Basic Research

Inhibitory effect of 1,25(OH)$_2$D$_3$ on proliferation of human laryngeal carcinoma cells and potential mechanisms

Lian-Jun Lu, Ding-Jun Zha, Tao Xue and Jian-Hua Qiu

[Abstract] Background and Objective; 1Alpha,25-dihydroxy vitamin D$_3$ [1,25(OH)$_2$D$_3$], the biologically active form of vitamin D$_3$, has antiproliferative activity against various tumor cells. This study was to explore the inhibitory effect of 1,25(OH)$_2$D$_3$ on human laryngeal carcinoma Hep-2 cells and potential mechanisms. Methods; Hep-2 cells were treated by 1,25(OH)$_2$D$_3$ (0, 1, 10 and 100 nmol/L) for 24, 48, 72 and 96 h, respectively. Cell proliferation was measured by MTT assay. Cell apoptosis was measured by flow cytometry (FCM). The expression and phosphorylation of ERK, p38MAPK, and JNK proteins were detected by Western blot. Results; 1,25(OH)$_2$D$_3$ significantly inhibited Hep-2 cell proliferation and induced cell apoptosis. 1,25(OH)$_2$D$_3$ increased p38MAPK phosphorylation but not ERK and JNK phosphorylation. The 1,25(OH)$_2$D$_3$-induced apoptosis of Hep-2 cells was partly blocked by p38 inhibitor SB203580. Conclusion; 1,25(OH)$_2$D$_3$ could induce apoptosis of Hep-2 cells and p38MAPK plays an important role in this process. Key words: laryngeal neoplasm, Hep-2 cell, vitamin D, mitogen-activated protein kinase

In addition to the regulatory role in the calcium-phosphorus metabolism balance, 1,25-dihydroxy vitamin D$_3$ [1,25(OH)$_2$D$_3$], the biologically active form of vitamin D$_3$, has extensive non-calcium-regulating effects such as inducing differentiation, inhibiting proliferation, and promoting apoptosis of tumor cells, which has become the hot spot in a field of tumor treatment. Up to now, the regulatory role of 1,25(OH)$_2$D$_3$ in human laryngeal carcinoma cells and its detailed anti-tumor mechanisms are unclear. The present study aimed to explore the inhibitory effect of 1,25(OH)$_2$D$_3$ on the proliferation of human laryngeal carcinoma Hep-2 cells and potential mechanisms, thereby providing new clues for the prevention and control of human laryngeal carcinoma.

Materials and Methods

Materials. Laryngeal carcinoma cell line Hep-2 was provided by Shanghai Institute of Cytology. Calf bovine serum was from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. RPMI-1640 was purchased from Gibco. 1,25(OH)$_2$D$_3$ was from Sigma Company, which was prepared into solutions at a concentration of 1 mmol/L with ethanol and stored at -80°C.

Page dimensions: 630.4x857.6
antibodies were purchased from Cell Signaling Company.

**Methods.** Cell proliferation in hibition experiment. Cells were cultured with RPMI-1640 medium containing 10% fetal bovine serum at 37°C in a 5% CO2 incubator, then seeded in 96-well plates (1×10^3 cells/well). When the cells reached a confluence of 80%, the culture medium was changed to 1,25(OH)_2D_3 of various concentrations (0, 1, 10 and 100 nmol/L). After interaction for various durations, each well was added with 15 μL MTT for another 4-hour culture, then the culture medium was removed and each well was added with 150 μL dimethyl sulfoxide, shaken for 10 min. The absorbance (A) of each well was measured with enzyme-linked reaction instrument at a wavelength of 490 nm. The inhibition rate of 1,25 (OH)_2D_3 on Hep-2 cell proliferation was calculated as follows: inhibition rate = (1 - mean A_000 of 1,25 (OH)_2D_3 group / mean A_000 of control group) 100%. Six-well repetition was set for the experiment.

Cell apoptosis detected with flow cytometry. Hep-2 cells were treated with 1,25 (OH)_2D_3 of various concentrations for 96 h, then harvested and washed with pre-cooled PBS, prepared into single cell suspension with 70% cool ethanol, and stained with PI. The apoptosis was detected with flow cytometry (FCM). This experiment was repeated three times.

Protein expression detected with Western blot. Hep-2 cells were treated with 1,25(OH) D_3 of various concentrations for various durations. Total protein were extracted from the cells, and transferred to nitrocellulose membrane through SDS-PAGE. After blocking the membrane, primary antibody and horseradish peroxidase-labeled secondary antibody were added. The chemiluminescence reagent was used to enhance reaction. The results were analyzed by gel imaging system. The experiment was repeated three times.

Statistical analysis. Data are expressed as mean SD, and were analyzed with SPSS11.0 software. The comparison of inter-group rates was performed using t test. The comparison of inter-group quantitative variables was performed using analysis of variance. The significant level was defined to be 0.05.

**Results**

**Inhibitory effect of 1,25 (OH)_2D_3 on Hep-2 cell proliferation.** MTT results showed that the inhibitory effect of 1,25 (OH)_2D_3 on Hep-2 cell proliferation was enhanced with increased concentration of drugs and prolonged interaction duration (Fig. 1), with significant differences among groups (P<0.05). The 50% inhibition concentration (IC_{50}) of 1,25(OH)_2D_3 for Hep-2 cells was 107.4 nmol/L. FCM results showed that the apoptosis rates of Hep-2 cells were (34.08±1.75)% in 10 nmol/L 1,25(OH)_2D_3 group and (54.48±2.36)% in 100 nmol/L, 25 (OH)_2D_3 group, which were significantly higher than that in control group (P<0.05) (Fig. 2).

**Role of p38 pathway inhibitor in 1,25 (OH)_2D_3 induced apoptosis of Hep-2 cells.** Western blot showed that 1,25(OH)_2D_3-induced p38 phosphorylation in Hep-2 cells was reduced to some extent by p38 pathway inhibitor SB203580 (Fig. 3). FCM results showed that the apoptosis rate of 10 nmol/L 1,25 (OH)_2D_3-treated Hep-2 cells decreased from (35.31±1.59)% to (11.93±1.39)% with the addition of SB203580 (Fig. 4).

Impact of 1,25 (OH)_2D_3 on the MAPK signaling pathway in Hep-2 cells. Western blot showed that, after Hep-2 cells were treated with 1,25 (OH)_2D_3, p38 phosphorylation level...
increased significantly with increased concentration of 1,25 (OH)\(_2\)D\(_3\), while the phosphorylation levels of ERK1/2 and JNK were not changed significantly (Fig. 5).

**Discussion**

Laryngeal carcinoma is a common neck and head malignancy, which requires comprehensive treatment including surgery, radiotherapy and chemotherapy. Although comprehensive treatment improves clinical outcome, controlling local recurrence and improving survival rate for advanced cancer patients are still challenging in the long run. Therefore, seeking new effective anti-tumor drugs is of clinical importance.

1,25 (OH)\(_2\)D\(_3\) is an open-ring steroid hormone that plays an important role in the calcium and bone metabolism, meanwhile, it is also a potential new anti-tumor drug. Experiments have demonstrated that 1,25 (OH)\(_2\)D\(_3\) could inhibit the growth and promote the differentiation of tumor cells in tumors of the breast, prostate, colon, gallbladder, thyroid gland, and pituitary gland, leukemia and lymphoma.\(^5\) In our study, MTT assay showed that 1,25 (OH)\(_2\)D\(_3\) inhibited the activity and proliferation of laryngeal carcinoma Hep-2 cells, indicating that 1,25 (OH)\(_2\)D\(_3\) is a potent inhibitor of laryngeal carcinoma growth.

Cell apoptosis is important for the development and homeostasis of multicellular organism, and its abnormality is critical in carcinogenesis of most human malignancies. The primary mechanisms by which 1,25 (OH)\(_2\)D\(_3\) takes its anti-tumor effect rely on inducing cell cycle arrest, promoting differentiation, and inducing apoptosis of tumor cells.\(^6\) In our study, FCM showed that 1,25 (OH)\(_2\)D\(_3\) induced apoptosis of laryngeal carcinoma Hep-2 cells.

Mitogen-activated protein kinase (MAPK), an intracellular serine/threonine kinase, is mainly composed of p38 MAPK, extracellular signal regulatory kinase ERK1/2 pathway and c-jun N-terminal kinase JNK/SAPK pathway, which are in close association with tumor cell apoptosis. Our results showed that 1,25(OH)\(_2\)D\(_3\) induced p38 phosphorylation, while had no impact on ERK and JNK phosphorylation. Previous studies demonstrated that, under various stimulations, p38 MAPK could modulate cell apoptosis, growth and differentiation via phosphating transcriptional factors, cellular skeleton-associated proteins and enzymes;\(^7\) in addition, it also took part in the regulation of cell malignant transformation and infiltration and metastasis of tumor cells, therefore, p38 MAPK plays
Inhibitory effect of 1,25(OH)\textsubscript{2}D\textsubscript{3} on proliferation of human laryngeal carcinoma cells and potential mechanisms

Figure 4 The role of p38 inhibitor SB2035080 in the 1,25(OH)\textsubscript{2}D\textsubscript{3}-induced apoptosis of Hep-2 cells

![Graph showing effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on cell proliferation](image)

**Figure 5** The effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on MAPK protein expression in Hep-2 cells

Lane 1: untreated Hep-2 cells; lanes 2 and 3: Hep-2 cells treated with 10 and 100 mmol/L 1,25(OH)\textsubscript{2}D\textsubscript{3}, respectively.

important roles in the regulation of cell cycle and apoptosis. Our results showed that p38MAPK signaling pathway specific inhibitor SB2035080 partly mitigated the apoptosis of laryngeal carcinoma Hep-2 cells induced by 1,25(OH)\textsubscript{2}D\textsubscript{3}, indicating that 1,25(OH)\textsubscript{2}D\textsubscript{3} induces the apoptosis of laryngeal carcinoma cells probably via the phosphorylation of p38 MAPK signaling pathway.

Our results showed that 1,25(OH)\textsubscript{2}D\textsubscript{3} could induce the apoptosis of in vitro cultured human laryngeal carcinoma cells in time- and concentration-dependent manners. However, whether the same effect exists in vivo requires further investigations. Because 1,25(OH)\textsubscript{2}D\textsubscript{3} is likely to cause hypercalcemia, it is valuable to find compounds that have synergistic effect with 1,25(OH)\textsubscript{2}D\textsubscript{3}, which in turn decreases the concentration of 1,25(OH)\textsubscript{2}D\textsubscript{3}, reduces the incidence of hypercalcemia, while increases the sensitivity of tumor cells to drugs.

**References**


