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Induction of the Connexin 32 Gene by Epigallocatechin-3-Gallate Potentiates Vinblastine-Induced Cytotoxicity in Human Renal Carcinoma Cells

Ayami Sato Miki Sekine Minako Kobayashi Nantiga Virgona
Masako Ota Tomohiro Yano

Research Group on Nutritional Sciences, Faculty of Life Sciences, Toyo University, Itakura, Japan

Key Words
Connexin 32 · Epigallocatechin-3-gallate · Epigenetics · Renal cell cancer · Vinblastine

Abstract
Background/Aim: Enforced expression of the connexin (Cx) 32 gene, a member of the gap junction gene family and a tumor suppressor gene in human renal cell carcinoma (RCC), enhanced vinblastine (VBL)-induced cytotoxicity in RCC cells due to suppression of multidrug resistance 1 (MDR1) expression. Furthermore, in RCC the Cx32 gene is silenced by hypermethylation of CpG islands in a promoter region of the Cx gene. In this study, we investigated if the green tea polyphenol epigallocatechin-3-gallate (EGCG) could enhance susceptibility of RCC cells (Caki-1, a human metastatic RCC cell) to VBL. ***Methods:*** The effects of EGCG on Caki-1 cells were estimated by WST-1 (cell viability), real-time RT-PCR (mRNA level) and immunoblotting (protein level). We estimated the methylation status in the promoter region of the Cx32 gene in RCC cells by methylation-specific PCR. Each protein function was inhibited by small interfering RNA (siRNA) and specific inhibitors. ***Results:*** The EGCG treatment elicited significant upregulation of Cx32 in Caki-1 cells, and the induction of the Cx led to the suppression of MDR1 mRNA expression through inactivation of Src and subsequent activation of c-Jun NH2-terminal kinase (JNK). Chemical sensitivity to VBL in Caki-1 cells was increased by EGCG pretreatment, and this effect was abrogated by siRNA-mediated knockdown of Cx32. ***Conclusion:*** This study suggests that the restoration of Cx32 by EGCG pretreatment improves chemical tolerance on VBL in Caki-1 cells via the inactivation of Src and the activation of JNK.

Introduction

Renal cell carcinoma (RCC) is the most common malignant tumor arising in the kidney accounting for 90–95% of renal cancers. In 2013, approximately 60,000 new cases and 13,000 deaths due to renal cancer are expected in the USA [1]. Approximately 30% of the patients will have metastatic disease at the time of diagnosis and most of these patients will have very low rates of long-term survival [1]. Furthermore, metastases will develop in 50% of the patients over the course of the disease [2]. Unfortunately, radiotherapy and available chemotherapeutic agents are ineffective for metastatic RCC and thus it remains a very lethal disease [2, 3]. For instance, vinblastine (VBL) is one of the few cytotoxic agents with reproducible activity in RCC, but the outcome of treatment of metastatic RCC with VBL alone is often disappointing [4, 5]. Other therapies have been tried in response to the poor

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Prof. Tomohiro Yano, PhD
Faculty of Life Sciences, Toyo University
1-1-1 Izumino, Itakura
Oura, Gunma 374-0193 (Japan)
E-Mail ya.tomohiro@gmail.com

results of chemotherapy, such as immunotherapy using interferon-α and interleukin-2, but the therapy has been effective in only a small percentage of patients with metastatic RCC [6]. These deficiencies in chemotherapy outcomes have been ascribed in some part to the multidrug resistance (MDR) exhibited by metastatic RCC. P-glycoprotein (P-gp), the MDR1 gene product, appears to function as an energy-dependent transport pump capable of decreasing the intracellular concentration of a wide range of anticancer agents such as VBL, which confers a chemoresistant phenotype on cancer cells [7]. Since overexpression of P-gp has been found in nearly 80% of RCCs, the chemoresistance of RCC has been linked mainly to P-gp [8]. Also, a recent study has clearly demonstrated that a potent MDR reversal agent improves chemoresistance in breast cancer cells [9]. Thus, P-gp seems to be an attractive target to improve chemotherapy in metastatic RCC [10, 11].

Connexins (Cx), the constituent proteins of gap junctions (GJ), mediate GJ communication, which is instrumental in the control of cell growth and differentiation. Impaired Cx expression or loss of function is associated with the development of cancers [12]. It is well known that Cx acts as a tumor suppressor gene by keeping electrical and metabolic cell homeostasis via GJ-dependent transfer of small molecules <1,500 Da among neighboring cells [13]. In addition to the GJ-dependent mechanism, the Cx gene exerts a tumor-suppressive effect in a GJ-independent manner [14]. As an important function of Cx genes in the GJ-independent mechanism, there is a potentiating effect of Cx on anticancer agent-induced cytotoxicity in cancer cells [15]. It has been reported that enforced expression of the Cx32 gene could enhance VBL-induced cytotoxicity against RCC via the reduction of the P-gp level in a GJ-independent manner [16]. Thus, the current report confirms that restoration of Cx32 gene expression by an adequate approach is an effective procedure to heighten chemical sensitivity to VBL in RCC. Methylation of CpG islands in the 5′ regions of tumor suppressor genes is known to inhibit transcription, leading to silencing of the corresponding genes. We have reported that Cx32 is downregulated in RCC because of hypermethylation of CpG islands in the promoter region [17, 18]. Subsequently, we have confirmed that zebularine and 5-aza-2′-deoxycytidine DNA-demethylating agents induced reexpression of the Cx32 gene in RCC cells in vitro [19] as well as in vivo [20]. Furthermore, we have reported that treatment with DNA-demethylating agents would reduce VBL resistance in RCC due to the restoration of Cx32-dependent tumor-suppressive effects [19]. However, DNA-methylating agents have severe toxic effects [21], so that clinical usage may be limited in some cases.

Recent research on the functional food component epigallocatechin-3-gallate (EGCG), a major and the most active constituent of green tea, has demonstrated the ability to modulate the expression of anticancer genes by reducing DNA methylation [22]. Considering the possibility to improve the overall effectiveness of RCC cancer treatment while minimizing toxicity, we examined whether EGCG could abrogate VBL resistance in RCC cells via the restoration of Cx32-driven tumor-suppressive effects.

Materials and Methods

Materials

All culture reagents were purchased from Invitrogen (Carlsbad, Calif., USA). VBL was obtained from Wako Pure Chemicals (Osaka, Japan). PP2 (an Src inhibitor) and PP3 (a negative control of PP2) were from Biomol (Plymouth Meter, Pa., USA). Nonspecific (NS) small interfering RNA (siRNA) and HP-validated siRNAs for Cx32 (catalog No. SI00003514) and HiPerfect transfection reagent were obtained from Qiagen Japan (Tokyo, Japan). PCR primers were purchased from Sigma Genosys (Hokkaido, Japan). Other chemicals were purchased from Sigma (St. Louis, Mo., USA) unless otherwise noted. Antibody against Cx32 was obtained from Zymed (San Francisco, Calif., USA). All other antibodies were purchased from Cell Signaling Technology (Danvers, Mass., USA).

Cell Culture and Treatment

A representative human metastatic RCC cell line (Caki-1), which was obtained from ATCC (Manassas, Va., USA), was routinely maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2. The cells were plated and treated with EGCG for 24 h, subsequently other chemicals were added to the culture system, and treatment was continued for the indicated times. Since several malignant phenotypes have been ascribed to Caki-1 cells in metastatic RCC, such as chemoresistance, we selected this cell line for the present study.

Cell Viability Assay

Cells were cultured on microtiter plates and treated with each chemical at the indicated doses for the indicated treatment periods. Cell viability was then determined using the cell proliferation assay kit with WST-1 reagent (Sigma) according to the manufacturer's protocol.

P-gp Functional Assay

The functional activity of P-gp in the cells was performed using rhodamine 123 (Rh123) [23]. After the treatment described in the figure legend, the culture medium was removed, Hanks' balanced salt solution (HBSS) containing 10 mM Rh123 was added to each well, and Rh123 was loaded onto the cells by incubation for 6 h at 37°C. After the incubation, cells were washed with HBSS, subsequently lysed with 0.3 M NaOH and neutralized with 0.3 M HCl.

Then, the accumulation of Rh123 in the lysate was measured on a fluorescence spectrophotometer (Hitachi, Ibaraki, Japan) at 492 nm excitation and 522 nm emission. Each value was normalized by cell viability in each group.

Methylation-Specific PCR

The chemical modification of cytosine to uracil by bisulfite treatment is a useful method to study DNA methylation. In this reaction, all cytosines are converted to uracil, but 5′-methylcytosines are resistant to modification and remain as cytosine. The GenElute mammalian genomic DNA kit (Sigma) was used to extract genomic DNA from cell lines as per the manufacturer's protocol. The bisulfite reaction was carried out on genomic DNA as follows: the bisulfite conversion reaction was carried out by incubating DNA with 5 M bisulfite solution and 100 mM hydroquinone, pH 5.0, at 50°C for 4 h. Surplus bisulfite was removed using the QIAEX II gel extraction kit (Qiagen Japan). To estimate the methylation status of the promoter region in the Cx gene, bisulfite-treated genomic DNA was amplified using methylated-specific primers for Cx32 and unmethylated-specific primers for Cx32, as previously reported [17]. The PCR product (245 bp) was electrophoresed on 2% agarose gel and stained with GelStar nucleic acid gel stain (Takara, Shiga, Japan).

Real-Time RT-PCR

Total RNA was isolated using ChargeSwitch® total RNA cell kits (Invitrogen) and cDNA was synthesized as previously described [19]. Real-time RT-PCR was performed using the ABI Prism 7000 sequence detection system (Applied Biosystems Japan Ltd., Tokyo, Japan) and SYBR® Premix Ex Taq™ (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturers' instruction. PCR was carried out using primers MDR1 (NCBI reference No. 42741658): sense primer (nucleotides 2779–2798) and antisense primer (nucleotides 2905–2924), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NCBI 7669491): sense primer (nucleotides 174–193) and antisense primer (nucleotides 313–332). The reaction was performed at 95°C for 10 s, followed by 40 cycles at 95°C for 5 s and then 60°C for 1 min. Abundance of amplified DNA was determined from the threshold cycle values and normalized to the values for the control gene GAPDH to yield the relative abundance. The values of MDR1/GAPDH were normalized to those of control.

Immunoblot Analysis

After each treatment, the cells were lysed in cell lysis/extraction reagent with protease inhibitor cocktail, and phosphatase inhibitor cocktails 1 and 2 (Sigma), and 15 μg protein extract from each sample were loaded onto 10% SDS polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes. The blots were incubated with each primary antibody. Each immunoreactive band was detected with an ECL system (Amersham, Little Chalfont, UK) and the cooled CCD camera-linked cool saver system (Atto, Osaka, Japan). Molecular sizing was performed with the Rainbow molecular weight markers (Amersham). Protein concentration was determined by the DC protein assay system (BioRad, Hercules, Calif., USA). In order to determine the activation or inactivation of each signal molecule, we checked the level of each phosphorylated plus unphosphorylated signal molecule (described as total in the figures) in addition to that of each phosphorylated signal molecule. Also, we confirmed equal loading of each sample, using β-actin as an internal standard.

Transfection of siRNA

Cx32 was downregulated by siRNA targeting Cx32. For transfection, the cells were seeded in a 6-well plate and transfected with HiPerfect transfection reagent according to the manufacturer's protocol. After pretreatment of the cells with siRNA for 24 h, combination treatment of EGCG and siRNA was further continued for 24 h. Then, knockdown of Cx32 by siRNA was confirmed by immunoblot analysis. The MDR1 mRNA level was determined by real-time RT-PCR. As a negative control, NSsiRNA was used.

Src Activity Assay

After the treatment, the cells were lysed and immunoprecitaed with anti-Src antibody as described previously [24]. The immune complex was used as an enzyme source to determine Src activity. The reaction mixture contained Src immune complex, 250 ng poly(Glu4-Tyr) peptide-biotin conjugate, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, 200 μM ATP and 20 mM Tris-HCl buffer (pH 7.4), and the reaction was performed at 37°C for 15 min. Src activity was then analyzed by an ELISA with a tyrosine kinase assay kit (Upstate, Charlottesville, Va., USA) according to the manufacturer's protocol.

Statistical Analysis

Data are expressed as the mean ± SE, and analyzed by one-way analysis of variance followed by Dunnett's t test or Student's t test. $p < 0.05$ was regarded as significant difference.

Results

Effect of EGCG on Cell Growth, Cx32 Expression and Src Activation

To evaluate if EGCG could suppress the growth of Caki-1 cells due to restoration of Cx32 expression, the cells were treated with EGCG. The EGCG treatment significantly suppressed cell growth by 50% compared with the control (fig. 1a), and, similarly, the treatment increased Cx32 levels (fig. 1b). Also, methylation-specific PCR analysis indicated that the treatment caused demethylation of a promoter region in the Cx32 gene (fig. 1c).

In a previous report [25], we have shown that enforced expression of Cx32 has negative growth control of RCC cells via the inactivation of Src. In this study, we showed that silencing of EGCG-induced Cx32 by siRNA treatment increased both cell viability and Src activity (fig. 2). These results indicate that EGCG exerted negative growth control of the cells based on the inactivation of Src by restoration of Cx32 expression.

Effect of EGCG on Chemical Tolerance on VBL in Caki-1 Cells

To test if EGCG could improve chemical tolerance in Caki-1 cells, we evaluated the effect of EGCG on VBL-induced cytotoxicity in Caki-1 cells. The EGCG treat-

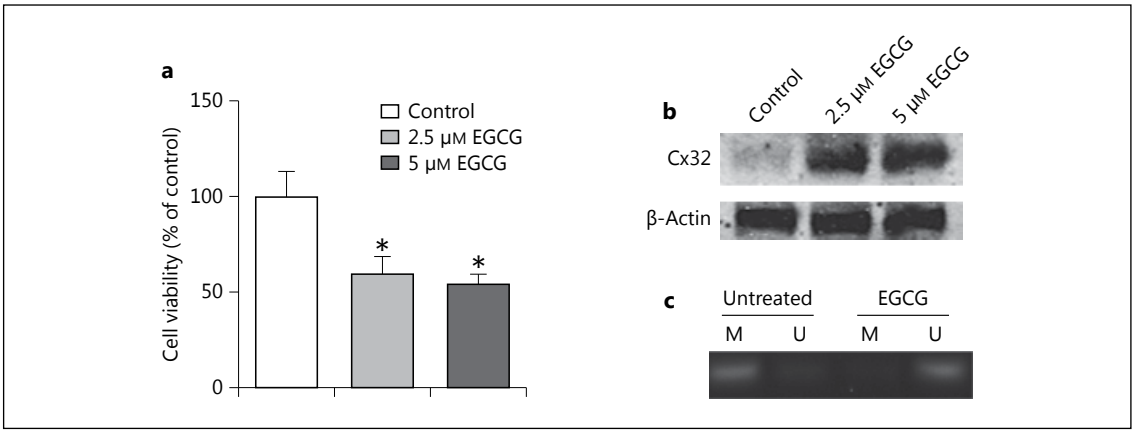

Fig. 1. Effect of EGCG treatment on cell viability and Cx32 level in Caki-1 cells. **a** Caki-1 cells were treated with EGCG at the indicated doses for 48 h, and subsequently cell viability was determined using WST-1 reagent. Each value is the mean of 5 determinations; vertical lines indicate SE. * $p < 0.05$ vs. control. **b** Restoration of the Cx32 protein level in Caki-1 cells after the treatment with EGCG for 48 h. β-Actin was used as an internal control. This result is representative of 2 independent experiments. **c** Methylation-specific PCR of Cx32 in Caki-1 cells. After EGCG treatment (2.5 μM) for 48 h, methylation-specific PCR analysis was performed as described in the Materials and Methods. The presence of a PCR product in M indicates the presence of the methylated Cx32 gene, the presence of a PCR product in U indicates the presence of the unmethylated Cx32 gene. This result is representative of 2 independent experiments.

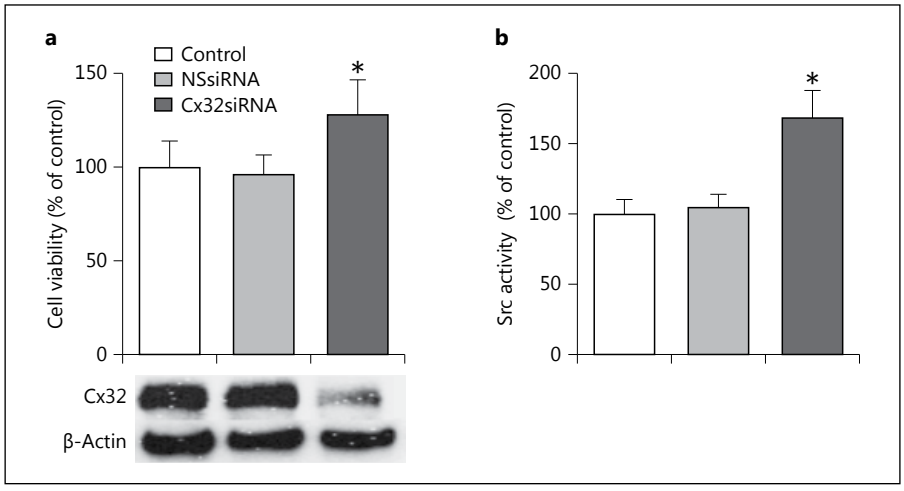

Fig. 2. Effect of Cx32 siRNA treatment on cell viability and Src activity in Caki-1 cells treated with EGCG. Caki-1 cells were treated with Cx32siRNA and EGCG as described in the Materials and Methods. **a** Cell viability was measured by the WST-1 method and Cx32 protein level was determined by immunoblot analysis. **b** Src activity was determined by an ELISA method. Each value in cell viability and Src activity is the mean of 5 determinants; vertical lines indicate SE. * $p < 0.05$ vs. control. The Cx32 protein level result is representative of 2 independent experiments.

ment significantly potentiated the VBL-induced cytotoxicity in Caki-1 cells, and this effect showed a dose dependency (fig. 3a). However, VBL treatment alone showed a slight reduction in the cytotoxicity only at the maximum dose (20 μM; fig. 3a).

Because resistance of VBL to RCC closely relates to the level of P-gp [9], we evaluated whether restoration of Cx32 by EGCG could affect the function of P-gp in Caki-1 cells. As shown in figure 3b, verapamil (VPL, a specific inhibitor of P-gp) significantly potentiated VBL-induced cytotoxicity in Caki-1 cells. Also, EGCG treatment significantly reduced the MDR1 mRNA level, and silencing of Cx32 by siRNA treatment restored the EGCG-reduced MDR1 mRNA level (fig. 3c, d). Finally, we confirmed that EGCG treatment suppressed P-gp function (fig. 3e). These results suggest that the EGCG treatment suppressed P-gp function mainly via the induction of Cx32.

Effect of EGCG on the Signal Pathway Regulating MDR1 mRNA Level in Caki-1 Cells

Src and c-Jun N-terminal kinase (JNK) have been documented to be major factors to regulate the MDR1 gene

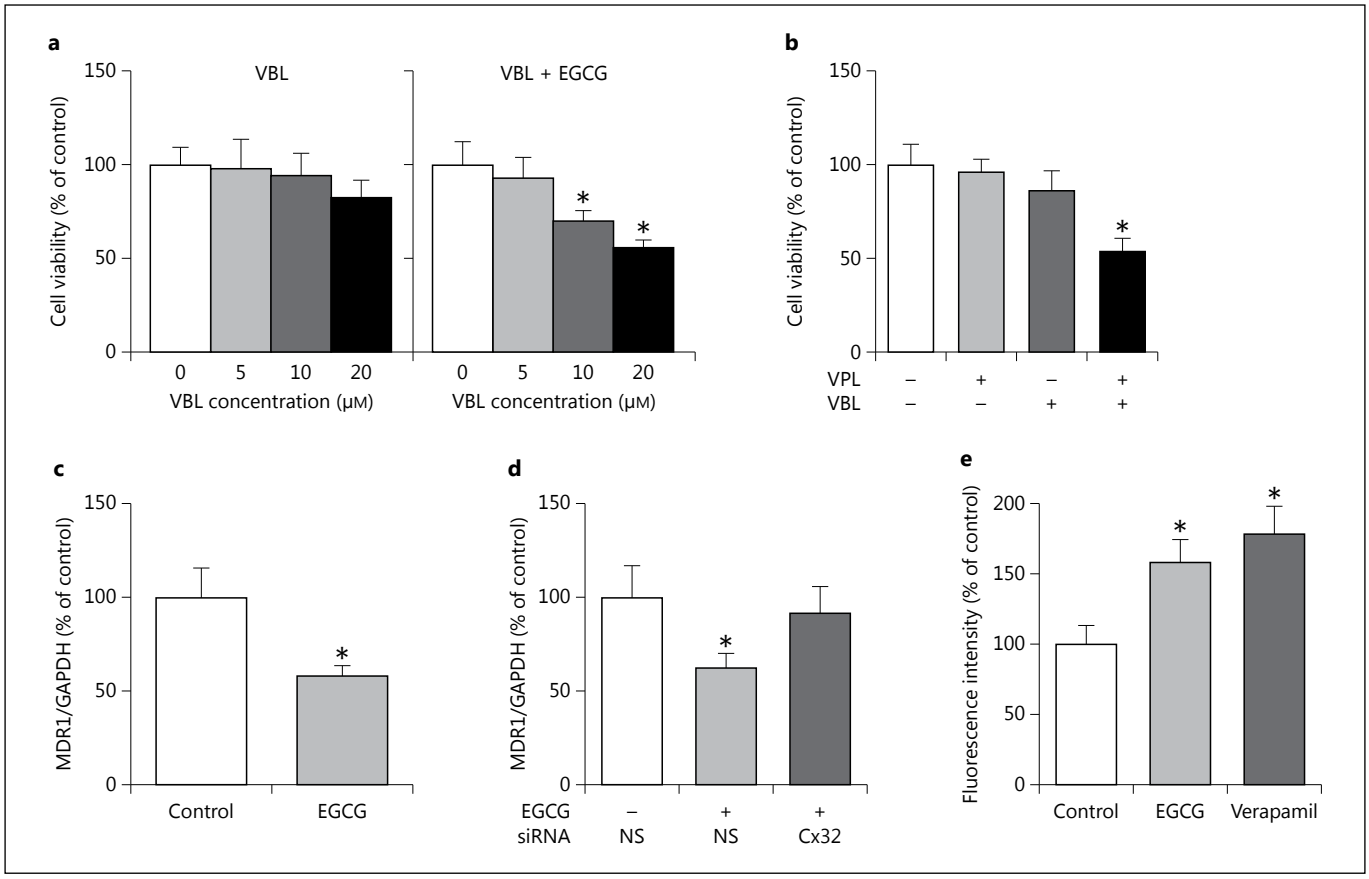

Fig. 3. Upregulation of Cx32 by EGCG enhances VBL-induced cytotoxicity in Caki-1 cells via the inhibition of P-gp. Each value is the mean of 5 determinations; vertical lines indicate SE. * $p < 0.05$ vs. control. **a**, **b** Cell viability was determined by WST-1 assay. **a** The cells were treated with 2.5 μM EGCG for 48 h and subsequently with VBL at the indicated doses for 48 h. **b** The cells were treated with 20 μM VBL and/or 10 μM VPL for 48 h. **c** The cells were treated with 2.5 μM EGCG for 48 h, and subsequently MDR1 mRNA level was determined by real-time RT-PCR. **d** After the cells were treated with siRNA and/or 2.5 μM EGCG for 48 h, the MDR1 mRNA level was measured by real-time RT-PCR. **e** After the treatment with 2.5 μM EGCG or 10 μM VPL for 48 h, Rh123 was loaded onto the cells as described in the Materials and Methods. Then, Rh123 accumulation in the cells was determined from each fluorescence density.

expression [26]. Hence, we determined whether EGCG treatment could regulate the MDR1 mRNA level due to the alteration of two molecules through the upregulation of Cx32. PP2 (an Src inhibitor) significantly reduced the MDR1 mRNA level in Caki-1 cells (fig. 4a), and PP3 (a negative control of PP2) did not affect the mRNA level (data not shown), indicating that Src is required for the induction of the MDR1 gene. Next, we proceeded to confirm the contribution of JNK activation to the suppression of the MDR1 mRNA level. As shown in fig. 4b, EGCG as well as PP2 induced the activation of JNK. Also, we showed that SP600125 (a JNK inhibitor) mostly abrogated the suppression of the MDR1 mRNA level by EGCG (fig. 4c). Also, we confirmed that Cx32siRNA caused the inactivation of JNK in Caki-1 cells treated with EGCG (data not shown). These results suggest that upregulation of Cx32 by EGCG suppressed MDR1 mRNA due to the inactivation of Src and the activation of JNK in Caki-1 cells.

Discussion

Epigenetic gene regulation has been recognized to play a role in the etiology of cancer. DNA methylation is one of the important epigenetic events in the regulation of gene expression and maintenance of cellular function. Abnormal DNA methylation is a characteristic of cancer and often mediates silencing of genes, particularly tumor suppressors, leading to cancer development and progres-

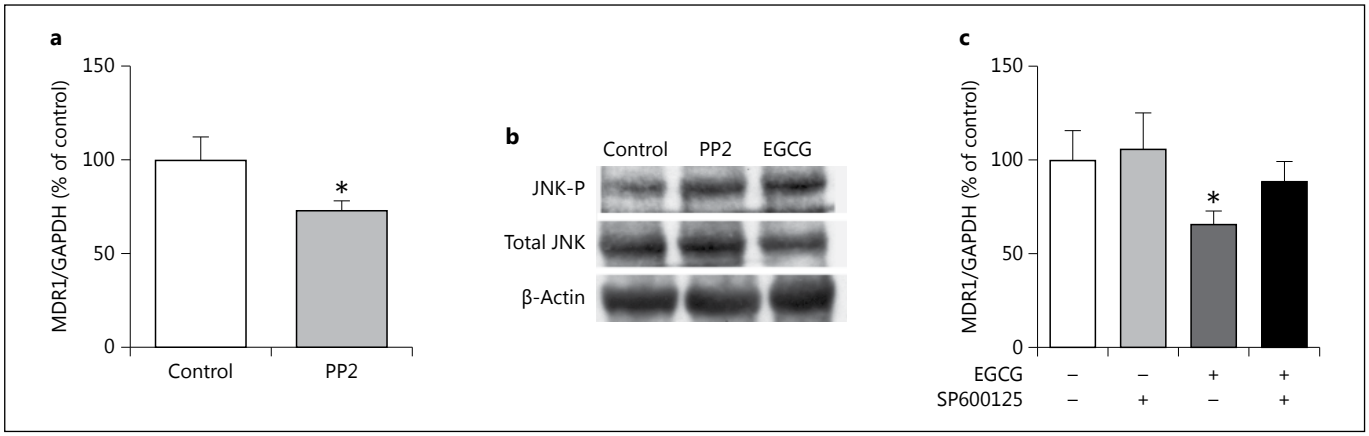

Fig. 4. EGCG-induced JNK activation contributes to the reduction in the MDR1 mRNA level in Caki-1 cells. **a** The cells were treated with 0.1 μM PP2 for 24 h and subsequently the MDR1 mRNA level was determined by real-time RT-PCR. **b** The cells were treated with 0.1 μM PP2 or 2.5 μM EGCG for 24 h and subsequently the activation of JNK was determined by immunoblot analysis. This result is representative of 2 independent experiments. JNK-P = Phosphorylated JNK. **c** The cells were treated with 2.5 μM EGCG for 24 h and subsequently 0.1 μM SP600125 for 24 h. After the treatment, the MDR1 mRNA level wad measured by real-time RT-PCR. **a**, **c** Each value is the mean of 5 determinations; vertical lines indicate SE. * $p < 0.05$ vs. control.

sion. Cx32, a member of the GJ protein, has been documented to be a tumor suppressor gene in tissue in which it is expressed and the loss of Cx function by mutation or deletion of DNA has been found to be a rare event in cancers [27]. In our previous studies, we have reported that Cx32 acts as a tumor suppressor gene in RCC, and loss of Cx32 function in RCC depends on methylation of the promoter regions [17]. Demethylation of the promoter regions by DNA methyltransferase inhibitors (decitabine and zebularine) can restore the Cx32 gene expression in RCC cells in vivo [20] as well as in vitro [19], although at the expense of severe toxicity against nontumorigenic cells. Alternatively, a natural ingredient, EGCG, is known to act as a DNA-demethylating agent [28] which might facilitate upregulation of Cx32, which can be induced at noncytotoxic levels against nontumorigenic cells.

In this study, we observed that EGCG treatment enhanced the cytotoxic effect of VBL in Caki-1 cells and that the restoration of Cx32 by EGCG closely related to the enhancing effect of EGCG using siRNA for Cx32. These results indicated that the combination of VBL and EGCG could be effective to regulate RCC. Furthermore, the treatment dose of EGCG (2.5 μM) was almost equal to the maxium upper level observed in human serum [29]. Also, with respect to the uptake of EGCG into cells, a recent report showed that the agent was incorporated into the cytoplasm of the cells and further translocated into the nucleus in a time-dependent manner [30]. These reports indicate that EGCG can actually inhibit DNA methylation in cancer cells in vivo. Thus, this combination strategy of VBL and EGCG may be effective in future clinical usage.

Next, in order to clarify the mechanism of the combination, we examined what critical event was related to the enhancing effect of Cx32. Since P-gp, an adenosine triphosphate-driven efflux pump for VBL, is in part a critical factor to determine resistance of RCC against this chemotherapeutic agent [31], we estimated the contribution of this molecule to the enhancing effect of VBL in Caki-1 cells. The result showed that P-gp function as well as its mRNA level were significantly reduced by EGCG treatment. As mentioned above, overexpression of P-gp closely relates to the appearance of resistance in cancer cells against VBL, so the reduction in P-gp function by EGCG may contribute to the enhancement of VBL-induced cytotoxicity. Also, knockout of EGCG-induced Cx32 by siRNA restored MDR1 mRNA levels. These results suggest that the restoration of Cx32 by EGCG potentiates the VBL-induced cytotoxicity in Caki-1 cells via the suppression of P-gp function.

Identification of activated signaling pathways in RCC has been one of many approaches to cancer therapy. The non-tyrosine kinase Src is known to have a role in signal transduction of multiple oncogenic cellular processes. Enforced expression of Cx32 has previously been shown to suppress Src activation and that the inactivation of Src by the Cx mainly contributes to the tumor-suppressive effects in Caki-1 cells [25]. Also, earlier reports have sug-

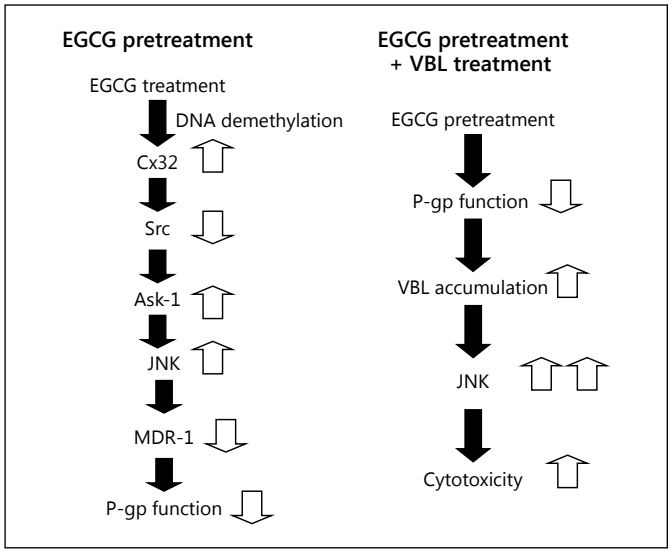

Fig. 5. Proposed mechanism of a possible scheme for how the restoration of Cx32 by EGCG treatment improves chemical tolerance on VBL in Caki-1 cells.

gested that Src indirectly regulates the level of P-gp [32]. These reports show a possibility that EGCG-induced expression of Cx32 could reduce the MDR mRNA level via the inactivation of Src signaling. The present experiment demonstrated that EGCG reduced the MDR mRNA level and P-gp function, and that the decreased MDR mRNA level was mostly recovered under knockdown of Cx32 by siRNA. Additionally, PP2 (an inhibitor of Src) significantly suppressed the MDR1 mRNA level. These observations completely support the above possibility.

Of molecules located in downstream Src signaling, we can identify JNK as a candidate regulating the expression of MDR1 due to the following results: (1) the inactivation of Src can induce the inactivation of Akt [33]; (2) the inactivation of Akt leads to release the suppression of Ask1 activity [34]; (3) the increase in Ask1 activity causes the activation of JNK [35], and (4) the enhancement of JNK activity contributes to the reduction in the P-gp level [36]. Actually, in this study, we observed that EGCG treatment as well as PP2 induced the activation of JNK. Also, we confirmed that the induction of the MDR1 gene by EGCG was mostly cancelled by the inhibition of JNK activity using JNK inhibitor SP600125. These results indicate that JNK can negatively regulate the level of P-gp in Caki-1 cells. Additionally, the activation of JNK-dependent apoptotic signaling mainly contributes to VBL-induced cytotoxicity in several types of cancer [37]. As described, pretreatment with EGCG before VBL administration suppressed P-gp function via Cx32-mediated activation of JNK signaling. Overall, we can speculate a possible scheme how the restoration of Cx32 by EGCG treatment improves chemical tolerance to VBL in Caki-1 cells: the restoration of Cx32 by EGCG pretreatment induces the inactivation of Src and subsequently suppresses P-gp function via the inactivation of JNK. As a consequence, the reduction in P-gp function finally enhances VBL-induced cytotoxicity in Caki-1 cells. This schema is shown in figure 5. In order to further establish the usefulness of the combination strategy in RCC therapy, we should confirm the effectiveness of this strategy in xenotransplant models as well as in other RCC cell lines.

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