Anticytoproliferative effect of Vitamin C on rat hepatic stellate cell

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Abstract: This study was conducted to investigate the potential therapeutical benefit of Vitamin (VC), a potent antioxidant, on suppressing proliferation of immortalized rat liver stellate cell line (HSC-T6) in vitro, and to discuss the underlying mechanism. HSC-T6 was co-treated with different concentrations of VC (50, 100, 200 μmol/L) on designed time points. Then, cell viability was assessed by using MTT analysis, and the changes of cytomorphology was observed with apoptosis-specific TUNEL and immunohistochemical stains, as well as the intracellular target genes was determined by using RT-PCR, respectively. As the outcomes, VC-treated HSC-T6 showed significantly inhibited cell growth in a dose-dependent manner when compared to the vehicle control. Cytologically, VC increased TUNEL-labeled positive cells in cultured HSC-T6, which the cell count was greater than vehicle control. Meanwhile, VC-treated HSC-T6 showed elevated immunoreactive for TGF-β1-labeled cells. Moreover, VC contributed to down-regulated expressions of intracellular c-myc, cyclin D1, mTOR mRNAs in HSC-T6. Collectively, these preliminary findings have demonstrated that VC-mediated anti-proliferative effect on HSCs is involved in molecular mechanisms of promoting apoptosis and blocking endogenous collagenation.

Keywords: Vitamin C, hepatic stellate cells, proliferation, collagenation

Introduction

Hepatofibrosis, also called liver fibrosis, is featured with plenty of depositional extracellular matrix that deposits in diseased liver, resulting in liver injury and dysfunction [1]. Pathologically, over-activation of hepatic stellate cells (HSCs) is a major culprit that impels liver fibrosis development via secreting collagen, in which its excess proliferation is responsible for forming scar tissue in response to hepatic impairment [2, 3]. As an invasive cell hallmark of fibrosis, normalization of HSCs mass in the liver can be regarded as a strategy for managing hepatofibrosis [4]. In clinical application, a majority of chemotherapy for hepatopathy is accompanied with undesired side effects [5]. Thus, seeking a new treatment substitute to control HSCs over-growth seems to be essential. Interestingly, Vitamin C (VC) serves as a vital antioxidant against oxidative stress. Mounting studies indicate that VC supplementation contributes to potential health benefits, such as preventing cancers, reducing the risk of cardiovascular diseases, slowing the progression of chronic diseases [6-8]. In our previous studies, VC supplementation shows pronounced hepatoprotective actions against chemical-damaged and immuno-deficient livers in mice via regulating intracellular signal pathways [9-11]. Therefore, we hypothesize that VC may contribute to block development of liver fibrosis, even to affect HSCs. In the present study, HSC-T6 was used to investigate the inhibitory effects on cell proliferation mediated by VC, and the underlying molecular mechanism would be further discussed.

Material and methodology

Materials

Rat HSC-T6 cell line was received as a gift from Prof. RB Huang of Guangxi Medical University.
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Commercially available VC was purchased from Succhi Shiqi Pharmaceutical Co. Ltd. (Guangdong, China). VC stock solution was prepared as 200 μmol/ml before delivery of treatment. Reagents and kits used were listed in the experimental sections, respectively.

Cells culturing and VC treating

HSC-T6 cells were cultured in DMEM-high glucose medium contained 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, within a incubator of 5% CO₂ at 37°C for cell growth.

MTT test

Once the cells grown stably, passaged cells were seeded in a 96-well plates with a density of 1 × 10⁵ cells/1 ml per well. After 24 h later, the cells were exposed to serial dilution of VC solutions (50, 100, 200 μmol/L) for 20 h. Subsequently, 20 μl MTT (Sigma, USA) buffer was added to each well for further 4 h incubation within a incubator. All the media was removed, and 100 μl DMSO was added in each well under gentle shaking for 10 min. The solution was collected for determining photometric analysis at 570/690 nm by using a spectrophotometer (INESA Instrument Co., Ltd., Shanghai). The final data were computed via the following formula:

\[
\text{Inhibitory rate (\%)} = \left(\frac{\text{OD}_{\text{blank}} - \text{OD}_{\text{treatment}}}{\text{OD}_{\text{blank}}} \times 100\%\right)
\]

TUNEL analysis

HSC-T6 cells were fixed with freshly-prepared 4% paraformaldehyde (pH 7.4) for 15 min at room temperature. When being washing with PBS for two times, the cells were post-fixed in ethanol/acetic acid (2:1, v/v) for permeabilization. After rinsing twice, the cells was added on equilibration buffer before immediately exposing to working strength TdT enzyme (Roche, Germany) for 1 h, followed by incubation with stop solution for 10 min. Anti-digoxigenen conjugate was added and placed in light-proof humidified chamber for 30 min, followed by sealed coverslip, imaged and calculated steps, respectively.

Immunohistochemical staining

Treated cells were attached to slice before immunostaining. The sections were subjected to different levels of xylene/ethanol (30%, 75%, 90%, 100%) for 10 min each time. After washing with PBST twice, 5% BSA blocking buffer was exposed to the samples for 1 h. Subsequently, goat-anti TGF-β1 antibody (1:500, Boster, Wuhan, China) was added on the slices for 1 h at 37°C, followed by rabbit-anti-goat secondary antibody (1:1000, Boster, Wuhan, China). After rinsing with PBST twice, a SABC kit (Boster, Wuhan, China) was used to bind substrate, and DAB was applied in chromogen before hematoxylin counterstaining on nuclei. Finally, the slices were mounted, imaged and calculated, respectively.

Real time-PCR assay

Fresh total RNAs were separated from HSC-T6 cells by using a commercially available Trizol reagent (Beyotime Biotechnology, China). All RNAs’ purity was verified under a 260 nm spectrophotometer. cDNA transcription from 500 ng RNA was manipulated by using a second strand cDNA synthesis kit (Beyotime Biotechnology, China) according to the manufacturer’s manual. The primers were designed by a biotechnology company (TianGEN Biotech, Shanghai, China), as shown in: c-myc forward primer, 5’ GAA ACG GCG AGA ACA GTT GA 3’, antisense primer: 5’ CCA AGG TTG TGA GGT TGA GCA GC 3’ (170 bp); cyclin D1 forward primer, 5’ ACC CTG ACA CCA TCC TTC TC 3’, antisense primer: 5’ CCT CGC AGA CCT CTA GCA T 3’ (159 bp); mTOR forward primer, 5’ CGC ATC ATT CAC CCG...
ATT GT 3’, antisense primer: 5’ TCT TCA TCA GCA AGC GTG TA 3’ (218 bp); GAPDH sense primer: 5’ GCA AGT TCA ACG GCA CAG T 3’, antisense primer: 5’ GCC AGT AGA CTC CAC GAC AT 3’ (140 bp). Briefly, PCR reaction system was included in 40 cycles, stepped by prenaturation for 3 min at 95°C, denaturation for 3 s at 95°C, annealing 45°C for 30 s, and elongation at 68°C for 1 min. As a result, data from each group was measured by normalizing to the β-actin following the 2−ΔΔCt format.

Statistical analysis

The final data were yielded by using the SPSS 16.0 software (Chicago, IL, USA). Differences between two groups were assessed through a one-way analysis of variance (ANOVA) with Bonferroni post tests for comparisons. Results were represented as mean ± SD. A P<0.05 was considered significantly.

Results

VC inhibited cell proliferation of HSC-T6

As displayed in MTT data, the inhibitory effect of VC treatments on HSC-T6 was statistically significant compared to vehicle control (P<0.05), in which the antiproliferative action was related to dose-dependent manner. If untreated, the HSC-T6 cells resulted in rapid growth with increased counts (Figure 1).

VC induced apoptosis of HSC-T6

The images from representative immunofluorescence staining showed that VC-treated HSC-T6 contributed to elevated TUNEL-labeled cells, in which the apoptotic counts were gradually increased following stepwise VC doses, in which proapoptotic outcomes were more greater than that of untreated control (P<0.05) (Figure 2).

VC reduced TGF-β1 expression of HSC-T6

As shown in histochemical stain, untreated HSC-T6 cells contained numerous TGF-β1 immunoreactivity in cytoplasm. Instead, the TGF-β1 immunoactive cells were progressively decreased after VC treatments, in which the counts were less than that of vehicle control (P<0.05) (Figure 3).

VC modulated target genes expression in HSC-T6

At gene levels, RT-PCR data exhibited that intracellular c-myc, cyclin D1, mTOR mRNAs in HSCs maintained in relative low expressions. Interestingly, VC treatments resulted in down-
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regulation of these gene expressions when compared to vehicle control \( (P<0.05) \) (Figure 4). The effective trend showed dose-dependently.

Discussion

Etiologically, over-growth of hepatic stellate cell (HSC) is responsible for development of fibrogenesis, eventually resulting in liver failure [12]. HSC regeneration causes hepatocellular structure changes, particularly in fibrosis or cirrhosis. In addition, uncontrolled proliferative HSC leads to excessive extracellular matrix (ECM) deposition that can affect functional liver cells [13, 14]. Alternatively, suppression of HCS over-activation may be an applicable measure for fibrogenic management in the liver. In this study, the MTT outcome showed that inhibitory effects of VC on HSC-T6 were significant, with a dose-dependent tendency. This preliminary data indicated antiproliferative role mediated by VC was one of the underlying mechanisms in vitro. In methodology, TUNEL represents a common technique for screening DNA fragmentation that triggers apoptotic cascade events. As a result, VC resulted in increased TUNEL-positive cells in HSC-T6. Interestingly, the cytological observations of VC-mediated proapoptosis were accordance with MTT-reflected antiproliferative effect.

TGF-β1 refers to a multifunctional cytokine that regulates cell growth, differentiation, and apoptosis [15]. Recently, increasing evidences suggest that TGF-β signaling induces fibrotic response via activating EMT deposition in the liver [16]. More specifically, TGF-β1 is crucial for HSC development and division, and therefore suppression of TGF-β1 signaling in HSC might effectively reverse hepatic fibrosis [17, 18]. Here we extrapolated VC-mediated anti-prolif-

Figure 3. VC lowered TGF-β1 expression of HSC-T6 (immunohistochemical stain, scale bar: 200 μm). Brown-stained cytoplasm represented TGF-β1 positive cells. The averaged data were produced from three independent tests. Results were analyzed by one-way ANOVA followed by Bonferroni post tests for comparisons, and final data were expressed as the Mean ± SD. Notes: vs. vehicle control, \( *P<0.05 \).

Figure 4. VC regulated target genes expression in HSC-T6 (RT-PCR assay). The averaged data were produced from three independent tests. Results were analyzed by one-way ANOVA followed by Bonferroni post tests for comparisons, and final data were expressed as the Mean ± SD. Notes: vs. vehicle control, \( *P<0.05 \).
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C-Myc is an important target effector of Wnt mitogenic signal, which its capability is to induce cell growth, proliferation and apoptosis [19]. As a downstream component of c-Myc pathway, amplification and overexpression of cyclin-D1 can cause cell cycle changes [20]. Mammalian target of rapamycin (mTOR) exerts the various functions of controlling cell growth and proliferation in response to mitogenic signals [21]. Based on these specific characteristics of apoptosis-regulatory molecules, they will assist to understand the further mechanism of VC-associated anti-proliferation against HSC. As a consequence, VC-treated HSC-T6 contributed to down-regulation of intracellular c-myc, cyclin D1, mTOR mRNAs in a dose-dependent manner, in which these outcomes were consistent with the ameliorations as mentioned above. These evidences illuminated the molecular mechanisms that VC promoted apoptosis in HSC-T6 via synergetic regulation of c-myc/cyclin D1 and mTOR pathways.

Taken together, our primary findings in cell model suggest that VC may be a potential candidate for managing liver fibrosis. However, further study in vivo should be conducted prior to making clinical evaluation.

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Disclosure of conflict of interest

None.

Abbreviations

VC, Vitamin C; HSC-T6, mmortalized rat liver stellate cell line; MTT, 4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; TGF-β1, transforming growth factor beta1; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; RT-PCR, reverse transcription polymerase chain reaction; mTOR, mammalian target of rapamycin; HSCs, hepatic stellate cells; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; ANOVA, one-way analysis of variance; ECM, extracellular matrix.

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References

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