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THE DIFFERENTIATION INDUCING EFFECT OF TANSHINONE AND RETINOIC ACID ON HUMAN CERVICAL CARCINOMA CELL LINE (ME180) *IN VITRO*

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The cervical carcinoma cell line, ME180 cells were treated with tanshinone (Tan) or retinoic acid (RA) in DMSO (final concentration 0.02%, V/V) on 4 successive days. The cells treated with the same concentration of DMSO alone served as control. Morphological studies with light and transmission electron microscopy showed that the cells treated with both Tan and RA became well-differentiated. The cellular growth and proliferation were suppressed (as revealed by cell counting. [^{3}H]-thymidine uptake and colony-forming assay). The number of nuclear organizer regions (AgNORs) in cells reduced and the distribution type returned nearly to normal type. The tumorigenicity in nude mice was reduced. The cell RNA dot hybridization showed that the expression of c-myc and c-Ha-ras mRNA was inhibited markedly. All above results showed that Tan and RA could reverse some malignant phenotype and possessed differentiation inducing activity on ME180 cell line. No significant difference was observed between the cells treated with Tan and RA.

Key word: Tanshinone, All trans retinoic acid, Cervical carcinoma cell line, Inducing-differentiation

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Study on induced differentiation or reversion of tumor cells is an important field of molecular biology of oncology at present. The Application of all trans retinoic acid to treating leukemia is a successful example of differentiation inducing therapy. Since many inducers of differentiation have too high toxicity and side effects to be used in clinical practice. Researchers are working for screening high effective and low toxicant inducers of differentiation. Tanshinone is an alcohol extract from the root of the traditional Chinese medicine-Salvia Miltiorrhiza Bunge. Previous Studies *in vitro* have shown that tanshinone possesses cytotoxic effect on tumor cells, and markedly inhibits DNA synthesis of tumor cells.[1,2] A study on differentiation inducing activity of tanshinone has not been previously reported. We studied the differentiation-inducing effect of tanshinone on the basis of using ME180 cell line as material and using all trans retinoic acid as a positive control.

MATERIALS AND METHODS

Cervical Carcinoma Cell Line

The cell line derived from human cervical carcinoma, ME180 (kindly provided by professor Fan

Weike, Chong Qing Medical University), was established by syker[3] in 1970. ME180 cells were cultured in the RPMR 1640 medium at 37 ℃ in a 5% CO_2 incubator.

Drugs and Treatment

Tanshinone (its abbreviation is Tan) was provided by Institute of Traditional Chinese Medicine of Sichuan (its content is 96%). It was dissolved in DMSO (final concentration is 0.22% V/V). The solution was filtered through a 0.22 μm microporefilter, then stored at 4 ℃ for use. ME180 cells were seeded, then treated with drugs. The control cells were added the equal quantity of DMSO for control experiment. The cells were treated with drugs or DMSO for 4 days continually. The usage of all trans retinoic acid (its abbreviation is RA, sigma's product) is equal to tanshione.

Cytotoxicity Assay

ME180 cells were treated with different concentration of Tan or RA respectively for successive 5 days, then cytotoxic reaction of the cells (degeneration, necrosis, scaling) was observed, and viability and quantity of the cells were assessed by trypan blue exclusion day by day.

Light and Transmission Electron Microscopy Observation

The treated cells were observed by inverse and light microscopy after HE method stain. On the other hand, the cells was made into ultra-slice by routine method, then observed by transmission electron microscopy.

Cellular Growth Properties Measurement

The cells treated with drugs and control cells were harvested by trypsinization, and the sum total of cells was counted by trypan blue exclusion day by day.

Colony-Forming assay

ME180 cells were treated with drugs for 4 days, and cultured in routine medium for 7 days continually. Then the cells were fixed by methanol, Giemsa staining. Finally, the sum total of colony was counted.

^{3}H-Thymidne Uptake Assay

The cells were treated with [^{3}H]-thymidine (final concentration is 7.4×10^7Bq. L^{-1}) for 4 hours before harvested, then digested, centrifuged and dissolved, and the cellular value of cmp of each group was measured by liquid scintillation counting.

AgNORs Analysis[4]

ME180 cells were harvested, smeared, fixated. Then the surface of slice was covered with mixed solution of gluey silver (2g gelatin, 100ml methanol, 50% silver nitrate solution) and stained with hematoxylin. Finally, 100 cells and the distribution type of AgNORs in each cell were observed, and the number of AgNORs of per cell was counted under oil immersion lens.

Tumorigenic Assay[5]

ME180 cells of each group were harvested and counted. The cells were injected separately into nude mice subcutaneously at 1×10^6 cells per animal. Three mice in each group were injected. The cells only treated with DMSO were used for negative control. The growth and tumorigenic state of nude mice were observed and recorded. The nude mice were put to death as long as 80 days after injection and then materials drew from the tumor of nude mice were prepared pathological section.

Cell RNA Blot Hybridization[6]

ME180 cells were harvested and counted. The cells of each group was plated onto nylon filters in a gradient of concentrations (1×10^9, 5×10^8, 2×10^8, 1×10^8. L^{-1}), then fixated with glutaraldeyhde, washed with buffer and digested with 20mg. L^{-1} proteinase K buffer (1 mg. L^{-1}RNase or DNase buffer was used as the control filter). The filter got dry in air for use. Probes of the oncogene (c-myc, c-Ha-ras) were labeled with biotin-14-dATP by "BioNick™ Labeling System" kit description. Hybridization of the filter and the probe biotin-labeled DNA was performed by routine method. The biotin-labeled DNA probes were conjugated with the binding streptavidin-alkaline phosphatase. Photogene™

Detection System measured the conjugated probe. Light emission from Photogene system has all been recorded onto the X-ray film, which was used for autoradiography. The X-ray film was measured by density scanner for quantitative analysis.

RESULTS

Dose of Drugs Used in Inducing Differentiation *in vitro*

The maximum of no-cytotoxic reaction was selected as the dose of inducing differentiation *in vitro*. The doses of Tan and RA were 1 mg. L^{-1} and 0.5 mg. L^{-1}, respectively.

Morphology of ME180 Cells Treated with Tan and RA

Light and electron microscopy observation: after induced treatment with Tan and RA, the round cells became spindle or polygon cells, cellular volume became accordant, tumor giant-cells were less, karyon and nucleolus became small. Karyon/cytoplasm ratio fell, well-differentiated organelle such as microfilament and Golgi complex occurred. Morphology of the cells trended towards benign differentiation compared with the control cells (Figure 1A and 1B).

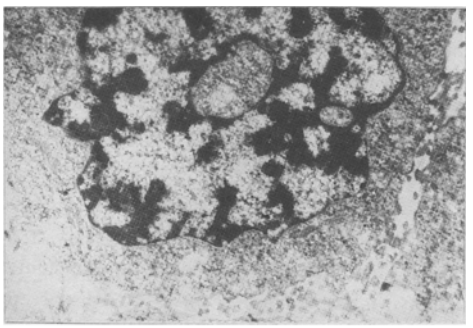
Growth Properties of ME180 Cells Treated with Tan and RA

It was observed that the growth of ME180 cells treated with Tan and RA was markedly inhibited. The doubling time of proliferation extended and the proliferation fold fell. The saturation density of the cells reduced (Table 1).

Table 1. Effect of Tan and RA on growth of ME180 cells

Group	Control	Tan	RA
doubling time of proliferation (hours)	35.0	47.8*	50.2*
proliferation fold (times)	7.0	4.7*	4.2
Saturation density (cells. L^{-1}	6.0×10^8	4.0×10^{8}*	3.6×10^{8}*
inhibition rat of growth(%)		33.3*	40.5*

*$P<0.01$ compared with control by t test or χ^2 test



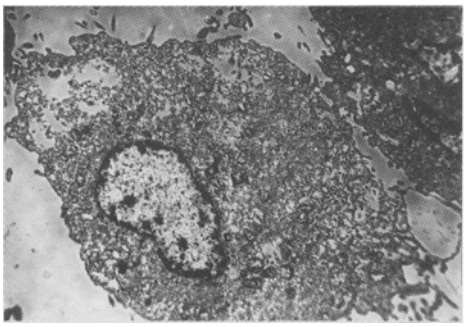


Fig. 1. Effect of tanshinone on ultramicro structure of ME180 cells

A: Control, nucleus/cytoplasm ratio rose and poor-differentiated organelle existed in cytoplasm.

B: Cell were treated with Tan 1 mg. L^{-1} for 4 days, nucleus/cytoplasm ratio fell and well-differentiated organelle existed in cytoplasm.

Cell Multiplication Kinetics of ME180 Cells Treated with Tan and RA

The capacity of colony-forming of the cells treated with Tan and RA both declined with colony-forming rate of 21.7% and 20.2% respectively (control group 38.2%, $P<0.01$); The rate of [^{3}H]-thymidine uptake also reduced with 59.5% (Tan), and 53.8% (RA) of the control group; The number of AgNORs decreased form 5.10 to 3.62 (Tan) and 3.47 (RA) per cell. 20% (Tan) and 21%(RA) of the cells recovered the normal number (1-2 per cell) of AgNORs. The distribution type also returned to normal type (Figure 2A and 2B).

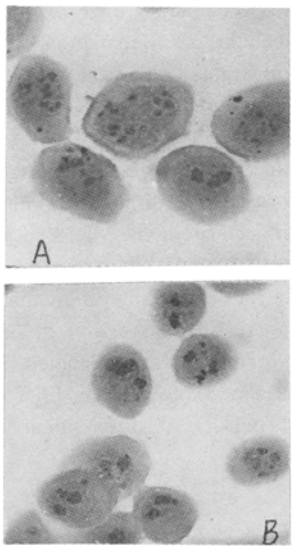


Fig 2. Effect of tanshinone on numbers and distribution of AgNOR in ME180 cells

A: Control, aggregated type

B: Cell were treated with Tan 1 mg. L^{-1} for 4 days, scattered type

Tumorigenicity in Nude Mice of ME180 Cells Treated with Tan and RA

The tumorigenic incubation period of ME180 cells treated with Tan and RA was extended to 18 days (Tan) and 20 days (RA) respectively (control group, 10 days) after injection for 80 days. The formed tumors grew slowly and their weight were 1.75g (Tan) and 1.25g (RA) respectively, reduced more markedly than which of control group (6.37g). The HE pathological section of the tumor tissue demonstrated that the tumor was derived from squamous epithelial tumor. In control group, no remarkable keratose tissue and integrant membrane were found in the tumor tissue, and arrangement of the tumor cells was crowded. But remarkable keratose tissue and integrant membrane were found in the tumor tissue from the groups treated with Tan and RA, and cellular inflammatory infiltration and necrosis in which were found, and only few tumor cells and tumor giant-cells were observed. The morphology of the cells and tissues trended towards benign differentiation by pathological examination.

C-myc and c-Ha-ras Oncogene Expression of ME180 Cells treated with Tan and RA

The results of cell dot hybridization revealed an obvious suppression of c-myc and c-Ha-ras oncogene mRNA expression. the inhibition rates of c-myc or c-Ha-ras were 56.8% or 46.0% (Tan) and 62.2% or 56.9%(RA), respectively. The hybridi-zation signal on the control filter digested by RNase became markedly weak (the inhibition rate was 82.8%), but the hybridization signal on the control filter digested by DNase was unaffected (the inhibition rate was 19.8%). The results indicated that the nucleic acid hybridized with the oncogene probe was indeed mRNA of cells (Figure 3A and 3B).

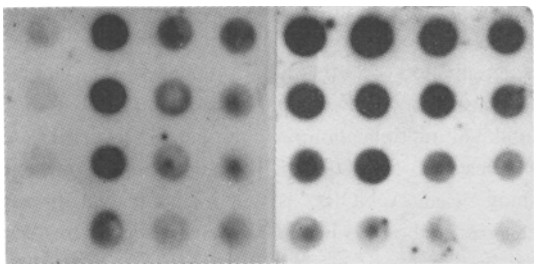


Fig 3. Effect of Tan an d RA on gene expression of c-myc and c-Ha-ras gene (cell RNA dot hybridization)

A: c-myc B: c-Ha-ras

A1: Control treated with RNase,

B1: Control treated with DNase,

2: Control,

3: Cell were treated with Tan 1 mg. L^{-1} for 4 days,

4: Cell were treated with RA 0.5 mg. L^{-1} for 4 days.

a ~ d: Cellular gradient of concentrations (1×10^{9}, 5×10^{8}, 2×10^{8}, 1×10^{8}. L^{-1})

DISCUSSION

Previous studies have shown that the experiment *in vitro* has more advantages than *in vivo*, and the former's result is consistent with the later's. At present, the study *in vitro* is a major method to study inducer of differentiation. All trans retinoic acid is an acknowledged, broad- spectrum inducer of differentiation. In our present study, it was used as a positive control to demonstrate the differentiation inducing activity of tanshinone on tumor cells *in vitro*. On the basis of cytotoxity assay and previous studies,[7] we chose the no-cytotoxic maximum in inducing-differentiation period as the dose of inducing-

differentiation *in vitro*.

Cellular differentiation includes morphological and functional differentiation. The change of cellular morphology is a phenotypic and basic characteristic of differentiation. Light and electron microscopy observation showed that Tan and RA induced cellular morphology to trend towards well-differentiation, and partial morphology of the induced cells became normal.

Cellular unlimited growth and proliferation is a main characteristic of malignant tumor cell. The measurement of cellular growth properties showed that Tan and RA markedly inhibited growth of ME180 cells. Trypan blue exclusion demonstrated the inhibiting effects of Tan and RA on ME180 cells weren't caused by cytotoxicity, but were by inducing-differentiation. ^{3}H-thymidine uptake and colony forming assay showed that Tan and RA obviously inhibited multiplication of tumor stem cells and DNA synthesis of ME180 cells. In addition, the present study used AgNORs as an index for observing changes of the cells induced differentiatiion. AgNORs is a sort of protein associated with forming nucleolus. It represents active ribosomal DNA, reflects changes of structure and function of nucleolus, and correlates with protein synthesis. Thus, AgNORs is a sensitive index for studying cellular multiplication. The experiment indicated that Tan and RA stopped ME180 cells in interphase of division, and inhibited cellular multiplication by preventing protein synthesis.

Tumorigenic assay of nude mice is a reliable index for demonstrating cellular benignity and malignancy. Our present study found the tumorigenic time in nude mice of ME180 cells treated with Tan and RA was extended, the growth of the tumor became slow, the tumor tissue and cells trended towards well-differentiation. these data illustrated that Tan and RA induced ME180 cells to reduce their malignant degree.

Recent studies on mechanism of inducer of differentiation have been shown that the effect of RA is related to inhibition and inactivation of some oncogene.[8] C-myc and Ha-ras oncogene are associated with multiplication and malignancy of cells.[9] Our previous studies have demonstrated that amplification of c-myc, point mutation of Ha-ras and their over-expression were associated with the cervical carcinoma.[10] Via the inhibition of transcription and expression of c-myc and Ha-ras oncogene, Tan and RA inhibited cellular growth and proliferation, promoted cellular differentiation, and induced morphological and biological characteristics of ME180 cells to trend towards benign or normal changes.

Aforeside data suggested that tanshinone and retinoic acid possess the differentiation inducing effect on human cervical carcinoma ME180 cell line *in vitro*. No significant difference was found between them. Their mechanism of inducing differentiation might be related to suppressing expression of oncogene, and which has yet to be further studied. We consider tanshinone is a new prospective, high effective and low toxicant inducer of differentiation for clinical application.

REFERENCES

1. 张玉五，高亚菲。活血化瘀药丹参抗肿瘤作用研究进展。西安医科大学学报 1990; 11: 191.
2. Chang WL, Chen CF. Cytotoxic activities of tanshinone against human carcinoma cell lines. Am J China med 1991; 19(3-4): 207.
3. Sykes JA, whitescaver J, Jernstrom P, et al. Some properties of a new epithelial cell line of human origin.. J Natl Cancer Inst 1970; 45: 107.
4. Crocker J, Nar P. Nucleolar organizer regions in lymphomas. J Pathol 1987; 151: 11.
5. Conway K, Morgan D, Phillips KK, et al. Tumorigenic suppression of a human cutaneous squamous cell carcinoma cell line in the nude mouse skin graft assay. Cancer Res 1992; 1152: 6487.
6. Paeratakul U, De-Stasio PR, Taylor MC, et al. A first and sensitive method for detecting specific viral RNA in mammalian cell. J Viral 1988; 62: 1131.
7. Stinson SF, alley MC, kopp WC, et al. Morphological and immunocytochemical characteristics of human tumor cell line for use in disease oriented anticancer drug screen. Anti Cancer Res 1992; 12: 1035.
8. Ai ZW, Zha XL, chen HL. Effect of retinoic acid on hepatocarcinoma cell line. J Tumor Marker Oncol 1990; 328.
9. Loodwood AH, Murphy SK, Borislow. Cellular signal transduction and the reveral of malignancy. J Cell Biochem 1987; 33: 237.
10. 黄光琦。宫颈癌中多癌基因变化及其临床意义。实用妇产科杂志 1994; 10(5): 231.