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Anti-cancer mechanisms of clinically acceptable colchicine concentrations on hepatocellular carcinoma

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ABSTRACT

Aims: This study was to investigate whether the clinically acceptable colchicine concentrations had anti-cancer effects on hepatocellular carcinoma (HCC) and their anti-cancer mechanisms.
Main methods: Two human HCC cell lines (HCC24/KMUH, HCC38/KMUH) and two human cancer-associated fibroblast (CAF) cell lines (F28/KMUH, F59/KMUH) were investigated by proliferative assay, microarray, quantitative reverse transcriptase-polymerase chain reaction, and nude mouse study using clinically acceptable colchicine concentrations.
Key findings: Both 2 and 6 ng/mL colchicine significantly inhibited the cellular proliferation of all cell lines tested ($P < 0.05$). The anti-proliferative effects of colchicine on F28/KMUH, HCC24/KMUH and HCC38/KMUH cells were dose-dependent. The anti-proliferative effects of 6 ng/mL colchicine on both HCC cell lines were similar to the effects of 1 μg/mL epirubicin. The anti-proliferative effects of colchicine on HCC cells could be partially explained by dose-dependent up-regulations of 2 anti-proliferative genes (*AKAP12*, *TGFB2*) in these cells. *TGFB2* was also up-regulated in CAFs but was not dose-dependent. Up-regulation of *MX1* which can accelerate cell death was a common effect of 6 ng/mL colchicine on both CAF cell lines, but 2 ng/mL colchicine down-regulated *MX1* in F28/KMUH cells. Nude mouse (BALB/c-nu) experiment showed that colchicine-treated mice (0.07 mg colchicine/kg/day × 14 days) had lower increased tumor volume ratios, slower tumor growth rates and larger percentages of tumor necrotic areas than control mice (all $P < 0.05$).
Significance: Clinically acceptable colchicine concentrations have anti-cancer effects on HCC. This drug has potential for the palliative treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer. Sorafenib is the approved molecular inhibitor for the treatment of advanced HCC. Despite the successful approval and extensive application of sorafenib, the prognosis for patients with advanced HCC remains poor and the benefits with sorafenib are modest (Xie et al., 2012; Zhu, 2012). On the other hand, the tumor microenvironment plays active roles in determining the malignant phenotype. Cancer-associated fibroblast (CAF) is one of the most crucial components of the tumor microenvironment to promote the growth and invasion of cancer cells (Lin et al., 2012; Mazzocca et al., 2011; Yang et al., 2011). Therefore, the strategy for the developing new molecularly targeted agents for the treatment of advanced HCC should focus on both cancer cells and CAFs.

The defining characteristics of cancer cells are a significantly increased rate of mitosis which means that cancer cells are significantly more vulnerable to mitotic poison than are normal cells. Microtubules have long been considered as an ideal target for anti-cancer drugs because of their essential roles in mitosis and forming the dynamic spindle apparatus. Colchicine is an alkaloid agent which has been used in medicine for a long time (Cocco et al., 2010; Finkelstein et al., 2010; Imazio et al., 2005, 2009; Kallinich et al., 2007). Colchicine is a microtubule destabilizer which has very strong binding capacity to tubulin to perturb the assembly dynamics of microtubules (Bhattacharyya et al., 2008; Lu et al., 2012; Stanton et al., 2011; Sivakumar, 2013). Colchicine also can increase cellular free tubulin to limit mitochondrial metabolism in cancer cells through inhibition of the voltage-dependent anion channels of the mitochondrial membrane (Maldonado et al., 2010). Although the clinical application of colchicine has been limited because of its toxicity, oral colchicine is a safe treatment when appropriately used and contraindications have been excluded (Cocco et al., 2010;

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Finkelstein et al., 2010; Imazio et al., 2005, 2009; Kallinich et al., 2007). A retrospective cohort study showed that long-term colchicine administration in patients with viral hepatitis-related cirrhosis could prevent and delay the development of HCC without significant side effects (Arrieta et al., 2006). This implies that colchicine may have potential as an anti-cancer drug for HCC. The peak plasma concentrations after oral administration of 0.6 to1 mg colchicine range from around 2 to 6 ng/mL (Ferron et al., 1996; Rochdi et al., 1994; Terkeltaub et al., 2010). The lowest reported lethal doses of oral colchicine are 7–26 mg and acute ingestions of colchicine exceeding 0.5 mg/kg have a high fatality rate (Finkelstein et al., 2010). The purpose of this study was to investigate whether the clinically acceptable colchicine concentrations had anti-cancer effects on HCC and their anti-cancer mechanisms. All gene names are according to the official symbols from the HUGO Gene Nomenclature Committee provided by the US National Center for Biotechnology Information.

Materials and methods

Cell lines

Two human HCC cell lines (HCC24/KMUH, HCC38/KMUH) and two CAF cell lines (F28/KMUH, F59/KMUH) originated from the fine-needle aspirated specimens of HCC tumor were investigated. Among these cell lines, F59/KMUH CAF cell line was newly established from HCC tumor in our institution. The remaining cell lines had been used in our previous studies (Lin et al., 2009, 2012). The detailed methodology for the establishment of these cell lines was described in our previous study (Lin et al., 2006). The F59/KMUH CAF cell line was verified by positive stain for fibroblast activation protein (ENZO Life Sciences International, Inc., Butler Pike, Plymouth Meeting, PA, USA), α-smooth muscle actin (Sigma-Aldrich, St. Louis, Mo., USA) and CXCL12 (R & D Systems, Inc., Minneapolis, MN, USA). All procedures to establish these cell lines were approved by the Institutional Review Board of our hospital and patients were given informed consent. All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The serum-containing culture medium consisted of 10% fetal bovine serum, 90% DME/HIGH glucose, supplemented with 20 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin (HyClone, Logan, Utah, USA). Colchicine and epirubicin were purchased from Sigma-Aldrich (St. Louis, Mo., USA).

Proliferation experiment

Each cell line seeded in 96-well culture plate was incubated with serum-containing medium for 24 h. Then the medium was replaced with serum-free medium with various concentrations of colchicine (0, 2, 6 ng/mL). The cells were incubated for further 72 h for proliferation assay. To compare the anti-proliferative effects of colchicine with epirubicin on HCC cells, the same procedure was repeated but the testing drugs were changed to 1 μg/mL epirubicin and 6 ng/mL colchicine. The premixed WST-1 cell proliferation reagent (Clontech Laboratories, Inc., A Takara Bio Company, Mountain View, CA, USA) was applied. The experimental procedures were carried out following the manufacturer's protocols. The cells were incubated with reagent for 3 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. After then, the results were analyzed by an automated microplate reader (MRX, Dynex Technologies, Inc., Chantilly, VA, USA). The absorbance was measured at 450 nm wavelength (reference wavelength 630 nm). In all experiments, 16 replicate wells were used for statistical calculation.

Microarray and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) experiments

Each cell line was seeded in three 25-cm^2 plastic culture flasks with serum-containing medium for 24 h. Then the medium was replaced with serum-free medium with various concentrations of colchicine (0, 2, 6 ng/mL). The cells were incubated for further 24 h. Then total RNA in each flask was extracted by Trizol® Reagent (Invitrogen™, Life Technologies Corporation, NY, USA), and followed by RNAeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). Purified RNA was quantified by OD260 nm by a ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE, USA) and qualified by Bioanalyser 2100 (Agilent Technology, Santa Clara, CA, USA). To investigate genes with consistently differential expression caused by different concentrations of colchicine, three microarrays (Agilent SurePrint G3 Human GE 8 × 60 k, Agilent Technologies, Santa Clara, CA, USA) were applied for F28/KMUH CAFs (6 ng/mL colchicine vs. control 2 ng/mL colchicine), HCC24/KMUH cells (2 ng/mL colchicine vs. control without colchicine) and HCC38/KMUH cells (6 ng/mL colchicine vs. control 2 ng/mL colchicine) respectively. Microarray experimental procedures were carried out following the manufacturer's protocols and the criteria for the selection of differentially expressed genes were the same as in our previous studies (Lin et al., 2009, 2012). For quantitative RT-PCR study, specific oligonucleotide primer pairs were selected from Roche Universal ProbeLibrary (Roche Diagnostics Ltd. Taipei, Taiwan). The procedures for real-time PCR reactions and the calculation of the fold expression or repression of the target gene were the same as in our previous studies (Lin et al., 2009, 2012). The housekeeping gene *TBP* (TATA box binding protein) was used as a reference gene. Three genes were selected for quantitative RT-PCR study. The PCR primers used were 5′-CCTCTCTATGG CAGGAAGACATTC-3′ sense primer and 5′-ATGGAATCGCAACTGTGAT GGC-3′ anti-sense primer for *AKAP12*, 5′-TGGCGGGATTGAAGGATGCTG-3′ sense primer and 5′-GCAAGGTGGAGCGATTCTGAG-3′ anti-sense primer for *MX1*, 5′-CTTTCTGGGGTTGGTTGTTTGGG-3′ sense primer and 5′-GAGGAGTCTGGTCTTGTAGGTAGC-3′ anti-sense primer for *TGFB2*, and 5′-CAATTTAGTAGTTATGAGCCAGAG-3′ sense primer and 5′-TTCTGCTCT GACTTTAGCAC-3′ anti-sense primer for *TBP*.

Nude mouse experiment

This study was approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University. All mice were kept in the Experimental Animal Center of our hospital. The feeding process was carried out by a qualified staff using a syringe connected with a feeder. Sixteen male nude mice (BALB/c-nu) purchased from The Taiwan Laboratory Animal Center of National Health Research Institutes were equally divided into A and B two groups. A mixture of 3.75×10^7 HCC38/KMUH cancer cells and 3×10^4 F28/KMUH CAFs suspended in 0.2 mL phosphate-buffered saline (HyClone, Logan, Utah, USA) was injected subcutaneously into the flank of each mouse in group A. The nude mice in group B received the same amount of cancer cells but the CAFs were changed to the equal amount of F59/KMUH CAFs. When tumors reached a diameter of 4–5 mm in largest dimension, four mice in each group were randomly assigned to treatment group and the remaining mice were assigned to control group. Each mouse in treatment group was continuously fed with 0.07 mg colchicine/kg dissolved in phosphate-buffered saline once per day for 14 days. All mice were sacrificed at the 15th day after the start of colchicine treatment. The tumor volumes were calculated by the formula: length × $width^2$ × 0.5 (Wu et al., 2012). The increased tumor volume ratio was calculated as following: tumor volume at day x (Vx) divided by baseline pretreatment tumor volume (V_0). The tumor growth rates were calculated by the formula: $\ln(V_0 - V_{14}) / (t_0 - t_{14})$, where V_0 and V_{14} are tumor volumes at the start of treatment and at the 14th day of treatment respectively and the t is the day for the measurement of tumor volume (Mehrara et al., 2007). All tumors were immediately fixed in 24% formalin after sacrifice of mice. After then, all tumors were embedded in paraffin within 24 h for further pathological study. Serial sections at a distance of 3 mm for all tumors were performed to detect the percentage of tumor necrosis and microvascular density.