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Anti-HCV activity of the Chinese medicinal fungus *Cordyceps militaris*

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ABSTRACT

Persistent hepatitis C virus (HCV) infection causes chronic liver diseases and is a global health problem. Although the sustained virologic response rate in the treatment of genotype 1 using new triple therapy (pegylated-interferon, ribavirin, and telaprevir/boceprevir) has been improved by more than 70%, several severe side effects such as skin rash/ageusia and advanced anemia have become a problem. Under these circumstances, a new type of anti-HCV oral drug with few side effects is needed. Our recently developed HCV drug assay systems, including the HuH-7 cell line-derived OR6 and AH1R, and the Li23 cell line-derived ORL8 and ORL11, allow genome-length HCV RNAs (several strains of genotype 1b) encoding renilla luciferase to replicate efficiently. Using these systems as anti-HCV candidates, we have identified numerous existing medicines that can be used against HCV with few side effects, such as statins and teprenon. To obtain additional anti-HCV candidates, we evaluated a number of oral health supplements, and found that the capsule but not the liquid form of *Cordyceps militaris* (CM) (*Ascomycotinanorth, North Chinese caterpillar fungus*), which is used as a Chinese herbal medicine, exhibited moderate anti-HCV activity. In combination with interferon-α or ribavirin, CM exhibited an additive inhibitory effect. Among the main components of CM, cordycepin, but not ergosterol, contributed to the anti-HCV activity of CM. In consideration of all these results, we suggest that CM would be useful as an oral anti-HCV agent in combination with interferon-α and/or ribavirin.

1. Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma. Since approximately 170 million people are infected with HCV worldwide, HCV infection is a serious global health problem [1]. HCV is an enveloped virus with a positive single-stranded RNA of the *Flaviviridae* family. The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acids, which is cleaved into 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [2,3].

Recently, a new therapy for hepatitis C (genotype 1) with a combination of pegylated-interferon (PEG-IFN), ribavirin (RBV), and telaprevir/boceprevir (inhibitor of HCV NS3-4A protease) has been started as a global standard therapy [4]. Although the sustained virological response (SVR) in this therapy has improved approximately 70–80% [5], this therapy has several problems, such as severe side effects (skin rash, ageusia, advanced anemia, etc.), emergence of resistant viruses, and high treatment cost [5,6].

Although cells derived from the human hepatoma cell line HuH-7 have been used as the preferred culture system for the study of HCV life cycles and for the development of anti-HCV drugs [7], we previously found a new human hepatoma cell line, Li23, that enables reproducibility of the HCV life cycle [8]. Using the Li23 cell line, we developed Li23-derived drug assay systems (ORL8 and ORL11) in which a genome-length HCV RNA (the O strain of genotype 1b derived from an HCV-positive healthy carrier) encoding renilla luciferase (RL) replicates efficiently [8], based on a method previously reported in the development of a HuH-7-derived drug assay system (OR6) [9]. Since we demonstrated that the gene expression profile of Li23 cells was distinct from that in HuH-7 cells [10], and that the anti-HCV targets in Li23-derived cells (ORL8 and ORL11) were distinct from those in HuH-7-derived cells (OR6 and AH1R, which was developed using the AH1 strain of genotype 1b) [11–14], we considered that we might find a new type of anti-HCV agent by conducting a search using these two kinds of cell-based HCV RNA-replication assay systems. Indeed, we recently found that the preclinical antimalarial drugs N-89 and N-251 [15,16] exhibited potent anti-HCV activities [17].

Here, we report the further discovery that an oral health supplement used as a Chinese herbal medicine, *Cordyceps militaris*

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(CM) (*Ascomycotinanorth*, *North Chinese caterpillar fungus*), exhibited moderate anti-HCV activity.

2. Materials and methods

2.1. Cell cultures

HuH-7-derived OR6 [9] and AH1R [12] cells harboring genome-length HCV RNA encoding RL and HuH-7-derived polyclonal sOR [18] cells harboring subgenomic HCV replicon RNA encoding RL were cultured in the medium used for HuH-7 cells in the presence of G418 (0.3 mg/ml; Geneticin, Invitrogen, Carlsbad, CA) as described previously [17]. Li23-derived ORL8 [8] cells harboring genome-length HCV RNA encoding RL and Li23-derived polyclonal sORL8 [8] cells harboring subgenomic HCV replicon RNA encoding RL were also cultured in the medium used for Li23 cells in the presence of G418 (0.3 mg/ml) as described previously [8].

2.2. Reagents

The capsule and liquid forms of CM were purchased from CAITAC (Okayama, Japan). RBV was kindly provided by Yamasa (Chiba, Japan). Human IFN-α and vitamin E (VE) were purchased from Sigma-Aldrich (St. Louis, MO). Cordycepin was purchased from Wako (Osaka, Japan). Ergosterol and cyclosporine A (CsA) were purchased from Tokyo Chemical Industry (Tokyo, Japan).

2.3. RL assay

The RL assay was performed as described previously [8,14]. Briefly, the cells were plated onto 24-well plates (2×10^4 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to luciferase assay using an RL assay system (Promega, Madison, WI). From the assay results, the 50% effective concentration (EC_{50}) of each reagent was determined.

2.4. WST-1 cell proliferation assay

The WST-1 cell proliferation assay was performed as described previously [14]. Briefly, the cells were plated onto 96-well plates (1×10^3 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. From the assay results, the 50% cytotoxic concentration (CC_{50}) of each reagent was determined.

2.5. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting analysis were performed as described previously [19]. The antibodies used in this study were those against HCV Core (CP11; Institute of Immunology, Tokyo, Japan), NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan), and β-actin (AC-15; Sigma–Aldrich) as the control for the amount of protein loaded per lane.

2.6. Selective index (SI)

The SI value of each reagent was determined by dividing the CC_{50} value by the EC_{50} value.

2.7. Statistical analysis

Determination of the significance of differences among groups was assessed using the Student's *t*-test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. The capsule form of CM, used as an oral health supplement, showed anti-HCV activity in both HuH-7- and Li23-derived HCV RNA-replicating cells

During the course of evaluating various oral health supplements for their anti-HCV activities using our previously developed HuH-7- and Li23-derived HCV assay systems, there was an opportunity to evaluate CM known as one of the Chinese herbal medicine. We first evaluated the anti-HCV activities of the capsule and liquid forms of CM using HuH-7-derived OR6 and AH1R assay systems and an Li23-derived ORL8 assay system, all of which enable monitoring of the replication of genome-length HCV RNA. The results revealed that the capsule form but not the liquid form of CM possessed moderate anti-HCV activities in all assay systems (Fig. 1A and B). The EC_{50} and SI values of the capsule form of CM were calculated in each assay system (EC_{50} 62 μg/ml, SI 1.9 in the OR6 assay; EC_{50} 54 μg/ml, SI >5.6 in the ORL8 assay; EC_{50} 31 μg/ml, SI 5.2 in the AH1R assay) (Table 1). The anti-HCV activities of the capsule form of CM found in the OR6, ORL8, and AH1R assays were confirmed by Western blot analysis of the HCV Core and NS5B (Fig. 1C). We next examined the activities of the capsule form of CM using HuH-7-derived polyclonal sOR and Li23-derived polyclonal sORL8 assay systems that enable monitoring of the replication of HCV subgenomic replicon RNA. These assays also showed that the capsule form of CM possessed anti-HCV activity with EC_{50} values less than those in the OR6 and ORL8 assays (Supplementary Fig. S1 and Table 1). Taken together, these results indicate that the anti-HCV activity of CM is not dependent on the specific cloned cell line, HCV strain, or HCV structural proteins.

3.2. Additive effect of the anti-HCV activities of CM in combination with IFN-α or RBV

To determine the intake effect of CM in the current HCV treatment, we examined the anti-HCV activity of the capsule form of CM in combination with IFN-α or RBV using an Li23-derived ORL8 assay system. The results revealed that the anti-HCV effects of CM plus IFN-α or RBV were additive (Fig. 2A and B). Although we observed that the anti-HCV activities of CM in combination with 4 IU/ml of IFN-α or 25 μM of RBV were greater than the expected sum of the constituent activities, these differences were not statistically significant (Fig. 2A and B). Therefore, these results suggest that the anti-HCV effects of CM do not interfere with those of IFN-α and RBV, and in fact may even augment them.

3.3. Cordycepin, but not ergsterol, is responsible for the anti-HCV activity of CM

We next examined which component of CM is responsible for the anti-HCV activity. The Japanese Food Research Laboratories (Tokyo, Japan) have reported that the main components of CM are as follows: β-glucan 8.40 g, cordycepin 4.95 g, mannitol 4.52 g, ergosterol 0.75 g, superoxide dismutase 860,000 U, copper 2.13 mg, zinc 17.1 mg, and selenium 80 μg per 100 g of CM (http://www.caitac.co.jp/matsubaratouchukasou/syouhin.html). From this information, we speculated that cordycepin or ergosterol might have anti-HCV activity (Fig. 3A). Thus, we evaluated

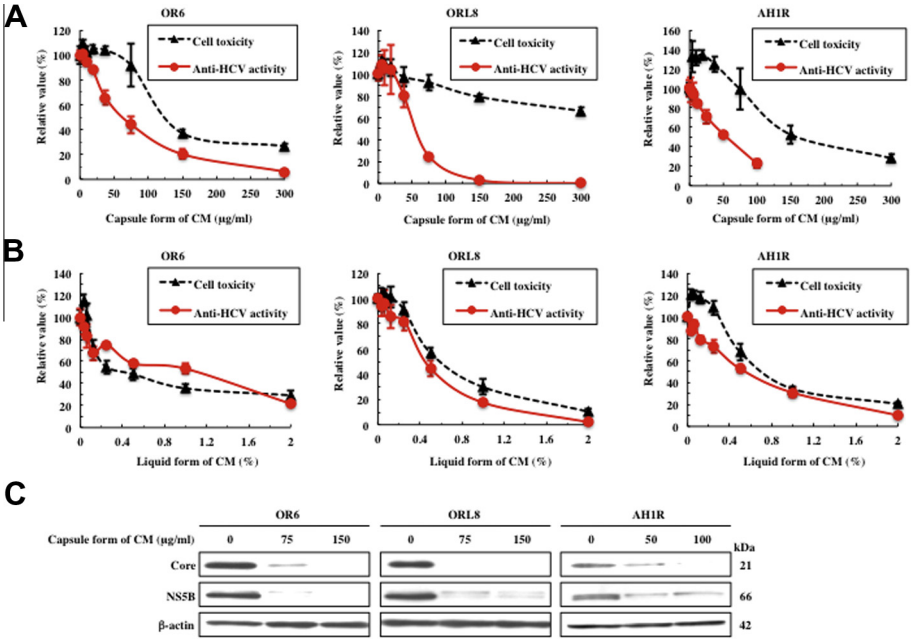

Fig. 1. Anti-HCV activities of the capsule form of CM detected in the OR6, ORL8, and AH1R assays. (A) Effects of the capsule form of CM on genome-length HCV RNA replication. OR6, ORL8, and AH1R cells were treated with the capsule form of CM for 72 h, followed by RL assay (red circles) and WST-1 assay (black triangles). The relative value (%) calculated at each point, when the level in non-treated cells was assigned as 100%, is presented here. Data are expressed as the means ± standard deviation of triplicate assays. (B) The liquid form of CM did not inhibit the genome-length HCV RNA replication. The RL and WST-1 assay were performed as described in (A). (C) Western blot analysis of the cells treated with the capsule form of CM. HCV Core and NS5B were detected using anti-core and anti-NS5B antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Anti-HCV activities of 4 reagents evaluated in this study.

Assay system	Genome-length HCV RNA									HCV subgenomic replicon					
Cell origin	OR6 HuH-7			ORL8 Li23			AH1R HuH-7			sOR HuH-7			sORL8 Li23		
HCV strain	O			O			AH1			O			O		
Compound (concentration)	EC_{50}	CC_{50}	SI	EC_{50}	CC_{50}	SI	EC_{50}	CC_{50}	SI	EC_{50}	CC_{50}	SI	EC_{50}	CC_{50}	SI
Capsule form of CM (μg/ml)	62	120	1.9	54	>300	>5.6	31	160	5.2	12	45	3.8	30	120	4.0
Liquid form of CM (%)	1.1	0.44	0.40	0.54	0.70	1.3	0.45	0.59	1.3	ND			ND		
Cordycepin (μg/ml)	2.6	3.5	1.3	3.8	3.6	0.95	0.58	1.9	3.3	1.7	3.0	1.8	21	19	0.90
Ergosterol (μg/ml)	>4.0	>4.0	<1.0	>4.0	>4.0	<1.0	>4.0	>4.0	<1.0	>4.0	>4.0	<1.0	>4.0	>4.0	<1.0

ND, not determined.

cordycepin and ergosterol using HuH-7-derived OR6 and AH1R assay systems. The results of both assays revealed that cordycepin, but not ergosterol, possessed anti-HCV activity (Fig. 3B and C). The EC_{50} and SI values of cordycepin were calculated in each assay (EC_{50} 2.6 μg/ml, SI 1.3 in the OR6 assay; EC_{50} 0.58 μg/ml, SI 3.3 in the AH1R assay) (Table 1). If all of the anti-HCV activity of CM was attributable to cordycepin (4.95% of content), the EC_{50} values, 62 and 31 μg/ml of CM, obtained by OR6 assay and AH1R assay would correspond to 3.0 and 1.6 μg/ml of cordycepin, respectively. Therefore, these results suggest that cordycepin is an integral component for the anti-HCV activity of CM. However, we were not able to confirm the anti-HCV activity of cordycepin in the Li23-derived ORL8 or sORL8 assay, although we did detect anti-HCV activity of cordycepin in the HuH-7-derived sOR assay (Supplementary Fig. S2 and Table 1). Ergosterol did not exhibit any anti-HCV activities in these assays (Supplementary Fig. S3 and Table 1). Taken together, these results suggest that cordycepin is a responsible compound for the anti-HCV activity of CM, although the anti-HCV activity of cordycepin may depend on the cell strain used in the assay, unlike the anti-HCV activity of CM.

4. Discussion

In the present study, using cell-based HCV RNA-replication assay systems, we found that CM, an oral health supplement, possessed moderate anti-HCV activity, and showed an additive inhibitory effect in combination with IFN-α or RBV. Furthermore, we identified cordycepin as a responsible component for the anti-HCV activity of CM.

It is interesting that the liquid form did not show any anti-HCV activity, while the capsule form did. Because cordycepin is probably present in both CM formulations, cordycepin may be unstable in the liquid preparation, or compounds that inhibit the anti-HCV activity of cordycepin may also be present in the liquid formulation. Therefore, the anti-HCV activity of CM may depend on the formulation method.

The molecular mechanism underlying the anti-HCV activity of CM is also interesting. Since cordycepin was found to be a responsible component for the anti-HCV activity of CM and cordycepin is known to be an analog of nucleoside, we can estimate that cordycepin inhibits the RNA-dependent RNA polymerase (NS5B) of HCV. Previously, we reported that anti-HCV agents could be

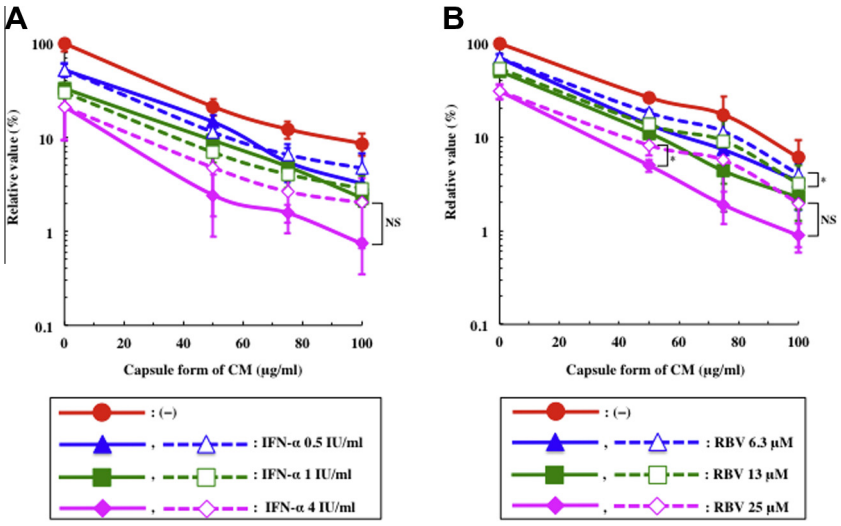

Fig. 2. Additive inhibitory effects of the capsule form of CM when used in combination with IFN-α or RBV on HCV RNA replication in ORL8 cells. Open symbols in the broken lines show the values expected as an additive anti-HCV effect and closed symbols in the solid lines show the values obtained by the ORL8 assay. ORL8 cells were treated with the capsule form of CM in combination with IFN-α (A) or RBV (B) for 72 h and subjected to RL assay. $^{*}P < 0.05$; NS, not significant.

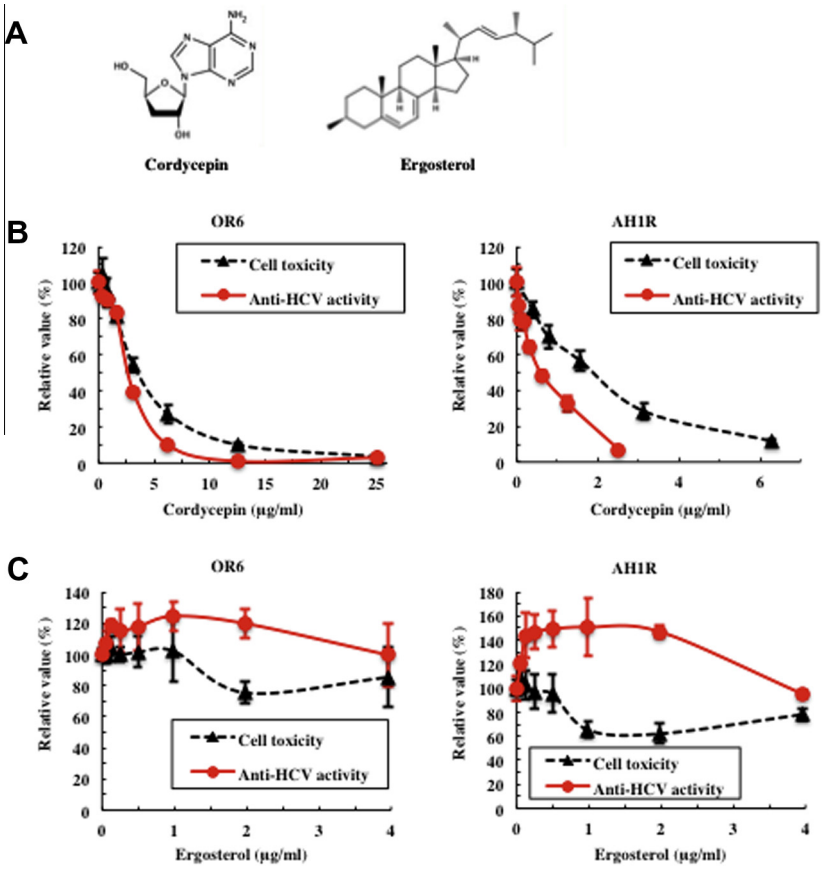

Fig. 3. Cordycepin is a responsible compound for anti-HCV activity of CM. (A) Structures of cordycepin and ergosterol. (B) Effect of cordycepin on genome-length HCV RNA replication. The RL and WST-1 assays using OR6 and AH1R cells were performed as described in Fig. 1A. (C) Ergosterol did not inhibit the genome-length HCV RNA replication. The RL and WST-1 assays using OR6 and AH1R cells were performed as described in Fig. 1A.

classified into two types: those whose anti-HCV activity is canceled by the antioxidant VE, and those whose activity is not canceled by VE [20]. To date, we have reported that CsA, N-251 (preclinical antimalarial drug), β-carotene, vitamin D2, and linoleic acid belong to the former group, and IFN-α, IFN-β, RBV, and statins belong to the latter [11,17,20]. We currently speculate that the oxidative stress induced by the former anti-HCV agents causes the anti-HCV activity via activation of the extracellular signal-regulated kinase signaling pathway [21]. Therefore, using the ORL8 assay system, we evaluated which group CM belonged to, and determined that the anti-HCV activity of CM was not canceled by VE, whereas the anti-HCV activity of CsA was completely cancelled by VE (Supplementary Fig. S4). These results suggest that the induction of oxidative stress is not associated with the anti-HCV

activity of CM, and support our initial estimation that cordycepin is a responsible component for the anti-HCV activity of CM and directly inhibits the NS5B polymerase.

In this study, we identified cordycepin as a responsible component of CM for the anti-HCV activity, because the EC_{50} value of cordycepin was comparable to the concentration calculated from the content of the capsule form of CM. However, the cell toxicity of cordycepin was stronger than that of the capsule form of CM. For example, in AH1R cells, the CC_{50} value of cordycepin was 1.9 μg/ml, whereas the value of the capsule type of CM was 160 μg/ml (equivalent to 7.9 μg/ml of cordycepin) (Table 1). Cordycepin is a promising preclinical drug that exhibits anti-tumor activities both *in vitro* and *in vivo* [22]. Since the cell lines that we established and applied to the HCV assay (OR6, ORL8, AH1R, etc.) were derived from HuH-7 or Li23 hepatoma cells, the obtained low CC_{50} values of cordycepin would be reasonable. Therefore, the high CC_{50} values obtained for the capsule form of CM are notable, and would seem to suggest that CM in the capsule form contains certain components that reduce the cytotoxicity of cordycepin. For this reason, we anticipate that the capsule form of CM will be useful as an oral supplement for the treatment of HCV with a minimal side effect profile.

In conclusion, we found that capsule form of CM, which is used as an oral health supplement, exhibited a moderate inhibitory effect on HCV RNA replication. This agent would therefore be useful as an additional component in an existing therapeutic regimen using HCV-specific inhibitors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.150.

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