Inhibitory effect of all-trans retinoic acid on human hepatocellular carcinoma cell proliferation

Yun-Feng Piao, Yang Shi, Pu-Jun Gao

Yun-Feng Piao, Yang Shi, Pu-Jun Gao, Department of Gastroenterology, the First Hospital of Jilin University, Changchun, 130021, Jilin Province, China
Correspondence to: Dr. Yang Shi, Department of Gastroenterology, the First Hospital of Jilin University, No.1 Xinmin Road, Changchun, 130021, Jilin Province, China. shiyangwhy@163.com
Telephone: +86-431-5612242  Fax: +86-431-5612542
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Abstract
AIM: To study the inhibitory effect of all-trans retinoic acid on human hepatocellular carcinoma cell line SMMC-7721 and to explore the mechanism of its effect.

METHODS: SMMC-7721 cells were divided into two groups, one treated with all-trans retinoic acid (ATRA) for 5 days and the other as a control group. Light microscope and electron microscope were used to observe the morphological changes. Telomerase activity was analyzed with silver-stained telomere repeated assay protocol (TRAP). Expression of Caspase-3 was demonstrated with western blot.

RESULTS: ATRA-treated cells showed differentiation features including small and pyknotic nuclei, densely stained chromatin and fewer microvilli. Besides, ATRA could inhibit the activity of telomerase, promote the expression of Caspase-3 and its activation.

CONCLUSION: Telomerase activity and Caspase-3 expression are changed in human hepatocellular carcinoma cell line SMMC-7721 treated with all-trans retinoic acid. The inhibition of telomerase activity and the activation of Caspase-3 may be the key steps through which ATRA inhibits the proliferation of SMMC-7721 cell line.

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INTRODUCTION
Several isomers have been found for retinoic acid (RA), an oxidative product of vitamin A, including all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9C-RA). ATRA has been used successfully in treatment of acute promyelocytic leukemia and other hematologic diseases[1-3]. It can induce cellular differentiation of many malignant tumors and inhibit their growth[4-11]. Morphological and biological changes were also observed in the human hepatocellular carcinoma (HCC) cell line SMMC-7721, treated with ATRA[12,13], but the mechanism remains obscure. For this reason, the changes in telomerase activity and Caspase-3 expression induced by ATRA were analyzed in SMMC-7721 in this study.

MATERIALS AND METHODS
Cell culture
The hepatocellular carcinoma cell line SMMC-7721, was kindly provided by the Hematology Institute of the First Hospital of Norman Bethune University of Medical Sciences, and cultivated in Iscove’s modified Dulbecco’s medium (IMDM, Gibco) containing 10 % fetal bovine serum at 37 °C in an incubator with 5 % CO₂. During the exponential stage, ATRA was added to the medium (10⁻⁵ mmol/L). Cells growing in the ATRA-free medium were used as the control group.

Morphological observation
The ATRA-treated cells and the control cells were observed everyday. After 5 days, the cells were collected, stained and observed under the light microscope. About 10⁶ cells were collected, washed with cold saline, and fixed with 4 % glutaral and 1 % osmium acid. After dehydration, embedding, sectioning and staining, the cells were observed under transmission electron microscope.

Activity of telomerase assayed by TRAP silver staining
About 2.5×10⁶ cells were collected, washed and homogenized. The telomerase activity was detected using a TRAP kit following instructions of manufactuer (Beijing Tiangekangning biotech institute). The reaction system, containing 25 μl TRAP agent, 0.2 μl Taq enzyme and 1 μl cell extract, was incubated for 30 min at 25 °C. Then 0.5 μl of primer was added and PCR was conducted for 30 cycles with denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, extending at 72 °C for 30 s. 15 μl PCR products was loaded onto a 9 % non-degenerative SDS gel, resolved through the SDS-PAGE, demonstrated by a reaction in 0.2 % silver nitrate for 15 min, and visualized by incubation in 30 g/L anhydrous sodium carbonate containing formaldehyde (1 ml/L). The activity of telomerase was indicated by the presence of a 6 hp-DNA ladder. The cell extracts inactivated by incubation at 75 °C for 10 min were used as the negative control.

Expression of Caspase-3 assayed by western blot
About 10⁶ SMMC-7721 cells were harvested, washed, and lyzed in the 5 volumes extract buffer (5 mM Tris-Cl (pH8.0), 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 100 μg/ml PMSF, 2 μg/ml aprotinin, 1 % NP-40) in the iced bath for 20 min. The supernatant was stored at -70 °C. After protein quantification, 80 μg of the extraction was subjected to SDS-PAGE. Proteins resolved on the gel were transferred to a nitrocellulose filter (Amersham) in the buffer containing 48 mmol/L Tris, 39 mmol/L glycerocol, 0.037 % SDS and 20 % methanol. After being blocked in phosphate buffer saline containing 5 % defatted milk, the blots were incubated with goat antibody against Caspase-3 (Santa Cruz). After being washed three times, the filter was incubated with HRP-labeled rabbit anti-goat immunoglobulin, and the reaction was visualized by incubation with a buffer containing DAB and H₂O₂.

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RESULTS

Morphological changes

ATRA-treated cells appeared spindle-shaped, rather than polygon-shaped as under normal conditions. Their nuclei became smaller and pyknotic. The cytoplasm was also stained densely. Cell shrinkage, loss of microvilli, chromatin clumps and reduction of the nuclear/cytoplasmic ratio were noted under the electron microscope. The mitochondria proliferation, enlargement of Golgi complex, cytoplasmic vacuolation and glycogen accumulation, as well as lipofuscin and tonofilaments were also observed (Figure 1).

Figure 1 Morphology of SMMC-7721 observed under transmission electron microscope. Untreated cells have many microvilli, and their nuclei have a great deal of incisure (A). ATRA-treated cells have smaller volume, fewer microvilli on the surface, denser chromatin and increased heterochromatin. With the plasma increased, the nuclear/cytoplasmic ratio decreased. The mitochondria also increased. The Golgi complex became bigger. More vacuole and glycogen were seen. There were also many lipofuscin and tonofilaments (B).

Activity of telomerase

The untreated cells showed a 6 bp ladder pattern, suggesting the active telomerase. So it served as the positive control (Lane 2). The negative control did not show the DNA ladder because the sample was inactivated at 75 °C (Lane 3). The ATRA-treated cells showed no ladder as the negative control (Lane 1).

Figure 2 Telomerase activity of SMMC-7721. The untreated cells showed 6bp ladder pattern, suggesting the active telomerase. So it served as the positive control (Lane 2). The negative control did not show the DNA ladder because the sample was inactivated at 75 °C (Lane 3). The ATRA-treated cells showed no ladder as the negative control (Lane 1).

Expression of Caspase-3

All the control groups showed weak signals at the position of 32 kD, suggesting the presence of Caspase-3 (Lane 2). For the ATRA-treated cells, the 32 kD signal was shown to be stronger. In addition, another signal at the position of 20 kD was detected. The latter represented the p20 subunit of Caspase-3, an active form of the molecule (Lane 1).

Figure 3 The expression of Caspase-3. All the control groups showed weak signals at the position of 32 kD, suggesting the presence of Caspase-3 (Lane 2). For the ATRA-treated cells, the 32 kD signal was shown to be stronger. In addition, another signal at the position of 20 kD was detected. The latter represented the p20 subunit of Caspase-3, an active form of the molecule (Lane 1).

DISCUSSION

It is estimated that about 437,000 people die of HCC every year worldwide, and that their 5-year survival rate is below 3%. It is believed that development of HCC is associated with many factors[14-18]. Several gene mutations have been proven to play some roles during this process[19]. Eukaryotic chromosomes are capped with repetitive telomere sequences that appear important for maintaining chromosomal integrity. In all normal somatic cells, each cycle of cell division and DNA replication results in the loss of 50-200 terminal nucleotides from each chromosome. This gradually results in instability of the chromosomes and cell death[20,21]. Telomerase is a type of reverse transcriptase being essential in many cases for telomere stability and cell proliferation, immortalization and transformation[22-35].

Recently, more than ten types of proteases with homology to ICE/CED-3 that is specific to aspartic acid have been found. The Caspase family plays key biological roles in inflammatory responses and in regulation of apoptosis of mammalian cells. Among them, Caspase-3 is known as a key protease whose activation can induce apoptosis of mammalian cells[36-42]. It lies in the upper stream of a series of cascade reactions. Therefore, it may be of some help to delineate Caspase-3 expression during the ATRA-associated cell differentiation and death for further understanding of its mechanism.

In the present study, a reduction of telomerase activity, upregulation of Caspase-3 expression and the activation of this molecule were linked to the ATRA-induced differentiation of...
SMCC-7721 cells. A few mechanisms have been proposed for the growth inhibition of HCC cells by ATRA. For example, ATRA can inhibit telomerase activity and shorten telomere length of cancer cells and disrupt the stability of the chromosomes. Alternatively, Caspase may also be involved in this process. ATRA activates Caspase-3, simulating a series of apoptotic signals and resulting in cell death. It is presumed that ATRA may directly or indirectly affect the function of Caspase-3 at the following three levels: 1) up-regulating the expression of Caspase-3; 2) indirectly acting on the upper stream regulator of Caspase-3; and 3) directly acting on Caspase-3 itself and promoting its activity. In conclusion, inactivation of telomerase and activation of Caspase-3 may be important pathways for the inhibition of HCC cell proliferation by ATRA.

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