-induced hepatic injury, and liver biopsy of the patient revealed hepatocytic necrosis and portal inflammation with eosinophils during long-term (49–60 days) and high-dose (2–3.5 g/day) therapy with MBZ. Rechallenge was followed by a marked elevation of the aminotranferase levels. Liver biopsy showed hepatocytic necrosis and portal inflammation with eosinophils (Junge and Mohr 1983), suggesting an immune-mediated drug response. Colle et al. (1999) also reported a case of granulomatous (immunoallergic) hepatitis with eosinophilia after the administration of MBZ.

It has been reported that the peak plasma concentrations of MBZ was 0.12 ± 0.08 μM 2 h after a single oral administration of 1000 mg MBZ in 16 healthy volunteers (Corti et al. 2009). Because it is very difficult to predict actual drug concentration in the liver and to extrapolate from an in vitro study to the in vivo condition in humans, it is better to test the drug effects in cell-based assays at a range of concentrations up to at least 30 times the efficacious concentration as reported by O'Brien et al. (2006). Thus, we conducted with up to 10 μM of MBZ. However, further study is necessary to clarify whether oral administration of MBZ stimulates the release of pro-inflammatory cytokines and chemokines in vivo.

We obtained ABZSO, an active metabolite of ABZ, which was investigated in this study as a structurally similar drug. ABZSO would be responsible for the systemic biological activity of ABZ, whereas ABZ sulfone is pharmacologically inert (Gottschall et al. 1990). A case report of acute hepatitis caused by ABZ was recently reported by Choi et al. (2008). In evaluating the relation of ABZ and ABZSO to the cytokine release, interestingly, pro-inflammatory cytokine release was found to be increased by treatment with ABZ, but not by ABZSO treatment in human monocytic cells (Figs. 2, 7). These results suggested that ABZ is a causal drug of hepatic injury. However, the activation of THP-1 cells by MBZ was much higher than that by ABZ.

In conclusion, we found that MBZ stimulated human monocytic THP-1 cells resulting in IL-8 and TNFα release. It is suggested that MBZ increases the pro-inflammatory cytokine release from monocytes and macrophages and activates the inflammatory response, which might result in immune-mediated hepatic injury. The findings presented here provide important insight concerning MBZ-induced liver injury.

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