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Effect of retinoic acid and its complexes with transition metals on human bladder cancer cell line EJ in vitro

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Abstract The aim of this study was to investigate the effect of retinoic acid (RA) and its complexes with transition metals on the bladder cancer cell line EJ. Retinoic acid complexes with transition metals Cu, Co, Zn, and Ni were prepared. Cell proliferation was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in the presence of RA or its complexes with transition metals Cu, Co, Zn, and Ni {$Cu(RA)_2{\cdot}3H_2O$, $Co(RA)_2{\cdot}3H_2O$, $Zn(RA)_2{\cdot}4H_2O$, and $Ni(RA)_2{\cdot}3H_2O$}. Colony formation in soft agar culture, A agglutination reaction, and lactic acid dehydrogenase isoenzyme assay were performed in the cells treated with these drugs to estimate the induced differentiation. p53 or c-Ha-ras expression in drug-treated cells was assayed by ABC immunocytochemistry technique. The results demonstrate that EJ cells treated with the drugs become less confluent and tend to exhibit normal characteristics. Although RA and its complexes showed inhibition to proliferation of EJ cells at the concentrations of 10^{-6} mmol/l, the inhibition induced by $Ni(RA)_2{\cdot}3H_2O$ was much more marked than that by RA. EJ cells were growth inhibited by RA or $Ni(RA)_2{\cdot}3H_2O$ from 48 to 96 h at the concentration of 10^{-8} mol/l. The levels of LDH4 and LDH5 in the cells were greatly increased by RA. Nevertheless, $Ni(RA)_2{\cdot}3H_2O$ did not affect LDH isoenzyme in EJ cells. The number of colony formations of EJ cells in soft agar culture was decreased by RA or $Ni(RA)_2{\cdot}3H_2O$. The percentage of colony formation in soft agar culture was much lower in EJ cells treated with $Ni(RA)_2{\cdot}3H_2O$ than with RA. The required concentration of A agglutination reaction was more increased for EJ cells treated with RA or $Ni(RA)_2{\cdot}3H_2O$ than for the control and was further increased in cells treated with $Ni(RA)_2{\cdot}3H_2O$. Mutant p53 expression was more decreased in the EJ cells treated with RA or $Ni(RA)_2{\cdot}3H_2O$ than in the control. Although RA at the concentration of 10^{-6} mmol/l caused lower p21 expression, $Ni(RA)_2{\cdot}3H_2O$ did not affect p21 expression in EJ cells. Therefore, RA and its transition metal complexes have a potential use in the treatment of bladder cancer.

Key words Retinoic acid · Transition metals · Bladder cancer · Proliferation · p53 · c-Ha-ras · Differentiation

Introduction

Retinoids are natural and synthetic derivatives of vitamin A, which have been shown to depress tumor incidence and size in animal models [25]. Studies with cancer cells demonstrate that retinoids induced growth inhibition [5–7], differentiation [8–9], and apoptosis [16] and modulated gene expression. Thus, administration of retinoids has become an established therapy of acute promyelocyte leukemia [25].

Individuals with low serum retinoid levels or low dietary intake of retinoids have an increased risk of bladder cancer and retinoids prevent the emergence of bladder cancer in carcinogen-treated mice [20]. Kolonel et al. [10] have described a slightly lower mean dietary intake of vitamin A in patients with bladder cancer than in an age-matched control. Mahmoud [13] has shown lower levels of serum retinol among patients with bladder cancer than in controls. Treatment of patients with bladder cancer with retinoic acid (RA) reduced the number of recurrent tumors compared with a group of individuals treated only with placebo [20]. However, Bonney concluded that 13cRA was unacceptably toxic and ineffective in some patients [20]. Therefore, there is merit in the investigation of newer, potentially stronger retinoids.

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Some metal elements which have pharmacological effects on humans have been widely studied. The rare earth elements (REE) thulium (Tm) and samarium (Sm), which have high affinity for cancer tissue, have been used in treatment and diagnosis of cancer. REE such as cadmium (Cd), cobalt (Co), platinum (Pt), and palladium (Pd) inhibited growth of some kinds of cancer [21]. Antitumor activity can be enhanced by inorganic drugs complex with Cd, Pt and Pd [23]. Nevertheless, the effect of RA complexes with transition metals has not been reported.

In the present study, RA complexes with transition metals Cu, Co, Zn, and Ni were prepared. In addition, the effects of these complexes and RA on proliferation induced differentiation and ras and mutant p53 gene expression in bladder cancer cells that were assayed.

Materials and methods

Chemicals

RA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *N*-methylphenazonium methyl sulfate (PMS), nicotinamide adenine dinucleotide (NAD), and L-glutamine were obtained from Sigma. Nitroblue terazolium (NBT) and agarose were obtained from Promega (USA). Retinoic acid complexes with transition metals Cu, Co, Zn, and Ni {$Cu(RA)_2{\cdot}3H_2O$, $Co(RA)_2{\cdot}3H_2O$, $Zn(RA)_2{\cdot}4H_2O$ and $Ni(RA)_2{\cdot}3H_2O$ } were prepared and kindly provided by the Department of Chemistry, Lanzhou University. RPMI 1640 medium was obtained from GIBCO. Fetal calf serum (FCS) was purchased from the Sino-American Biotechnology Company. Murine antihuman p53 and c-Ha-ras monoclonal antibody were purchased from DAKO Company (Denmark) and an ABC immunocytochemistry kit was purchased from Vector Lab (USA).

Cell culture

Human bladder transitional cell line EJ was kindly provided by the Institute of Urology, Beijing Medical University. The cells were maintained in complete medium (CM) which consisted of RPMI 1640, 100 U/ml penicillin, 100 U/ml streptomycin, 50 U/ml gentamicin, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate and 10% heat-inactivated FCS at 37 °C in 5% CO_2 air environment. Tumor cells were harvested by overlaying the monolayer with a solution of 0.05% trypsin (Sigma) and 0.53 mmol/l ethylenediaminetetraacetic acid (EDTA) and then resuspended in CM. The cells were plated in 96-well plates at 3×10^3 cells per well in 150 µl CM for proliferation assay.

Proliferation assays

The medium of EJ cells in 96-well plates was replaced after 48 h by 250 µl test medium which contained CM supplemented with different concentrations ($0–10^{-6}$ mol/l) of RA, $Cu(RA)_2{\cdot}3H_2O$, $Co(RA)_2{\cdot}3H_2O$, $Zn(RA)_2{\cdot}4H_2O$, and $Ni(RA)_2{\cdot}3H_2O$. The proliferation of EJ cells was determined by MTT assay every day. After culture for 24 h, the medium was removed and plates were washed with RPMI 1640 and 2% FCS. 100 µl RPMI 1640 medium which contained 0.5% MTT and 0.5% FCS was then added in each well. After incubation for 4 h, the medium was replaced by 100 µl DMSO (Sigma). The absorbance (A) at 570 nm in each well was determined by microplate autoreader (Nanjing, China). The proliferation assay was carried out in four wells and repeated four times.

Colony formation in soft agar culture

The experiment was performed in a 24-well plate containing 1 ml CM supplemented with 0.6% low melting point agar. The cells were plated in 24-well plates at 100 cells per well in 1 ml CM supplemented with 0.35% low melting point agar and RA or $Ni(RA)_2{\cdot}3H_2O$. The plates were incubated in 5% CO_2 air environment for 14 days and colony formation in each well was then counted. The assays were carried out in six wells and repeated three times.

A agglutination reaction

Cells were pretreated with RA or $Ni(RA)_2{\cdot}3H_2O$ for 48 h and were then plated in 96-well plates at 4×10^4 cells per well in 200 µl CM supplemented with various concentrations of concanavalin A (ConA; 0–50 mg/l). The plates were vibrated in an oscillator at 37 °C for 10 min. A agglutination reactions were observed in each well. The tests were carried out in four wells and repeated three times.

Lactic acid dehydrogenase isoenzyme assay

Lactic acid dehydrogenase isoenzyme in the cells treated with RA or $Ni(RA)_2{\cdot}3H_2O$ for 48 h was assayed by polyacrylamide gel electrophoresis [12].

Immunocytochemistry

The EJ cells treated with RA or $Ni(RA)_2{\cdot}3H_2O$ for 48 h were harvested by overlaying the monolayer with a solution of 0.05% trypsin and 0.53 mmol/l EDTA and washed with Hank's balanced salt solution three times. The cells were then resuspended in 70% ethanol and added to slides treated with 0.1% gelatin and 0.01% chrome alum. Slides were incubated at 70 °C for 1 h and then treated with methanol/ethanol solution (3:1) at 0 °C for 10 min, 70% acetic acid for 20 s, washed with water and treated with degradation ethanol. Endogenous peroxidase activity was blocked by incubating the slides in methanol containing 0.3% hydrogen peroxide for 30 min. The sections were then incubated with normal rabbit serum diluted in phosphate-buffered saline (PBS) for 30 min at room temperature. The primary monoclonal antibodies, which specifically recognize p53 or C-Ha-ras proteins, were used at dilutions of 1:50 in PBS and incubated for 2 h at 37 °C. The slides were incubated with biotinylated rabbit anti-rat IgG at a 1:200 dilution in PBS for 40 min at 37 °C and then with avidin-biotin-peroxidase complex. Between each antibody incubation, slides were washed three times with PBS. Immobilized peroxidase was visualized by incubation with 0.25 mg/ml of its substrate 3,3′-diaminobenzidine tetrahydrochloride in 0.05 mol/l tris-HCl buffer, pH 8, containing 0.03% hydrogen peroxide for 7 min. Finally, the sections were counterstained with hematoxylin and mounted.

Negative and positive controls were used with each staining batch. Positive controls were tumor sections known to be positive for the antigen used. As negative controls we used normal rabbit cells, irrelevant antibodies instead of primary antibody, or similar sections treated in the same way except for the primary antibody step. Positive samples were defined as cells showing immunostaining in the nucleus and cytoplasm (Fig. 1). Immunostaining for p53 or p21 was independently evaluated by two observers. An immunostaining of less or more than 25% experimental cells in comparison to control cells was considered as decreased or increased expression.

Statistical analysis

Statistical analysis of cell proliferation and the required concentration of A agglutination reaction was performed by analysis of variance. The statistical analysis of gene expression, LDH isoenzyme, and colony formation in soft agar culture was carried out by the χ^2 test.

Results

Proliferation of EJ cells treated with drugs

Although RA, $Cu(RA)_2{\cdot}3H_2O$, $Co(RA)_2{\cdot}3H_2O$, $Zn(RA)_2{\cdot}4H_2O$, and $Ni(RA)_2{\cdot}3H_2O$ all showed inhibition to proliferation of EJ cells at concentrations of 10^{-6} mol/l, the inhibition induced by $Ni(RA)_2{\cdot}3H_2O$ was much more marked than that by RA. Unlike all other drugs, the proliferation of EJ cells was not inhibited by $Cu(RA)_2{\cdot}3H_2O$ when the concentrations were below 10^{-6} mol/l (Table 1). EJ cells were growth inhibited by RA or $Ni(RA)_2{\cdot}3H_2O$ from 48 to 96 h at the concentration of 10^{-8} mol/l (Figs. 2, 3).

Lactic acid dehydrogenase isoenzyme

EJ cells were treated with RA or $Ni(RA)_2{\cdot}3H_2O$ at the concentration of 10^{-8} mol/l for 96 h and lactic acid dehydrogenase isoenzyme in the cells was assayed. The levels of LDH4 and LDH5 in the cells were greatly increased by RA ($P < 0.05$). Nevertheless, $Ni(RA)_2{\cdot}3H_2O$ did not affect LDH isoenzyme in EJ cells (Table 2).

Colony formation in soft agar culture

The number of colony formations of EJ cells in soft agar culture was decreased by RA or $Ni(RA)_2{\cdot}3H_2O$ at the concentration of 10^{-6} mol/l (Fig. 4). The percentage of colony formation in soft agar culture was much lower in EJ cells treated with $Ni(RA)_2{\cdot}3H_2O$ than with RA.

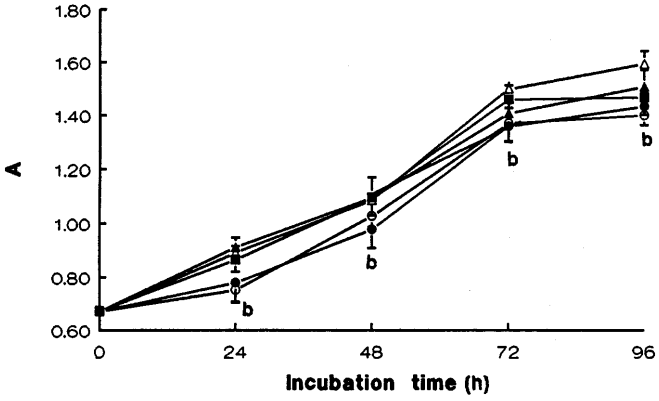

Fig. 2 Effect of retinoic acid (RA) on EJ cell proliferation. The cells were treated with RA at the concentrations of 0 mol/l (Fi), 10^{-6} mol/l (mo), 10^{-7} mol/l (mo), 10^{-8} mol/l (mo), and 10^{-9} mol/l (mo). The experiments were conducted in four wells and repeated four times. $^bP < 0.05$ vs control

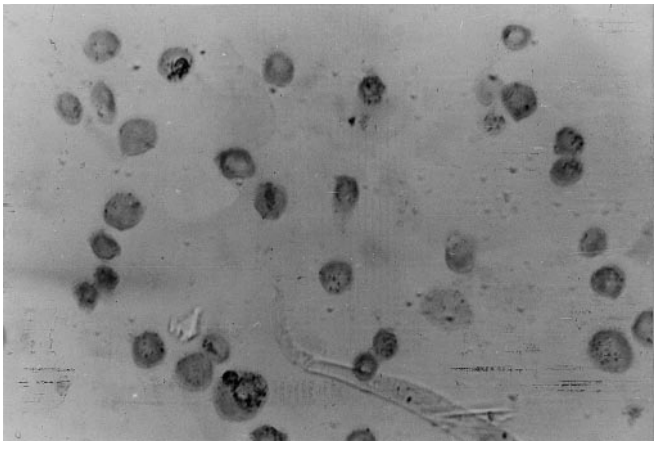

Fig. 1 p53 expression in EJ cells treated with $Ni(RA)_2{\cdot}3H_2O$ at the concentration of 10^{-6} mol/l for 48 h. ABC immunocytochemistry technique, counterstained with hematoxylin and observed under Olympus microscopy (BH2), original magnification ×400

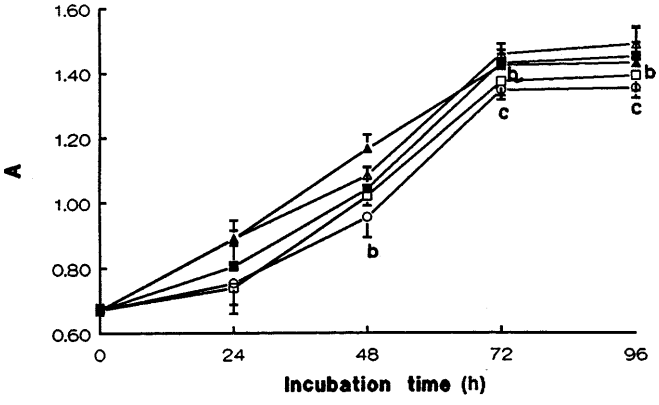

Fig. 3 Effect of $Ni(RA)_2{\cdot}3H_2O$ on EJ cell proliferation. The cells were treated with $Ni(RA)_2{\cdot}3H_2O$ at the concentrations of 0 mol/l (O), 10^{-6} mol/l (mo), 10^{-7} mol/l (mo), 10^{-8} mol/l (mo), and 10^{-9} mol/l (mo). The experiments were conducted in four wells and repeated four times. $^bP < 0.05$, $^cP < 0.01$ vs control

Table 1 Proliferation of EJ cells treated with drugs. The cells were cultured for 48 h in the presence of retinoic acid (*RA*) or *RA* complexes with Cu, Co, Zn and Ni. Each concentration group had five wells in five independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ vs control; $^{***}P < 0.05$ vs RA

Treatment	10^{-6} mol/l	10^{-7} mol/l	10^{-8} mol/l	10^{-9} mol/l
Control	1.09 ± 0.02	1.09 ± 0.02	1.09 ± 0.02	1.09 ± 0.02
RA	1.03 ± 0.02*	1.10 ± 0.08	0.98 ± 0.06*	1.10 ± 0.07
$Cu(RA)_2{\cdot}3H_2O$	1.02 ± 0.01*	1.06 ± 0.03	0.95 ± 0.01	1.08 ± 0.02
$Co(RA)_2{\cdot}3H_2O$	0.94 ± 0.04**	0.94 ± 0.05	0.97 ± 0.02*	1.05 ± 0.01*
$Zn(RA)_2{\cdot}4H_2O$	0.98 ± 0.02**	0.99 ± 0.03*	1.09 ± 0.01	1.11 ± 0.02
$Ni(RA)_2{\cdot}3H_2O$	0.95 ± 0.02**,***	1.01 ± 0.04*	1.02 ± 0.03	1.04 ± 0.06

Table 2 LDH Isoenzyme (%) in EJ cells treated with retinoic acid (*RA*) or $Ni(RA)_2{\cdot}3H_2O$. *$P < 0.05$ vs control

Treatment	LDH1	LDH2	LDH3	LDH4	LDH5
Control	22.4 ± 1.3	32.2 ± 1.7	26.6 ± 2.4	5.6 ± 1.6	8.8 ± 2.5
RA	24.1 ± 1.8	27.0 ± 2.6	25.9 ± 1.7	10.8 ± 1.4*	14.3 ± 1.6*
$Ni(RA)_2{\cdot}3H_2O$	20.3 ± 2.5	30.8 ± 3.6	36.4 ± 8.3	6.0 ± 1.9	4.7 ± 3.7

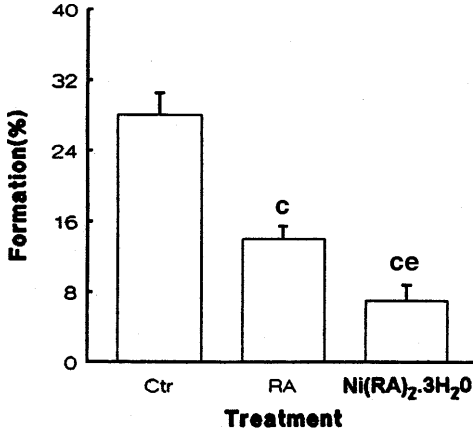

Fig. 4 Colony formation of EJ cells treated with retinoic acid (RA) or $Ni(RA)_2{\cdot}3H_2O$ in soft agar culture. The cells were treated with RA or $Ni(RA)_2{\cdot}3H_2O$ at the concentration of 10^{-6} mol/l for 14 days. Assays were carried out in six wells and repeated three times. $^cP < 0.01$ vs control; $^eP < 0.05$ vs RA

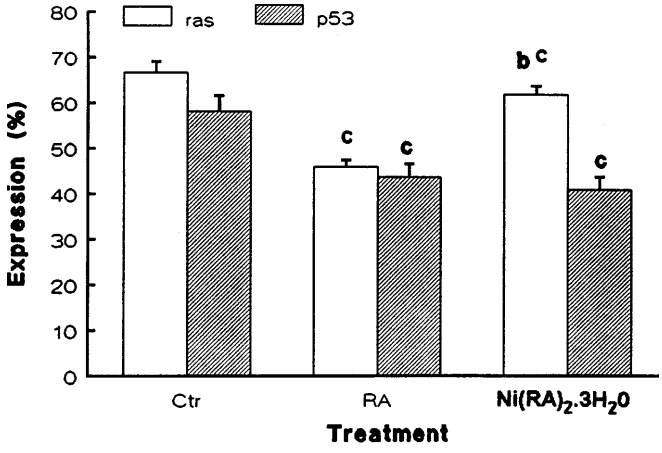

Fig. 5 Expression of mutation of p53 or c-Ha-ras in EJ cells treated with retinoic acid (RA) or $Ni(RA)_2{\cdot}3H_2O$. The cells were treated with RA or $Ni(RA)_2{\cdot}3H_2O$ at the concentration of 10^{-6} mol/l for 48 h. $^bP < 0.05$, $^cP < 0.01$ vs control; $^eP < 0.05$ vs RA

Required concentration of A agglutination reaction

The required concentration of A agglutination reaction was more increased for EJ cells treated with RA or $Ni(RA)_2{\cdot}3H_2O$ than for the control (3.125 μg/ml) ($P < 0.01$). Moreover, the required concentration of A agglutination reaction for EJ cells treated with RA at the concentration of 10^{-6} mol/l was 6.25 μg/ml, which was much lower than 12.5μg/ml for cells treated with $Ni(RA)_2{\cdot}3H_2O$ at the same concentration.

Mutation p53 or p21 expression

Mutant p53 expression was more decreased in the EJ cells treated with RA or $Ni(RA)_2{\cdot}3H_2O$ than in the control. Although RA at the concentration of 10^{-6} mmol/l caused lower p21 expression, $Ni(RA)_2{\cdot}3H_2O$ did not affect p21 expression in EJ cells (Fig. 5).

Discussion

A number of studies have demonstrated growth inhibitory effects of retinoids in cancer. Retinoids reduce the occurrence and progression of human bladder cancers in intact experimental animals and inhibit the formation of bladder cancer [20]. However, there have been some reports that no dramatic differences in recurrence rate were seen between patients treated with RA and placebo [20]. Toxicity occurred in 22% of patients and therefore some of them could not continue to accept the treatment [20]. Some new kinds of RA derivative have been developed in order to reduce toxicity and increase the effect [24]. Oridate [14] compared RA and N-(4-hydroxyphenyl)retinamide (4HPR) and demonstrated that 4HPR caused apoptosis in several cervical carcinoma cell lines and was more potent in this effect than RA. In this study, we compared RA and retinoic acid complexes with transition metals Cu, Co, Zn, or Ni and found that although both RA and $Ni(RA)_2{\cdot}3H_2O$ can inhibit the proliferation of EJ cells, the inhibition induced by $Ni(RA)_2{\cdot}3H_2O$ was more dramatic. This indicates that $Ni(RA)_2{\cdot}3H_2O$ has a potential use in chemotherapy of bladder cancer. Nevertheless, the toxic effects of retinoic acid complexes with transition metals should be tested in normal bladder transitional cells in vitro or in animals in vivo before using it clinically.

The observations that RA and $Ni(RA)_2{\cdot}3H_2O$ can downregulate proliferation of EJ cells raise a question of its possible molecular mechanisms. Redfern [16] found that neuroblastoma cells differentiated in response to RA in vitro. There are several methods to determine the cell differentiation induced by drugs. Decreased colony formation in soft agar culture can be considered an index of differentiated tumor cells in which marked morphological changes may be noted at the same time [3, 15]. In addition, the reduced degree of malignancy found in differentiated bladder cancer cells could be determined by a decreased capacity for agglutination by concanavalin A (ConA) [4, 18, 19]. The characteristic LDH isoenzyme profiles may provide another good marker for the investigation of

human cancer cell differentiation [1, 12]. Like normal hepatocytes, differentiated cancer cells contain solely isozyme 5 of lactate dehydrogenase (LDH) [22]. The analysis of LDH isoenzyme showed that no LDH1 and LDH2 expression was detected in some differentiated cancers treated with RA [11]. In our study, the number of colony formations of EJ cells in soft agar culture was decreased. In addition, the required concentration of A agglutination reaction as well as the levels of LDH4 and LDH5 in the cells were more increased in EJ cells treated with RA or $Ni(RA)_2{\cdot}3H_2O$ than in the control, indicating that EJ cells become more differentiated after treatment with the drugs. These results suggest that differentiation may be involved in the inhibition of growth by RA or $Ni(RA)_2{\cdot}3H_2O$. However, why $Ni(RA)_2{\cdot}3H_2O$ did not affect LDH isoenzyme in EJ cells is not very clear and needs to be investigated further.

Another explanation for the inhibition of proliferation in EJ cells treated with RA or $Ni(RA)_2{\cdot}3H_2O$ is regulation of gene expression. Changes associated with RA treatment of tumor cells include decreases in steady-state mRNA expression of the proto-oncogenes N-myc, c-myc, and c-Ha-ras [2]. The half-life of wide type p53 protein is too short to be assayed in cells and p53 protein is mutated in cancer cells. The half-life of mutated p53 protein is prolonged, so the protein which can be detected by immunocytochemistry is almost mutated p53 protein. Our results demonstrated that mutant p53 expression was more decreased in the EJ cells treated with RA or $Ni(RA)_2{\cdot}3H_2O$ than in the control while RA at the concentration of 10^{-6} mol/l also caused lower p21 expression.

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