MAPK p38経路を介する肝細胞のルブラトキシンB誘導アポトーシスを茶カテキン、エピガロカテキン-3-ガレート、が阻害する

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Tea catechin, epigallocatechin-3-gallate, inhibits rubratoxin B-induced apoptosis of hepatocytes through MAPK p38 pathway

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Summary

Epigallocatechin-3-gallate (EGCG), exhibited protective effect against rubratoxin B (RB)-induced apoptosis in cultured rat hepatocytes. In this study, the mechanism of hepatoprotection by EGCG was investigated. Hepatocytes were concomitantly treated with RB and EGCG and then the expression levels of mitogen-activated protein kinase (MAPK) p38, caspase-3 and caspase-8 genes were measured by reverse transcriptase-polymerase chain reaction (RT-PCR). p38 was induced by RB, indicating that RB-induced apoptosis was mediated by p38 pathway. On the contrary, the elevation of p38 by RB was diminished by EGCG co-treatment. The analysis of caspase-3 mRNA expression showed the same tendency as observed for the p38. To further elucidate, the expression level of mitogen-activated kinase kinase 6 (MKK6) gene, upstream signaling factor of p38, was analyzed by RT-PCR. EGCG also suppressed the expression of MKK6 mRNA. These results suggest that EGCG protects hepatocytes from RB toxicity by the suppression of the gene expression in p38 and caspase pathway at any points examined.

Key words : rubratoxin B, EGCG, p38, MKK6

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Introduction

Rubratoxin B (RB) is a potent hepatotoxic mycotoxin[1,2] produced by certain Penicillium fungi[2,3]. RB-induced liver failure produces fulminant hepatitis and induces cell apoptosis[2,4,5]. The RB model is a promising experimental basis for understanding the mechanism of clinical liver complaints. However, the mechanism of RB-induced cell death has not been clarified, yet.

To elusidate the mechanism of RB-induced cell death and develop the hepatitis treatment, we have investigated hepatoprotective agents[6-7]. In these studies, green tea catechin, especially (-)-

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epigallocatechin-3-gallate (EGCG), was found to have strong hepatoprotective activity and suppressed RB-induced cell death. The mechanism of RB-induced cell death was considered to be explained through the study of hepatoprotecting mechanism of EGCG against RB.

MAPK pathway plays an important role in the regulation of cellular functions, such as inflammation, cell growth, cell differentiation, the cell cycle and cell death[9,10]. To clarify the mechanism of hepatoprotective effect of EGCG, we analyzed the mitogen-activated protein kinase (MAPK) pathway.

In this paper, we report the role of p38 pathway in RB-induced cell death and the suppressive effect of EGCG on p38 pathway.

Materials and methods

Chemicals

(-)-Epigallocatechin-3-gallate (EGCG) was isolated from green tea leaf. According to high performance liquid chromatography (HPLC) analysis, the purity of EGCG is over 99 %. RB was purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). SB203580 [4-(4'-Fluorophenyl)-2-(4'-methylsulfinylphenyl)-5-(4'-pyridyl) imidazol], a p38 specific inhibitor, was purchased from Promega (Madison, WI, USA). Other chemicals were obtained from local commercial sources, and used without further purification.

Hepatocytes isolation and treatment

Hepatocytes were isolated from male Sprague-Dawley rats weighing 150-200 g by perfusing the liver with collagenase (from *Clostridium histolyticum*; Sigma-Aldrich Chemicals) using the method of Seglen[11]. Hepatocyte culture and RB-treatment of cells were done according to our methods previously reported[6-8]. Effects of EGCG on RB-treatment of cells were also examined according to our methods previously reported[6-8]. The used concentration of EGCG and RB were 0.2 mM and 77.1 μM, respectively.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs were isolated from hepatocytes treated with chemicals for 6, 12, 18 and 24 h using Sepasol-RNA I Super (Nacalai tesque, Kyoto, Japan). Then, reverse transcription (RT) of 1 μg of total RNA was performed using TaKaRa RNA PCR Kit (AMV) Ver. 2.1 (Takara, Shiga, Japan) and oligo-dT primers. Obtained cDNAs were subjected to PCR using TaKaRa Ex Taq™ (Takara) and corresponding gene primers. The used gene primers were designed based on the information in gene bank database, and listed in Table 1. PCR was performed 30 cycles (annealing temperature, 62 ℃).

The amount of PCR products was quatified densitometrically using Scion Image software (version Beta 4.0.2, Frederick, MD) after agarose gel electrophoresis.

Results and discussion

In our previous report[6], we found that EGCG protects hepatocytes from RB-induced apoptosis by the suppresson of the activation of caspase 3. Further detailed mechanism of RB-induced apoptosis, however, has not been clarified yet. It can be considered that the mechanism of RB-induced apoptosis

Table 1. List of gene primers used in RT-PCR experiments

Gene	Sequence
p38(sense)	5'-CCATCATTCACGCCAAAAGGTC-3'
p38(antisense)	5'-ATGATGCAGCCCACGGACCAAATA-3'
caspase-3(sense)	5'-GGAAGATCACAGCAAAAGGAGC-3'
caspase-3(antisense)	5'-CACGGGATCTGTTTCTTTGCATGG-3'
caspase-8(sense)	5'-TCGTAGAGATGGAGAAGAGGAC-3'
caspase-8(antisense)	5'-TTGCCCTTGTGGTCCTTGCTTT-3'
MKK6(sense)	5'-TAATAGCCAGGAGCAGAAACGG-3'
MKK6(antisense)	5'-CGGCTCTTCTACCACCTGTTTT-3'
ß-actin(sense)	5'-CATCCCCCAAAGTTCTAC-3'
ß-actin(antisense)	5'-CCAAAGCCTTCATACATC-3'

is also understood through the analysis of hepatoprotective mechanism of EGCG against RB. In our DNA microarray study using mice dosed RB, the expression level of p38 gene was increased 3-fold in liver (data not shown). This result suggested that p38 could be implicated in RB-induced apoptosis. To understand the detailed mechanism of RB-induced apoptosis, the effect of RB was examined *in vitro*. SB203580 (10 μM) was added to medium cultivating primary hepatocytes together with RB to confirm the participation of p38 in RB-induced apoptosis. After 24 h of the treatment, cell viability was determined as shown in Fig. 1, in which the cell viability of inoculated cells was expressed as 100 %. The treatment of SB203580 alone did not show any cytotoxic effects on hepatocytes. While, in the presence of RB, SB203580 protected cells from RB-induced apoptosis. This result shows that RB-induced cell death occurs via p38 pathway. As shown in Fig. 1, although EGCG did not affect the cell viability, RB-induced apoptosis was efficiently suppressed by addition of EGCG.

To investigate the effect of EGCG on RB cell death signaling pathway including p38, we examined whether EGCG affected the expression of p38, caspase-3 and caspase-8 genes. Fig. 2 (a) shows photograph of agarose-gel electrophoresis of RT-PCR product of p38, caspase-3 and caspase-8 genes at 6 hr after the treatment with RB and EGCG, and Fig. 2 (b) shows the densitogram of (a). As the preliminary test showed that the transcriptional activation of these genes reached maximum at 6 h (data not shown), treatment time with RB and EGCG was determined for 6 h in this study. The values of ordinate axis were standardized using that of house keeping gene (ß-actin). The results suggest that RB induces expression of p38 and caspase-3 genes and EGCG suppresses the expression of these genes. Effect on caspase-8 gene was unclear in this study. We must investigate the effect of EGCG and RB on casoase-8 mRNA expression in future study.

We also examined the expression of mitogen-activated kinase kinase 6 (MKK6), upstream signaling factor of p38 by RT-PCR. Fig. 3 (a) shows photograph of agarose-gel electrophoresis of RT-PCR product of MKK6 and Fig. 3 (b) shows the densitogram of (a). RB induced slightly the expression of MKK6, while EGCG suppressed the expression of MKK6 regardless of the presence of RB.

In conclusion, this study showed that RB induces cell death in primary hepatocytes via p38 pathway. This result is very important information for clarifying the mechanism of RB-induced apoptosis. In primary cultured cells, implication of p38 in RB-induced cell death has not been reported so far. Thus, this is the first report. It should be examined whether this mechanism is also shared among other types of cells. Although protecting mechanism of EGCG against RB remains

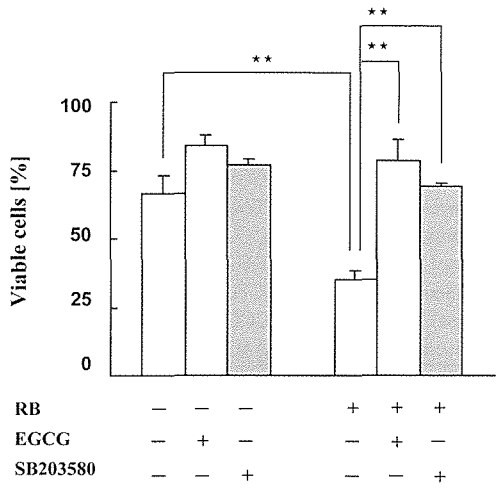

Fig. 1. Effects of SB203580 and EGCG on RB cytotoxicity against primary hepatocytes.
Hepatocytes were treated with 77.1 μM RB in combination with 10 μM SB203580 or 0.2mM EGCG for 24 h.
After 24 h treatment, cell viability was measured by trypan blue exclusion assay. Inoculated cell number (1 X 10^5 cells/cm^2) is expressed as 100 %.
** $p<0.01$

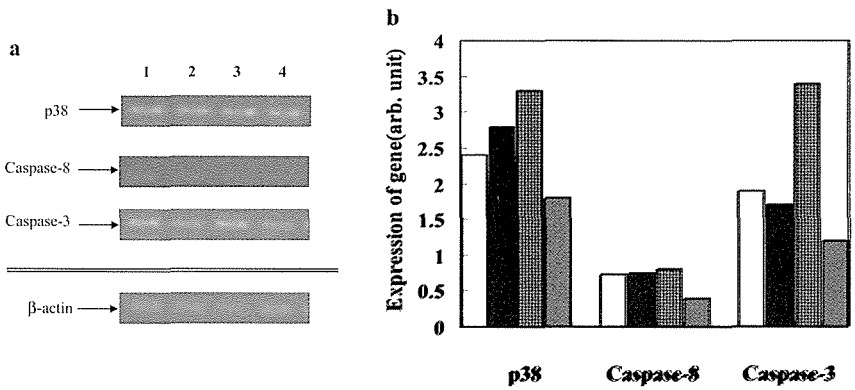

Fig. 2. Effect of RB and EGCG on expression levels of p38, caspase-3 and caspase-8 genes in hepatocytes.
Hepatocytes were treated 77.1 μM RB with or without 0.2 mM EGCG for 6 h and extracted total RNAs. The expression of level of p38, caspase-3 and caspase-8 genes was analyzed by RT-PCR.
(a) is photograph of agarose-gel electrophoresis. Lane 1 corresponds to control, non-treated with both RB and EGCG. Lane 2 corresponds the cell treated with EGCG. Lane 3 corresponds to the cells treated with RB. Lane 4 corresponds to the cells treated with both RB and EGCG.
(b) is the densitogram of (a). Open bars correspond to untreated control, non-treated with both RB and EGCG. Closed bars correspond to the cells treated with EGCG. Lattice bars correspond to the cells treated with RB. Hatched bars correspond to the cells treated with both RB and EGCG.

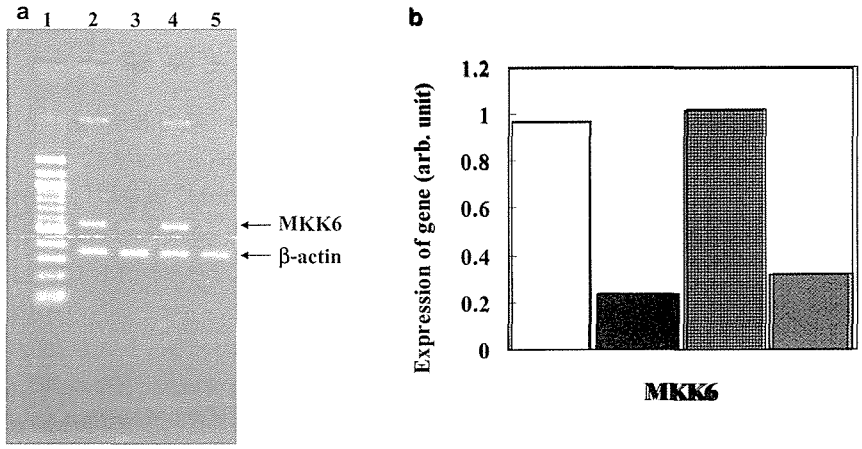

Fig. 3. Expression level of MKK6 gene in hepatocytes simultaneously treated with RB and EGCG.
Hepatocytes were concomitantly treated with RB and EGCG for 6 h and extracted total RNAs. Expression level of MKK6 was analyzed by RT-PCR.
(a) is photograph of agarose-gel electrophoresis. Lane 1 corresponds to 100 base pair ladder marker. Lane 2 corresponds to control, non-treated with both RB and EGCG. Lane 3 corresponds the cell treated with EGCG. Lane 4 corresponds to the cells treated with RB. Lane 5 corresponds to the cells treated with both RB and EGCG.
(b) is the densitogram of (a). Open bar corresponds to control, non-treated with both RB and EGCG. Closed bar corresponds the cell treated with EGCG. Lattice bar corresponds to the cells treated with RB. Hatched bar corresponds to the cells treated with both RB and EGCG.

unclear until now, in this study, we show that EGCG protects hepatocytes from RB toxicity by suppression p38 signaling pathway.

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MAPK p38 経路を介する肝細胞のルブラトキシン B 誘導アポトーシスを茶カテキン，エピガロカテキン-3- ガレート，が阻害する

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エピガロカテキンガレート（EGCG）はラット初代肝細胞においてルブラトキシン B（RB）誘導アポトーシスに対する保護効果を示した．RB と EGCG で肝細胞を処理し，MAPK・p38 とカスパーゼ-3 および－ 8 の発現を RT-PCR 法により調べた．RB により p38 が誘導され，RB 誘導アポトーシスが p38 経路を介することがわかった．一方，EGCG は RB による p38 の発現誘導を抑えた．カスパーゼ 3 についても同様の結果が得られた．また，p38 を活性化する MKK6 についても同様の検討を加えたところ，EGCG により p38 と同じく遺伝子発現が抑えられていた．本研究により，EGCG は p38 経路とカスパーゼの発現に影響を与えることで，RB の毒性より細胞を保護していることが分かった．

キーワード：ルブラトキシン B，EGCG，p38，MKK6