

Vitamin D inhibition of lung adenocarcinoma cell proliferation *in vitro*

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Received: 22 September 2013 / Accepted: 17 December 2013 / Published online: 3 August 2014
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Abstract Vitamin D has the capability to inhibit tumor cell proliferation and promote tumor cell apoptosis but whether this mechanism exists in lung adenocarcinoma cells remains to be studied. Our objective is to explore whether vitamin D has the capability to inhibit lung adenocarcinoma cell proliferation and synergize with cisplatin. Our method was to explore the effect of different concentrations of 1,25(OH)₂D₃ with or without cisplatin on lung adenocarcinoma cells by detecting cell proliferation rates at different time points. 1,25(OH)₂D₃ was encapsulated with nanomaterial before acting on lung adenocarcinoma cells, and cell proliferation rates at different time points were detected with the CCK-8 method. When vitamin D was applied at a concentration of 1×10^{-7} and 1×10^{-6} mol/L on A549, PC9, SPC-A1, and H1650 cells for 72 h, no inhibition occurred on cell proliferation. Between the concentrations of 1×10^{-5} and 0.5×10^{-5} mol/L, inhibition on cell proliferation increased with drug action time. Between the

concentration of 2.5×10^{-5} and 0.03×10^{-5} mol/L, inhibition on cell proliferation increased with increasing drug concentration. Analysis using bivariate correlations showed that the correlation coefficient of the proliferation inhibition rate and drug content was 0.580 ($p < 0.0001$). The correlation coefficient of proliferation inhibition rate and the drug action time was 0.379 ($p = 0.01$). The combined use of vitamin D and dichlorodiammine-platinum(II) (DDP) significantly increased the inhibition rate on A549 cell proliferation, which peaked after culturing for 96 h (Table 4). Further analysis using bivariate correlations showed that the correlation coefficient between proliferation inhibition rate and DDP concentration was 0.319 ($p < 0.0001$). The correlation coefficient of the proliferation inhibition rate and vitamin D concentration was 0.269 ($p < 0.0001$). The correlation coefficient of proliferation inhibition and drug action time was 0.221 ($p = 0.003$). Vitamin D encapsulated with nanomaterial (5 ng/ml) on PC-9 cells for 72 h did not inhibit cell proliferation, while after 10 days, the content of crystal violet dissolved decreased by 6.3 ± 3.2 % for the nonleaded nanomaterial group and decreased by 45.8 ± 10.9 % for the nanomaterial-encapsulated vitamin D group ($p < 0.0001$). Vitamin D has the capability to inhibit the proliferation of lung adenocarcinoma cells, synergistically inhibit the proliferation of lung adenocarcinoma cells with DDP, and when encapsulated with nanomaterial can significantly inhibit the proliferation of lung adenocarcinoma cells.

Statement of translational relevance Treatment of lung cancer has not received a good result; our study results suggest that: vitamin D is likely to increase the efficacy of chemotherapy for lung cancer and have a synergistic effect of cisplatin. The results will help clinicians to use vitamin D plus platinum-based chemotherapy.

All authors have read and approved the manuscript.

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Keywords Lung cancer · Vitamin D · Proliferation ·
Cisplatin · Adenocarcinoma

Introduction

Lung cancer is a disease severely threatening human health, and adenocarcinoma is its main cause [1]. Unrestricted proliferation of cancer cells leads to tumor enlargement and

subsequent compression on peripheral organs. Inhibition of tumor cells is an effective measure to treat malignant tumors. Although radiotherapy, chemotherapy, and targeted therapy can also inhibit tumor cell proliferation, recurrence and metastasis will occur eventually.

Epidemiologic study revealed that patients with malignant tumors are usually deficient in vitamin D [2]. Our research indicated that Chinese patients with lung cancer are also deficient in vitamin D [3]. Squamous cell carcinoma and adenocarcinoma are the two major pathological types of lung cancer. Afzal et al. [4] found that lower plasma 25(OH)D was associated with higher risk of tobacco-related cancers, while Cheng and Neuhausser found that serum 25(OH)D concentration was inversely associated with lung cancer mortality in nonsmokers [5]. As the incidence of lung adenocarcinoma is increasing, so we focused on adenocarcinoma. Recent studies indicated that vitamin D has the capability to inhibit tumor cell proliferation and promote tumor cell apoptosis [6], but whether this mechanism exists in lung adenocarcinoma cells still needs to be studied. Therefore, this study was conducted to investigate the inhibitory effects of vitamin D on the proliferation of lung adenocarcinoma cells.

Materials and methods

Experimental methods

Lung cancer cells SPC-A1, PC9, H1650, H1975, and A549 were obtained from Shanghai Cancer Institute. The cell lines have been tested and authenticated by STR 3 months before. In order to understand the different effect of vitamin D on different adenocarcinoma cell lines, we conducted the following experiment. 1,25(OH)₂D₃ was diluted with 10 % FCS-DMEM to obtain 1×10⁻⁷ and 1×10⁻⁶ mol/L solutions. SPC-A1, PC9, H1650, H1975, and A549 cells were cultured to a logarithmic phase, then transferred into 24-well culture plates (10⁴ cells/well; three repetitive wells for each group). Cell culture medium of the same volume was used as control. Each well was treated with 10 μL of CCK-8 after 72 h in culture. One hour later, the optical density (OD) values were measured with a microplate reader at a wavelength of 450 nm. After completion of the above experiment, in order to clarify the role of different concentration on cell proliferation, we conducted the following experiment. 1,25(OH)₂D₃ was dissolved in absolute

ethyl alcohol (purchased from Sigma, D1530) and diluted with 10 % FCS-DMEM cell culture medium to prepare solutions of the following concentrations: 1×10⁻⁵, 5×10⁻⁶, 2.5×10⁻⁶, 1.25×10⁻⁶, 0.62×10⁻⁶, 3.125×10⁻⁷, 1.56×10⁻⁷, and 7.87×10⁻⁸ mol/L. A549 cells at logarithmic phase were transferred into 24-well culture plates (10⁴ cells per well). The wells were treated with 1,25(OH)₂D₃ of different concentrations and were cultured for 24, 48, and 72 h, respectively. Each group had three repetitive wells, with the cell culture medium of the same volume as control. Each well received 10 μl of CCK-8 (purchased from Sigma), and 1 h later, the OD values were measured with a microplate reader at a wavelength of 450 nm.

To further clarify the role of different concentration on cell proliferation, we conducted the following experiment. 1,25(OH)₂D₃ dissolved in absolute ethyl alcohol was diluted with 10 % FCS-DMEM cell culture medium to prepare solutions of the following concentrations: 1×10⁻⁶, 2×10⁻⁶, 3×10⁻⁶, 4×10⁻⁶, and 5×10⁻⁶ mol/L. A549 cells at logarithmic phase were transferred into 24-well culture plates (10³ cells per well). The wells received 1,25(OH)₂D₃ of different concentrations and were cultured for 24, 48, 72, 96, and 120 h, respectively. Each group had three repetitive wells, with the cell culture medium of the same volume as control. Each well received 10 μl of CCK-8 (purchased from Sigma), and 1 h later, OD values were measured with a microplate reader at a wavelength of 450 nm.

To clear synergies of vitamin D and dichlorodiammine-platinum(II) (DDP) on cell proliferation, we conducted the following experiment. 1,25(OH)₂D₃ dissolved in absolute ethyl alcohol was diluted with 10 % FCS-DMEM cell culture medium to prepare solutions of the following concentrations: 1×10⁻⁶, 2×10⁻⁶, 3×10⁻⁶, 4×10⁻⁶, and 5×10⁻⁶ mol/L. DDP was diluted with 10 % FCS-DMEM cell culture medium to prepare solutions with concentrations of 10 mg/L and 5 mg/L. A549 cells at logarithmic phase were transferred in 24-well culture plates. The wells received 1,25(OH)₂D₃ and DDP of different concentrations and were cultured for 24, 48, 72, 96, and 120 h, respectively. Each group had three repetitive wells, with the cell culture medium of the same volume as control. Each well received 10 μl of CCK-8, and 1 h later, OD values were measured with a microplate reader at a wave length of 450 nm.

In order to clarify the effect of nanomaterial-coated vitamin D on cancer cell proliferation in 72 h, we conducted the

Fig. 1 Vitamin D effect on lung adenocarcinoma cell proliferation for 72 h

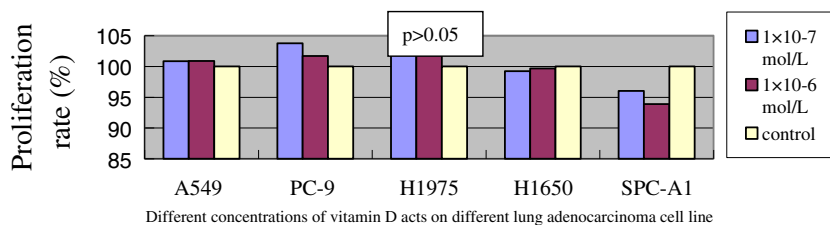


Table 1 1,25(OH)₂D₃ inhibit proliferation of A549 cells

Drug concentration ($\times 10^{-5}$ mol/L)	Inhibition rate (mean \pm SD)				<i>p</i>
	24 h	48 h	72 h		
1.00	0.84 \pm 0.001	0.84 \pm 0.006	0.91 \pm 0.003		<0.0001
0.50	0.55 \pm 0.002	0.65 \pm 0.032	0.82 \pm 0.003		<0.0001
0.25	0.34 \pm 0.003	0.03 \pm 0.073	0.38 \pm 0.025		<0.0001
0.13	0.25 \pm 0.001	-0.11 \pm 0.004	0.20 \pm 0.036		<0.0001
0.06	0.20 \pm 0.005	-0.12 \pm 0.022	0.12 \pm 0.003		<0.0001
0.03	0.13 \pm 0.012	-0.04 \pm 0.039	0.12 \pm 0.033		0.001
0.02	0.16 \pm 0.002	0.11 \pm 0.103	0.08 \pm 0.015		0.364
0.01	0.20 \pm 0.090	0.12 \pm 0.225	0.08 \pm 0.015		0.63
	<i>p</i> <0.0001	<i>p</i> <0.0001	<i>p</i> <0.0001		

Statistical methods: ANOVA

following experiment. Five micrograms of 1,25(OH)₂D₃ was capsulated with nanomaterial to obtain a final concentration of 1.7 g/ml. VD3-nanomaterial was diluted with cell culture medium to 10 ng/ml. PC9 cells at logarithmic phase were transferred into 96-well plates (100 μ L, 5,000 cells/well) and cultured overnight. One experimental group received 100 μ L of free 1,25(OH)₂D₃ (1×10^{-6} mol/L) and the 1,25(OH)₂D₃ concentration in the extracellular fluid was 0.5×10^{-6} mol/L. Another experimental group received 100 μ L of 1,25(OH)₂D₃-nanomaterial in each well; a final experimental group received cell culture medium of the same volume and served as a control. After being cultured for 72 h, each well received 10 μ L of CCK-8 and 1 h later, OD values were measured with a microplate reader at a wavelength of 450 nm. Each group had three repetitive wells. In order to clarify the effect of nanomaterial-coated vitamin D on cancer cell proliferation in 10 days, we conducted the following experiment. Five micrograms of 1,25(OH)₂D₃ was capsulated with nanomaterial to obtain a final concentration of 2.2 g/ml. A 6-well plate received 0.7 ml of 10 % FCS-DF12 culture medium and 0.1 ml cells (1×10^3 /well). In experimental groups, each well received 100 μ L of 1,25(OH)₂D₃-nanomaterial (1,25(OH)₂D₃ concentration, 1×10^{-7} mol/L) or nonloaded nanomaterial and were cultured for 3 days, while

wells of the control group received 100 μ L of culture medium. In studies using nanomaterials, each well received 100 μ L of 1,25(OH)₂D₃-nanomaterial (1,25(OH)₂D₃ concentration, 1×10^{-7} mol/L) or nonloaded nanomaterial, and after being cultured for 6 days, the supernatant was removed. Each well received 2 mL of culture medium, and after being cultured for 10 days, each well received 1 mL of 4 % triformol for fixation (10 min). After fixation, each well received 1 mL of crystal violet staining solution and a digital camera was used to take photos. After adding 1 mL of 3 % acetic acid (97 ml of double distilled water plus 3 mL of 36 % glacial acetic acid) to dissolve out crystal violet, 100 μ L of solution was taken to measure the OD at the wavelength of 570 nm. Each group had four repetitive wells.

Statistical method

Cell proliferation inhibition rate = (Blank group OD value - Treatment group OD value)/Blank group OD \times 100%. $\lg IC_{50} = X_m - I(P - (3 - P_m - P_n)/4)$. The differences of inhibition rates at different time and different drug concentrations were analyzed with one-way analysis of variance. The correlations of proliferation inhibition

Table 2 Correlation between proliferation inhibition rate and drug concentration in the role of vitamin D in A549 at different effect time

Concentration (mol/l)	Inhibition rate (mean \pm SD)				
	24 h	48 h	72 h	96 h	120 h
1×10^{-6}	0.26 \pm	0.07 \pm 0.04	0.01 \pm 0.01	0.11 \pm 0.03	0.15 \pm 0.03
2×10^{-6}	0.26 \pm	0.07 \pm 0.03	0.01 \pm 0.00	0.13 \pm 0.04	0.12 \pm 0.06
3×10^{-6}	0.27 \pm	0.10 \pm 0.01	0.02 \pm 0.02	0.15 \pm 0.04	0.11 \pm 0.06
4×10^{-6}	0.25 \pm	0.19 \pm 0.00	0.05 \pm 0.04	0.27 \pm 0.01	0.09 \pm 0.01
5×10^{-6}	0.30 \pm	0.16 \pm 0.01	0.12 \pm 0.02	0.35 \pm 0.03	0.15 \pm 0.11
<i>p</i>	0.443	<0.0001	<0.0001	<0.0001	0.733

Statistical methods: ANOVA

Table 3 Correlation between proliferation inhibition rate and effect time in the role of vitamin D in A549 at different drug concentration

Concentration (mol/l)	Inhibition rate (mean ± SD)				<i>p</i>
	48 h	72 h	96 h		
1×10^{-6}	0.07±0.04	0.01±0.01	0.11±0.03		0.007
2×10^{-6}	0.07±0.03	0.01±0.00	0.13±0.04		0.01
3×10^{-6}	0.10±0.01	0.02±0.02	0.15±0.04		0.002
4×10^{-6}	0.19±0.00	0.05±0.04	0.27±0.01		<0.0001
5×10^{-6}	0.16±0.01	0.12±0.02	0.35±0.03		<0.0001

Statistical methods: ANOVA

rate, drug concentration, and action time were analyzed with bivariate correlations. Statistical analysis was performed with statistical software SPSS 13.0.

Results

Vitamin D at concentrations of 1×10^{-7} and 1×10^{-6} mol/L for 72 h had no significant inhibition on the proliferation of A549, PC9, SPC-A1, and H1650 cells (Fig. 1). As the drug concentration gradient changed, vitamin D exerted different effects on the proliferation of A549 cells. When drug concentration was in the range of 1×10^{-5} and 0.5×10^{-5} mol/L, as drug action time increased, cell proliferation inhibition rate increased accordingly. The inhibition rate increased with the drug concentration as well (Table 1). When the drug concentration was in the range of 2.5×10^{-5} and 0.03×10^{-5} mol/L, the inhibition rate increased with drug concentration (Table 1). When the drug concentration was in the range of 0.02×10^{-5} and 0.01×10^{-5} mol/L, the inhibition rate on A549 cell

proliferation had no significant changes as the action time and drug concentration increased (Table 1). Using the formula $\lg IC_{50} = X_m - I(P - (3 - P_m - P_n)/4)$, the IC_{50} of $1,25(OH)_2D_3$ acting on A549 cells for 72 h was 2.5×10^{-6} mol/L.

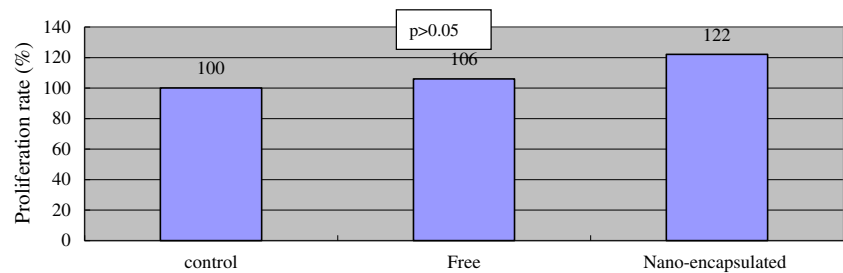
When the samples were cultured for 24 and 120 h, the inhibition of vitamin D on the proliferation of A549 cells had no significant difference (Table 2). When the samples were cultured for 48 to 96 h, as the drug concentration increased proliferation inhibition increase, 96 h treatment had significantly higher inhibition effects than that for 48 h (Table 3). Results of analysis of bivariate correlations are as follows: the correlation coefficient of the proliferation inhibition rate and the vitamin content was 0.580 ($p < 0.0001$); the correlation coefficient of the proliferation inhibition rate and the vitamin action time was 0.379 ($p = 0.01$). The combined use of vitamin D and DDP significantly increased the inhibition rate on A549 cell proliferation, which reached a peak after samples were cultured for 96 h (Table 4). Results of analysis of bivariate correlations showed that the correlation coefficient of the proliferation inhibition rate and DDP concentration was

Table 4 Vitamin D combined with DDP inhibit A549 proliferation

DDP (mg/l)	Vitamin D (mol/l)	Inhibition ratio (mean ± SD)				
		24 h	48 h	72 h	96 h	120 h
5	0	0.28±0.02	0.16±0.04	0.05±0.04	0.31±0.04	0.16±0.06
5	1×10^{-6}	0.36±0.05	0.14±0.03	0.10±0.03	0.51±0.03	0.35±0.04
5	2×10^{-6}	0.36±0.02	0.17±0.01	0.17±0.05	0.40±0.01	0.32±0.02
5	3×10^{-6}	0.33±0.01	0.19±0.02	0.21±0.02	0.42±0.02	0.29±0.02
5	4×10^{-6}	0.35±0.02	0.25±0.04	0.23±0.01	0.40±0.04	0.22±0.00
5	5×10^{-6}	0.33±0.01	0.26±0.04	0.15±0.05	0.42±0.03	0.19±0.02
	<i>p</i>	0.014	0.004	<0.0001	<0.0001	<0.0001
10	0	0.31±0.021	0.17±0.017	0.19±0.041	0.40±0.051	0.34±0.030
10	1×10^{-6}	0.34±0.01	0.14±0.034	0.23±0.058	0.36±0.021	0.45±0.009
10	2×10^{-6}	0.41±0.004	0.17±0.009	0.21±0.021	0.38±0.062	0.47±0.041
10	3×10^{-6}	0.41±0.002	0.19±0.018	0.23±0.022	0.53±0.02	0.44±0.014
10	4×10^{-6}	0.40±0.001	0.25±0.041	0.39±0.032	0.54±0.01	0.44±0.030
10	5×10^{-6}	0.48±0.137	0.26±0.037	0.41±0.035	0.49±0.01	0.42±0.037
	<i>p</i>	0.047	0.001	<0.0001	<0.0001	0.002

Statistic method: ANOVA

Fig. 2 Nano-encapsulated vitamin D effect on PC-9 proliferation for 72 h



0.319 ($p < 0.0001$). The correlation coefficient of the proliferation inhibition rate and vitamin D concentration was 0.269 ($p < 0.0001$). The correlation coefficient of proliferation inhibition rate and the drug action time was 0.221 ($p = 0.003$).

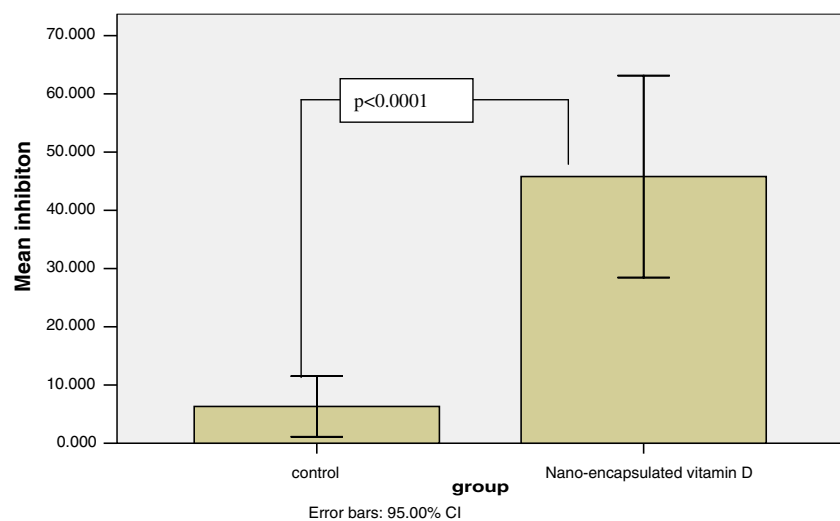
Nanomaterial-capsulated vitamin D (5 ng/ml) for 72 h did not inhibit cell proliferation (Fig. 2).

When PC-9 cells were treated with nanomaterial-capsulated vitamin D for 10 days, the content of the crystal violet dissolved out in the cells decreased by $6.3 \pm 3.2\%$ for the nonloaded nanomaterial group and decreased $45.8 \pm 10.9\%$ for the nanomaterial-capsulated vitamin D group ($p < 0.0001$) (Fig. 3).

Discussion

Lung cancer has a poor prognosis. Unfortunately, even in stage I non-small cell lung cancer (NSCLC), 5-year survival rates are in the range of 55 to 72%. For unresectable disease in stages IIIB and IV, 5-year survival rates are $< 5\%$. DDP is one of the main drugs to treat lung cancer. Unfortunately, the platinum doublet's therapeutic efficacy has reached a plateau [7]. Based on plateau chemotherapy, formulation of an individualized treatment plan that combines other drugs deserves further discussion.

Fig. 3 Nanomaterial-encapsulated vitamin D inhibit PC-9 cells proliferation after 10 days



In clinical studies, significantly increased overall cancer risk was observed among men with low 25(OH)D [8]. Serum 25(OH)D concentration was inversely associated with lung cancer mortality in nonsmokers [5]. Vitamin D is associated with tumor proliferation and apoptosis. A double-blind, randomized, and controlled study revealed that vitamin D regulated the expression of 66 genes, of which 17 played an important role in transcriptional regulation, immune function, response to stress, and DNA repair [9].

At present, there are still no reports on whether there is synergy between vitamin D and cisplatin in the treatment of lung cancer. One study revealed that there was an effect of ApaI T>G polymorphisms of the VDR gene on the platinumiferous plan chemotherapy response in patients with NSCLC as well as a prognostic role of VDR gene polymorphisms in Chinese patients with advanced NSCLC [10]. This study suggested that vitamin D and cisplatin may have synergic effects in the treatment of lung cancer.

Our current study indicated that when vitamin D at concentrations of 1×10^{-7} and 1×10^{-6} mol/L was applied to A549, PC9, SPC-A1, and H1650 cells for 72 h, no obvious inhibition was achieved on cell proliferation. However, between the concentrations of 1×10^{-5} and 0.5×10^{-5} mol/L, inhibition on cell proliferation increased with increasing drug action time. Between the concentrations of 2.5×10^{-5} and 0.03×10^{-5} mol/L, inhibition on cell proliferation increased

with the increase of drug concentration. Results of analysis of bivariate correlations showed the correlation coefficient of proliferation inhibition rate and the drug content to be 0.580 ($p < 0.0001$). The correlation coefficient of the proliferation inhibition rate and the drug action time was 0.379 ($p = 0.01$). These results suggest that vitamin D has an inhibitory effect on the proliferation of lung adenocarcinoma cells, but this effect is weak under physiological concentrations. Increasing drug concentration and drug action time could enhance its inhibitory effect on the lung adenocarcinoma cells.

The combined use of vitamin D and DDP significantly increased the inhibition rate of A549 cell proliferation, which reached a peak after being cultured for 96 h (Table 4). Results of analysis of bivariate correlations showed the correlation coefficient of the proliferation inhibition rate and DDP concentration to be 0.319 ($p < 0.0001$). The correlation coefficient of the proliferation inhibition rate and the vitamin D concentration was 0.269 ($p < 0.0001$). Finally, the correlation coefficient of the proliferation inhibition rate and the drug action time was 0.221 ($p = 0.003$). These results revealed that vitamin D and DDP have synergistic inhibition effects on the proliferation of lung adenocarcinoma cells. They also demonstrate that inhibition strength on cancer cell proliferation in a multi-drug treatment plan depends mainly on cisplatin concentration, secondly on the vitamin D concentration, and thirdly on the drug action time.

Although the effect of vitamin D in lung cancer cell apoptosis is still not really understood, there is evidence that vitamin D can induce apoptosis in breast cancer. Thyer et al. [11] believe when the active vitamin D (1,25(OH)(2)D3) binding with vitamin D receptor (VDR) and the vitamin D-binding protein-derived macrophage activating factor (GcMAF), GcMAF will stimulate macrophages then induce apoptosis. What Thyer et al. found support that vitamin D can introduce cancer cell apoptosis just as what we found.

Because the safe dosage of vitamin D under physiological conditions is narrow, increasing drug concentration became very difficult in clinical application. Nanomaterial-capsulated vitamin D may reduce the side effects of high-dose vitamin D by increasing the intracellular drug concentration.

The results of this study revealed that the action of vitamin D capsulated with nanomaterial (5 ng/ml) on the PC-9 cells for 72 h did not inhibit the cell proliferation, while after 10 days of treatment, the content of the crystal violet dissolved by these cells decreased by 6.3 ± 3.2 % for the nonloaded nanomaterial group as compared to a decrease of 45.8 ± 10.9 % for the nanomaterial-capsulated vitamin D group ($p < 0.0001$). These results suggest that nanomaterial-capsulated vitamin D can significantly inhibit the proliferation of lung adenocarcinoma cells. Nanomaterial-capsulated vitamin D needs 10 days (versus three) to play its role of inhibiting the proliferation of lung adenocarcinoma cells, an increase

from the action time of free vitamin D. This may be because nanomaterial-capsulated vitamin D needs to be taken into the cell first and then released before the vitamin can play its biological role.

Conclusion

Vitamin D has the capability to inhibit the proliferation of lung adenocarcinoma cells by itself and synergistically with DDP. Nanomaterial-capsulated vitamin D can significantly inhibit the proliferation of lung adenocarcinoma cells as well.

Acknowledgments This work was funded by grants 81101770 and 81201839 of the National Natural Science Foundation of China.

Conflicts of interest None

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