MOLECULAR STUDIES ON GROWTH ARREST AND APOPTOSIS INDUCED BY ANDROGRAPHOLIDE IN HUMAN HEPATOMA HEPG2 CELLS

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穿心莲内酯诱导人肝癌细胞 HepG2 的生长抑制和凋亡的分子学研究

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ABSTRACT

Cell cycle regulation and apoptosis are two important control mechanisms for the proliferation of cells. These two processes are cooperatively interlinked so that tissue homeostasis and normal development of organisms could be effectively maintained. Loss of cell cycle control or apoptosis will result in many diseases, and cancer is somewhat one of the most jeopardous examples. Hepatocellular carcinoma (HCC) is a liver cancer prevalent in Asia, especially in the Mainland China and in Hong Kong. The major modalities of treatment for HCC include liver transportation, chemotherapy and radiation, all of which have some limitations. Nowadays, chemotherapy involving the use of Traditional Chinese Medicines (TCMs), however, deserves exploration for the little side effects.

Andrographolide (Andro) is the main bioactive component of *Andrographis paniculata* (AP; Traditional Chinese: 穿心蓮; pinyin: Chuan Xin Lian) and has been reported to inhibit the proliferation of some tumor cell lines. However, there is no report about the anticancer effect of Andro to HCC cells although it has been claimed to be hepatoprotective and immunostimulative, both of which are supposed to be beneficial to normal liver cells.

This study reports for the first time that Andro is the most potent cytotoxic component among five analogues to two HCC cell lines. The IC50 to human hepatoma HepG2 cells was 40.2 ± 9.6 μM and to rat hepatoma H4IIE cells was 66.2 ± 14.6 μM after 48 h of treatment. Compared to its cytotoxicity to normal liver L02 cells (IC50 at 48 h was 135.1 ± 30.7 μM), Andro was found preferential targeting to tumor cells. When cell cycle and apoptotic study was monitored, it was found HepG2 cells was arrested at gap 2/mitosis (G2/M) phase of the cell cycle that subsequently

led to a late apoptosis. Fragmentation of chromosomal DNA was observed if the cells were treated with 200 μM Andro for 48 h or with 50 μM Andro for a prolonged period of 96 h. Analysis of apoptotic signal indicated that caspase-3 was activated during the process. Flow cytometric analysis of the treated cell after fluorescein isothiocyanate (FITC)-phospho-histone H3 plus propidium iodide (PI) staining revealed that the mitotic index increased from 5.5 to 49.1%, which was about 10 times of the control. As the G2/M phase cells were increased from 17% to 62.7%, these results mean that the ratio of cells at interphase G2 changed little upon Andro exposure while the arrest took place mainly at mitosis.

Immunoblotting analysis showed that upon Andro treatment, the tumor suppressor p53 (also known as a nuclear transcription factor), was up-regulated to almost 2-fold of the control. The activation of p53 subsequently turns on the transcription of the target Bax, responsible for causing apoptosis. Western blot analysis also showed that phosphorylated form of $p34^{cdc2}$ at Thr161 was up-regulated to 3-fold of the control. This activation was confirmed by the enhanced expression of Cdk7, a cyclin-dependent kinase which is in control of the Thr161 phosphorylation after combined with Cyclin H. Because Thr161-phosphorylated $p34^{cdc2}$ can form a complex named M-phase promoting factor (MPF) with Cyclin B and trigger cells pass through the G2/M checkpoint, this means that cells could enter into mitosis after Andro treatment. During mitosis, there is another cell cycle checkpoint, the metaphase-to-anaphase transition. At this transition, the MPF activates another complex - the anaphase-promoting complex (APC), which destroys Cyclin B reciprocally. From our result, the Cyclin B level was determined stable after Andro treatment, indicative of inactivation of APC thus cells could not pass through this transition and were arrested at metaphase. Studies with synchronized cells revealed

that the cells arrest at G2 phase were more sensitive to Andro treatment, followed by M phase cells. These results confirmed our hypothesis that upon Andro treatment, cells pass through the G2/M checkpoint and accumulate at M phase, during which apoptosis occurs.

Many previous studies indicated that the hepatoprotective effect of Andro was mainly due to the antioxidant effects. But in this study, we found that Andro caused the overproduction of hydrogen peroxide, a main form of reactive oxygen species (ROS) in HepG2 hepatoma cells. On the contrary, the level of superoxide radicals was decreased upon Andro treatment in both time- and concentration-dependent manners. When some well-known antioxidants were added prior to Andro treatment, it was found that N-acetylcysteine (NAC, a non-toxic precursor of GSH), catalase (an enzyme that degrading hydrogen peroxide) or reduced glutathione (GSH) inhibited the cytotoxic effect of Andro. Through an in vitro test, we observed that Andro could rapidly scavenge superoxide radicals generated by a KO_2 system. Cellular SOD activity was enhanced after Andro treatment, indicating that Andro may has some SOD-like activity. Because the GSH system contains the glutathione peroxidase which is one of the most important hydrogen peroxide-removing enzymes in mammalian mitochondria, the elevated level of hydrogen peroxide implies that GSH depletion might take place by Andro in the cells. Therefore, the intracellular GSH level was determined and it was found that the GSH system was destroyed by glutathione depletion after the addition of Andro. This depletion was confirmed by that Andro could react directly with GSH under certain conditions. When a GSH peroxidase (Gpx) mimicking antioxidant named ebselen was added prior to Andro, the cytotoxicity was also greatly attenuated.

Changes in the redox status after the addition of Andro also coupled with the

collapse of the mitochondrial membrane potential (MMP). This MMP loss, however, could be eliminated by NAC. On the other hand, the overproduction of hydrogen peroxide could not be attenuated by cyclosporine A (CsA), a well-known mitochondrial permeability transition pore (MPTP) inhibitor. Taken all the data present, it indicates that Andro treatment triggered changes in a redox signaling pathway via mitochondrial depolarization. Induction of cell cycle arrest at M phase was the main cause of growth inhibition and eventually led cells into apoptosis.

Finally, the redox variations of hepatoma HepG2 cells and normal liver L02 cells after Andro treatment was investigated. Less redox change was noted in L02 cells. Our results thus suggest that a redox signaling pathway played an important role in the cytotoxic effect of Andro to tumor cell and provide some valuable knowledge in the understanding of cell-type specific response to this drug.

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