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Parthenolide Suppress Bcl Expression and Induce Bax, Bim, and p53 Expression in order to Promote Caspase Cleavage in Nicotine Caused Lung Cancer

Hongxuan Li

Shanghai Southwest Weiyu Middle School, Shanghai 200135, China.

Abstract

Non-small cell lung cancer is manifested to have direct relationship with nicotine intake. Nicotine's pro-tumor activity may lead to destruction of cell metabolism, promoting growth and proliferation. Nicotine acetylcholine receptor can activate several signaling pathways causing tumorigenic effects. Through Ras-Raf-ERK signaling pathway, nicotine contributes to increasing cancer cell proliferation as well as apoptosis and proapoptosis inhibition. To find specific treatment, we are going to test Parthenolide's effect on inducing cancer cell apoptosis, both intrinsic and extrinsic, and its ability of anti-apoptosis inhibition. Parthenolide will decrease Erk activation and increase p53 expression caspase expression and cleavage. While Parthenolide exert its function, nicotine concentration affects the efficiency of Parthenolide. Parthenolide does have effect on inhibiting cell proliferation and increasing cell death, but in order to figure out its optimal effect while encountering nicotine dependent lung cancer, we should test how it associate with nicotine. In this article, groups with different concentration of Parthenolide and nicotine are discussed and both negative and positive control will involve in comparison. A549 and H526 cell lines are used to measure cell death by annexin V/PI FACS, MTT assay, cell counts (hemocytomer or coulter counter) and Erk activation by phospho-Erk western blots with Parthenolide with or without nicotine.

Keywords

Parthenolide; Nicotine; Metabolic & Signaling Pathway; Non-small Cell Lung Cancer.

1. Introduction

Associating with increasing chance of smoking, lung cancer, both small-cell and non-small cell, currently is a severe issue worldwide, causing the most cancerous death; among all cancerous patient, lung cancer dominates approximately 27%. Annually, about 1.8 million people die due to lung cancer, with 2.1 million people diagnosed for lung cancer. Among these people, frequent and consecutive smoking is a custom for 90% of them.[1] Hence, understanding lung cancer mechanism and treatment are crucial. Nicotine in cigarette is manifested contribute to lung cancer. It can induce angiogenesis and inhibit apoptosis through nicotinic acetylcholine receptors.[2] Parthenolide may be a plausible substance for lung cancer treatment. Parthenolide may down-regulates Ras-Raf-ERK signal pathway and the relating activity of P53 and CASP gene expression, hence down-regulating nicotine dependent cell proliferation gene. Early studies using different cell lines in lung cancer have shown that water-soluble Parthenolide inhibit tumor growth in vivo through targeting NF-KB, also inducing p53-dependent cell cycle arrested.[3,4] In cells, inhibition of NF-KB activates mitochondrial death pathways, promoting the release of cytochrome C and the activation of caspase 3 and 9.[5] In this article, we are going to briefly introduce how nicotine and Parthenolide influence the cancerous cell proliferation. The dependent variable and negative control will be set to guarantee the experiment viability.

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1.1 Research question

In non-small cell cancer, how Parthenolide, nicotine, and the combination of Parthenolide affects the Ras-Raf-ERK signal pathway and the relating activity of P53 and related CASP which together reflect the level of cancer cell proliferation?

1.2 Hypothesis

Predict that increasing amounts of Parthenolide will decrease Erk activation and increase caspase expression plus cleavage and p53 expression regarding the concentration of nicotine, leading to cell death in A549 and H526 lung cancer cells. We will measure cell death by annexin V/PI FACS, MTT assay, cell counts (hemocytomer or coulter counter) and Erk activation by phospho-Erk western blots with different amounts of parthenolide with or without nicotine. Negative control is no parthenolide and positive control is doxorubicin.

2. Materials and methods

2.1 Reagent and Cell culture

Parthenolide is purified directly from the plant. Non-small lung cancer cell A549 and H526 were cultured using RPMI with 7% percent fatal serum combining with 10% amino acids, vitamins, inorganic salts, and glucose without any growth factor and L-glutamine, gentamycin, and penicillin/streptomycin. Temperature is set at 37 degree Celsius; Carbon dioxide is 5% and no hydrogen and extra oxygen are supplied in the culture. Then, the two cell lines will expose to different concentration of Nicotine, Parthenolide, and their combination.

2.2 MTT assay and western blot

To figure out cell viability and Parthenolide related signaling pathway, we will use MTT assay and western blot. After cultured human lung cancer cells (A549 and H526) for 24 hours, the activated cells in mitosis state were collected and tested for their viability, with a density of 10,000 cells/Wells incubated overnight. After cultivation, the cells were treated (in three batches) with an increase in Parthenolide concentration (0.78-100nM), an increase in nicotine content (0.78-100 nM) and various combinations of the two compounds. In combination trials, Parthenolide concentration was fixed at 100 nM and nicotine concentration increased with the concentration (60-200 nm). The cells were incubated for 48h. Through measuring Caspase-3 antibody, anti-Bcl2, and Bax/Bam specific antibody by western blot, we will find the relationship between nicotine and Parthenolide concentration which leads to different degree of cell proliferation and apoptosis. MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide directly measures cell death which bolsters and generalizes western blot result.

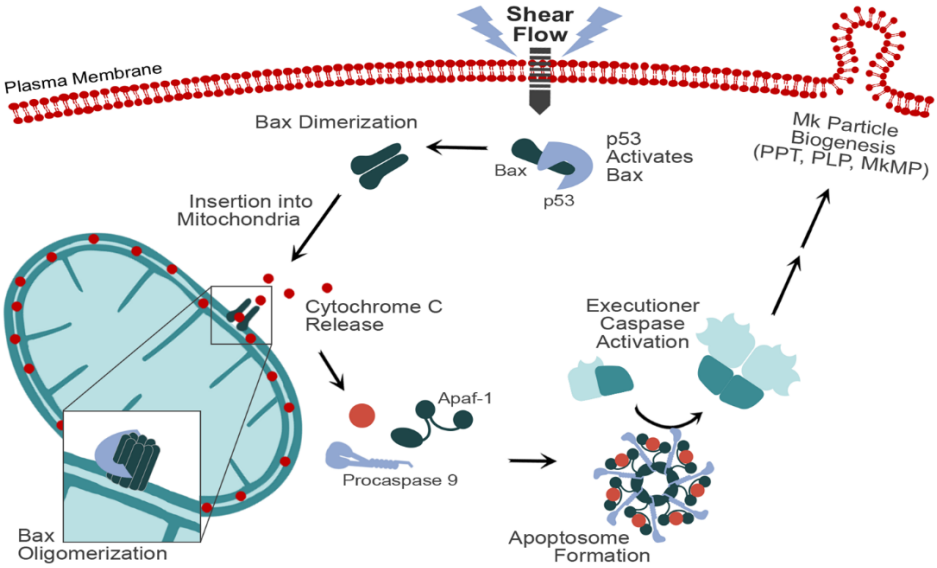

Figure.1 Effect of Parthenolide to lung cell

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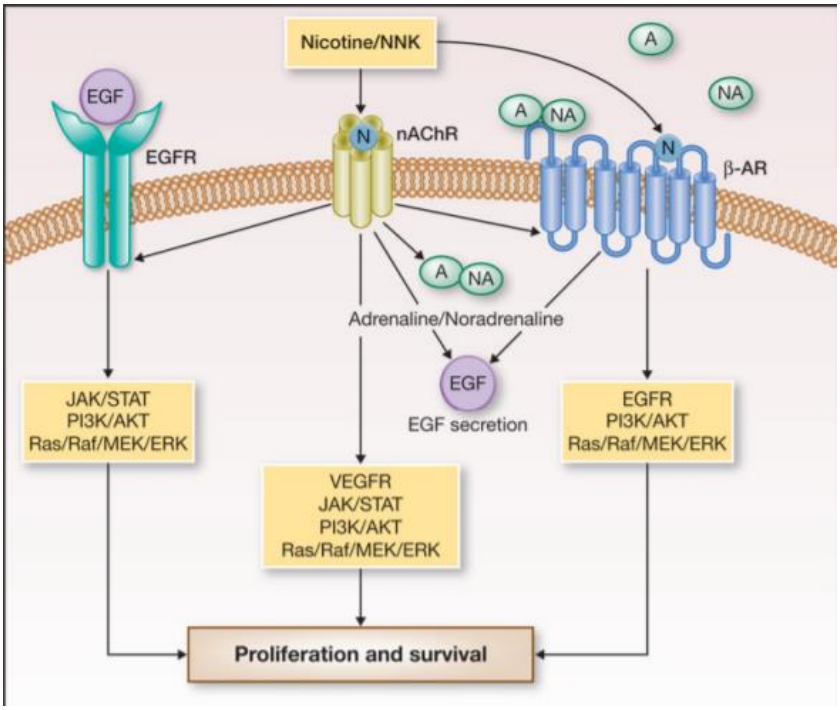

Figure.2 Effect of nicotine to lung cell

2.3 Experiment

2.3.1 Group 1

The Caspase-3 activity are measure under Parthenolide exposure, nicotine exposure, their combination, and negative control. Increase caspase activity in cell indicate the decrease cell proliferation and increase apoptosis. Nicotine exposure has no significant effect on caspase activity comparing with negative control group, while Parthenolide exposure and combination exposure has significant difference. But two possibility exist regarding the effect of combination group and Parthenolide group.

Nicotine: 50nM Parthenolide: 50nM

Table 1. The Caspase-3 activity under Parthenolide exposure, nicotine exposure, their combination, and negative control.

Result one	Caspase activity(Fold increasing)	0.7(±0.05)	2.2(±0.1)	2.1(±0.05)	1(±0.5)
Result two	Caspase activity(Fold increasing)	0.7(±0.05)	2.2(±0.1)	2.7(±0.05)	1(±0.5)
	substance	nicotine	Parthenolide	combination	control
	Significance	-	-	+	-

Result 2 best supports the hypothesis. The margin of error of combination group and Parthenolide group do not overlap, showing significant difference. Parthenolide exert its function better in the presence of nicotine. Parthenolide down regulates Bcl expression (Bcl is anti-apoptotic factor) and up-regulate p53 expression, which cause the expression and cleavage of caspase3&9, and Bim&Bax to initiate apoptosis, while nicotine increase Bcl expression and inhibit caspase response.[6] To be specific, apoptosis , including intrinsic pathway and extrinsic pathway, is a complex process involving the activation of several tumor suppressor genes and the suppression of several oncogenes.. The intrinsic pathway begins with the release of cytochrome C from mitochondria into the cytoplasm, which is regulated by Bcl-2, a family of proteins that control the release of cytochrome C out of the mitochondria outer membrane.[2][5] Bcl-2 aims to keep these channels closed, keeping cytochrome C locked up in the mitochondria. On the other hand, the function of Bax&Bim is to open mitochondrial channels and promote the flow of cytochrome C to the cytoplasm. In the cytoplasm, cytochrome C

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binds with apAF-1 protein to form apoptotic complex, and the apoptotic complex continues to activate Procaspase 9 and converts it to active caspase 9.[7] Caspase 9 activates the caspase 3, 6, and 7, initiating apoptosis. Nicotine causes DNA damage which induce the p53 expression responsible for caspase formation and cleavage, but apoptosis cannot be achieved without assistance from Bim and Bax, proapoptosis proteins using intrinsic apoptosis pathway. Bim and Bax plays dominate role in caspase apoptosis signaling pathway, bolstering and cooperating with p53 apoptosis pathway. Parthenolide enhance Bax and Bim expression to open ion channel gate which release cytochrome C produced during metabolic pathway in mitochondria into cytosol and inactivate NF-kb as allosteric non-competitive inhibitor of nicotine.[8] NF-kb level directly influences Bcl-2 gene expression. NF-kb inhibition contributes to lower Bcl-2 expression. Releasing cytochrome c initiate caspase cleavage. Hence, when nicotine only presents, Bcl blocks Bim and Bax production and mitochondria ion channel so that caspase made from p53 gene and mitochondria hardly cleave; when Parthenolide only presents, not as many p53 gene expression happens as nicotine presents so that little caspase is made. However, a combination of them obtain both condition optimal for apoptosis. As a result, result 1 partially support our hypothesis: it supports the prediction that Parthenolide has effect on inhibiting cancer cell proliferation and promoting apoptosis, but undermines the prediction that Parthenolide has certain relationship with nicotine. This result is not possible.

2.3.2 Group 2

Increase amount of Parthenolide and fixed amount of nicotine.

Cell viability is measured under increasing amount of Parthenolide and fixed amount of nicotine. Nicotine concentration is 50nM; concentration of three added substance is ranged from 1 to 100nM; cell viability at beginning is 91.6% in selected serum.

Table 2. Cell viability under increasing amount of Parthenolide and fixed amount of nicotine

Parthenolide	100nM	70nM	40nM	20nM	5nM	2nM	1nM
Cell viability	15% (±1.1)	20% (±1.3)	30% (±0.9)	60% (±1)	70% (±1)	80% (±1)	92% (±1)

Table 3. Cell viability under increasing amount of Doxorubicin and fixed amount of nicotine

Doxorubicin (positive control)	100nM	70nM	40nM	20nM	5nM	2nM	1nM
Cell viability	19%(±1.1)	23%(±1.3)	29%(±0.9)	67%(±1)	74%(±1)	87%(±1)	91%(±1)

Table 4. Negative Control

Placebo (negative control)	100nM	70nM	40nM	20nM	5nM	2nM	1nM
Cell viability	91%(±1.1)	92%(±1.3)	91%(±0.9)	91%(±1)	90%(±1)	92%(±1)	92%(±1)

Table 5 Cell viability in Parthenolide, Doxorubicin, and Placebo

	Parthenolide	Doxorubicin (positive control)	Placebo (negative control)
Cell Viability	Decreasing	Decreasing	unchanged
Average rate of change	0.77	0.72	0.01
Average value of cell viability (Euler method result)	37.82%	38.41%	91.3%

Table 6 Statistical significance

	Parthenolide&Doxorubicin	Parthenolide&Placebo	Doxorubicin&Placebo
Significance	-	+	+

Increase amount of Parthenolide increases apoptosis due to the increase expression of P53 and caspase3&9. This result is possible and support the hypothesis. Fixed amount of nicotine has a fixed

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Ras-Raf-Erk2 pathway, while Bcl-2 expression still depends on Parthenolide amount.[9] When Parthenolide increase, the Bax and Bim production and is direct to cytochrome C, reflecting the fact that Parthenolide down regulated Bcl-2 level since nicotine level is unchanged as a control. mRNA transcription of p53, Bax, Bim, GADD45 increases comparing with negative control in group one whereas relative increase rate is slower than group one. Comparing with positive control, the Parthenolide has similar effect as Doxorubicin; there is no significant difference comparing their margin of error so that Parthenolide does have effect on cancer cell.

2.3.3 Group 3

Increasing amount of nicotine and fixed amount of Parthenolide(50nM)

Result one:

Table7 Cell viability under increasing amount of nicotine and fixed amount of Parthenolide

Nicotine and 50nM Parthenolide	100nM	70nM	40nM	20nM	5nM	2nM	1nM
Cell viability	16%(±1.1)	26%(±1.3)	30%(±0.9)	35%(±1)	41%(±1)	44%(±1)	45%(±1)

Table 8 Negative control

Placebo (negative control)	100nM	70nM	40nM	20nM	5nM	2nM	1nM
Cell viability	91%(±1.1)	92%(±1.3)	91%(±0.9)	91%(±1)	90%(±1)	92%(±1)	92%(±1)

Result two:

Table 9 Cell viability under increasing amount off nicotine and fixed amount of Parthenolide

Nicotine and 50nM Parthenolide	100nM	70nM	40nM	20nM	5nM	2nM	1nM
Cell viability	47%(±1.1)	48%(±1.3)	47%(±0.9)	48%(±1)	46%(±1)	48%(±1)	47%(±1)

Table 10 Negative control

Placebo (negative control)	100nM	70nM	40nM	20nM	5nM	2nM	1nM
Cell viability	91%(±1.1)	92%(±1.3)	91%(±0.9)	91%(±1)	90%(±1)	92%(±1)	92%(±1)

Table 11 Comparison between Parthenolide group and Negative control group

	Decreasing Viability	
	Result one	Result two
Nicotine and 50nM Parthenolide	+	-
Nicotine and Placebo (negative control)	-	-

Result one is possible. However, if without Parthenolide in this group, the result is not clear so that only nicotine is not considered, but Parthenolide is a decisive substance associating with nicotine to completely change the signaling pathway. In group three, nicotine increase certainly increase cell proliferation through Bcl expression and caspase cleavage inhibition, also enhancing growth factor dependent DNA replication. These contribute to the increase cell division so that cell viability is likely to be high. But considering the effect of Parthenolide on intrinsic apoptosis pathway, NF-kb, caspase cleavage, cytochrome C release and relating Bax&Bim expression both alter or inhibit certain nicotine induced pathway. Bcl and NF-kb pathway is directly inhibited by Parthenolide that inhibit cell proliferation. Ras-Raf-ERK2 pathway is interrupted through destruction and dephosphorylation of relay molecule as well as binding with nACE2 receptor to inhibit growth factor formation which also lead to decrease proliferation. Nicotine itself will not inhibit caspase formation but inhibit caspase cleavage. A lot of caspase is likely to be produced through DNA damage while cell expose to nicotine. The aim of this group is to enhance the hypothesis so that high amount of Parthenolide is

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added in order to ensure all reaction happens when nicotine amount is increasing. This result is also an alternative explanation for group one and the hypothesis.

Result two completely undermines the hypothesis and is not possible. Increasing amount of Parthenolide has no effect while exposing to Parthenolide. The fate of cell is determined through the ratio between proapoptotic factor and anti-apoptotic factor. Inhibition of NF-kb and ERK directly lower anti-apoptotic factor. More caspase, the proapoptotic factor, is made under the exposure from nicotine. The caspase are precursor for apoptotic complex so that once present, it only requires activation. The ratio of proapoptotic factor to anti-apoptotic factor is the greatest in combination group. Hence, result two is not a plausible result

3. Discussion

In order to clearly understand the role of Parthenolide in inducing apoptosis, we evaluated the expression of several apoptosis-related genes. Topo I and II (DNA topoisomerases) are ribozymes necessary for DNA replication and mitosis. They increase in expression in actively dividing cells and induce transient DNA breaks during DNA replication and transcription to overcome topological problems. Inhibition of these enzymes leads to permanent DNA fragmentation and apoptosis induction. In our study, the expression levels of both enzymes did not decrease after Parthenolide treatment compared with the negative control. This suggests that Parthenolide alone induced apoptosis without changing the expression level of DNA topoisomerase, which was achieved by changing other factors in the process of apoptosis. E2f1 up-regulates P53 and inhibits cell proliferation, leading to p53-dependent apoptosis. P53 is an important transcription factor that induces apoptosis by controlling the transcription of hundreds of genes, including genes of some Bcl-2 family and many Caspases. In our study, we observed increased expression of E2f1 and P53 in cells treated with Parthenolide and in cells treated with Parthenolide and nicotine. In addition, both treatments showed overexpression of downstream genes (GADD45, BAX, BIM, CASP7, CASP8, CASP9, and CASP3) and low expression of Bcl-2. These results showed that single Parthenolide could induce p53-dependent apoptosis and confirmed that a only Parthenolide could exclude the anti-apoptotic effect of nicotine. Growth arrest and DNA damage induction (GADD) gene GADD45 is a p53 reactive stress protein that can be activated by a variety of DNA damage factors. In many cancers, low Gadd45 expression is associated with increased resistance to topoisomerase inhibitors. The Bcl-2 family includes anti-apoptotic (e.g., Bcl-2) and pro-apoptotic (e.g., Bax and Bim) members.[7]

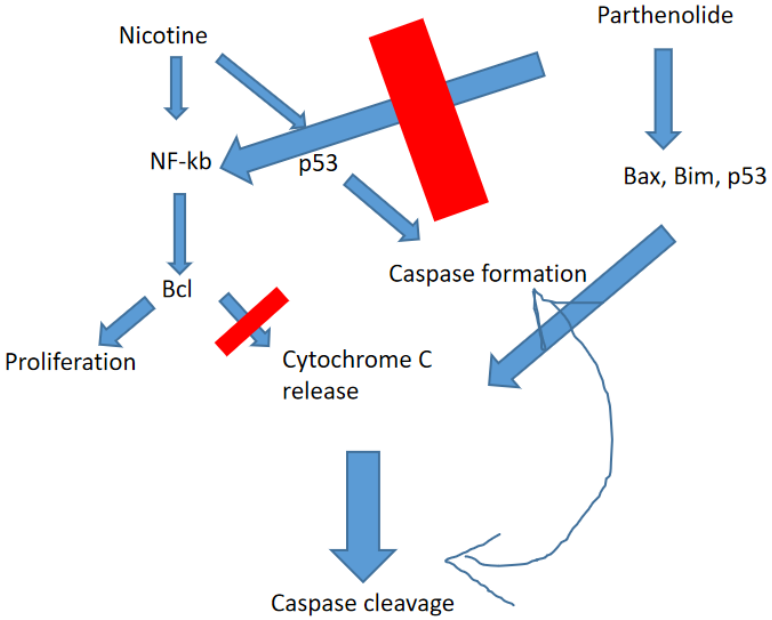

Figure.3 Signaling pathway under nicotine and Parthenolide

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4. Conclusion

The fate of cells (live or die) depends on the ratio of pro-apoptotic cells to anti-apoptotic cells. In this study, Bax and Bim were measure as the representatives of pro-apoptotic members, and Bcl-2 was selected as the anti-apoptotic members. On one hand, Parthenolide enhanced Bax and Bim expression, which further induced apoptosis by activating the caspase-dependent pathway. On the other hand, Parthenolide decreased NF-kb gene expression, hence preventing the production of anti-apoptotic Bcl-2 which would inhibit cytochrome C release. Hence, if Bcl-2 were not released, caspase would be able to cleave so that apoptosis could be triggered. Our results showed that Bax/Bim was up-regulated and Bcl-2 was down-regulated in Parthenolide treated cells. This reaction was also observed in cells treated with Parthenolide under the action of nicotine. The results simultaneously confirmed that p53-dependent apoptosis is induced by Parthenolide and Parthenolide can counteract the anti-apoptotic effect of nicotine.

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