THE EFFECTS OF KOREAN RED GINSENG SAPONIN ON THE GROWTH AND DIFFERENTIATION OF HUMAN PERIODONTAL LIGAMENT CELL IN CULTURE

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I. Introduction

Periodontal disease is characterized by a loss of attachment apparatus, loss of alveolar bone from teeth. With the onset of periodontal disease, pockets develop, with associated loss of periodontal ligament(PDL) and alveolar bone[1]. Therefore, the goal of periodontal therapy is to regenerate the loss of periodontal attachment apparatus[2].

In the previous study, the PDL cells possess the ability to reestablish connective tissue attachment[3]. Melcher[4] suggested that the design of surgical procedures was allowed colonization of wounds coronal to the alveolar crest by cells derived from PDL and bone rather than by cells derived from lamina propria of gingiva or bone alone. Therefore, PDL contributes to regeneration of the periodontium. The cells of PDL have been target subjects to study on the aspect of periodontal regeneration. Arnold and Baram[5] have attempted the *in vitro* cultivation of cells of PDL which have been successfully isolated from *Macaca multatta*(rhesus monkey) tissue utilizing the explant technique. Ragnarsson et al.[6] have reported to provide a rapid, repeatable method to obtain fibroblast cell culture from the PDL of single human teeth. Some investigators have reported the culture of PDL cell with other in fluencing factor, such as growth factor, toxic molecules hand cytotoxic substance. For example, fibronectin and endothelial growth factor have enhanced PDL cell proliferation and migration[7] and platelet-dervied growth factor-AA(PDGF-AA) and PDGF-BB is major mitogens for human PDL cells *in vitro*[8].

This experiments were designed to difine the growth and differentiation of PDL cells. Using the Korea red ginseng saponin, which is known to world-wide and the effects of it have been investigated by many reserchers for years. Ginseng saponin, one of the major components of Korea ginseng(Pannax ginseng, C. A. Meyer) root, has many various biologic effects, such as cytotoxic effect[9,10], tumoricidal activity[11], protein biosynthesis[12] and membrane modifying effect[13].

The present investigation was undertaken to determine the effects of Korean red ginseng saponin on the growth and differentiation of PDL cells according to the investigation of cell proliferation, alkaline phosphatase activity using histochemistry and biochemistry and *in vitro* bone nodule formation.

II. Materials and Methods

1. Isolation of PDL cells

PDL cells were isolated by a modification of the method of Ragnarsson et al.[6] Periodontally healthy premolars extracted for orthodontic reasons were obtained fresh from the oral Maxillofacial Surgery Department at Dankook dental hospital. The teeth were washed 3 times with 5ml of Hank's Balanced Salt Solution (HBSS, Gibco). The crown portion was dipped in a microtube with 5% sodium hypochlorite solution for 2min to romove bacteria and remaining gingival tissue. The teeth were washed 3 times with 5ml of HBSS. Each tooth was placed in a 15ml centrifuge tube(Corning) with 5ml of 0.05% trypsin(Gibco), 0.5 mM ethylenediamine tetraacetic acid(EDTA, Gibco) and 0.1% collagenase. The tube was placed in water-bath at 37℃ for 2 hours with vigorous shaking. After incubation the isolated cells were centrifuged at 200xg for 6min. After the supernatant was discarded, the cell pellet was resuspended in 4ml of Dulbecco's modified eagle medium(DMEM, Gibco) containing 10% fetal bovine serum(FBS, Gibco) in the tube. And the cells were plated into 35mm culture dishes(Corning). The dishes were incubated in a humidified environment of 95% air, 5% CO_2 at 37℃ for overnight to allow attachment and then the dishes were incubated with 2ml of DMEM containing 10% FBS to change every other day.

2. Cytotoxic effect of total saponin in cultured PDL cell

Total saponin was supplied from Korea Ginseng and Tobacco Reserch Institute(KGTRI). Stock solution of total saponin was prepared in Dulbecco's phosphate buffered saline (DPBS, Gibco) to make final concentration of each 100mg/ml.

When confluence was attained, the cells were subcultured by trypsinization. The cells were transfered to 15ml centrifuge tube and centrifuged at 800xg for 6 min. The cell pellet was resuspended in 12ml of DMEM containing 10% FBS and plated into a 24-well plate (Corning) at a density of 6×10^3 cells per well. The cells in 24-well plates were incubated for 24 hours to allow attachment and then the plate was added to each total saponin concentration(0-1mg/ml). After the cells were incubated for 24 hours, added to 0.2% trypan blue then the cells were counted at randomly selected field under the light microscope.

3. Effect of total saponin on the cell viability of PDL cell

PDL cells were plated into a 24-well plate at 6×10^3 cells/well and cultured with 1ml of DMEM to change every other day for 1 week. Then cells were treated with 0.01, 0.1, 1 and 10μg/ml of total saponin for 1 week. Then the cells were stained with 0.2% trypan blue to count the survival and total cell number under the light microscope.

4. Effect of total saponin on the proliferation of PDL cell

1) Cell proliferation by various concentration

PDL cells were plated into a 24-well plates at 5×10^2 cell per well and cultured with 1ml of DMEM to change every other day for 1 week. Then 0.1, 1, 10 and 100μg/ml of total saponin and the media was changed every other day for 1 week. Two weeks after seeding, PDL cells were trypsinized and cell number was counted with hemocytometer.

2) Time course of cell proliferation

PDL cells were seeded into a 24-well plates at 9×10^2 cells per well and cultured with 1ml of DMEM to change every other day for 1

week. Cells were cultured in the presence or absence of total saponin(1μg/ml). After culture of 1, 3, 5, 7 and 9th days, the cells were trypsinized and added to 0.2% trypan blue then the cell number was counted with hemocytometer.

5. Effect of total saponin on ALP activity of PDL cell

PDL cells were seeded into a 24-well plate at 9×10^2 cell per well and cultured until confluence was obtained. Then the cells were cultured with 1ml of DMEM containing with total saponin(0.1-10μg/ml) to change every other day for 4 days. After removal of the culture media from the wells, the cells in each well were washed twice with DPBS. The cells were trypsinized, ultrasonicated and centrifuged at 1500xg for 15min. After centrifugation, the supernatant was used to ALP assay. In each well was added to 50μl of 15mM p-nitrophenylphosphate(p-Npp, Sigma) and allowed to proceed at 37℃ for 30min. To terminate the enzyme reaction, the each well was added to 100μl 1N NaOH, followed by spectrophotometer at 410nm to determine the absorbance of released p-nitrophenol. The protein content was measured by the method of Lowry using bovine serum albumin(BSA, Gibco) as a standard.

6. Effect of total saponin on ALP activity of PDL cell by histochemistry

PDL cells were seeded into a 24-well plate at 9×10^2 cell per well and cultured with 1 ml of DMEM to change every other day for 1 week. Then the cells were treated with 1 μg/ml of total saponin for 1 week. After culture, the cells were washed with DPBS and were fixed with the citrate-acetone-formaldehyde and were stained with alkaline phosphatase kit(Sigma). Naphthol AS-BI phosphate was used as substrate and the fast blue BB as coupling factor. After the reaction was proceeded at room temperature for 15min, the cells were counter stained with neutral red solution(Sigma). The cells were observed at ×100, ×200 by light microscope.

7. Effect of total saponin *in vitro* bone nodule formation of PDL cell

PDL cells were plated into two 35mm culture dish at 5×10^3 cell per dish and cultured for 2 weeks. Each dish was added to 3ml of DMEM containing 50μg/ml ascorbic acid, and 10mM β-glycerophosphate. The cells of the control group was added to 10μl DPBS and the cells of the test group was added to 1μg/ml total saponin. The dishes were changed media contained with 1μg/ml of total saponin every other day and cultured for 3 weeks. To observe the produced calcified materials, Each dish was stained by von Kossa's staining. After the media was discarded. Each dish was washed 2 times with 2ml of DPBS. And each was added to 2ml of 2% paraformaldehyde(PFA, Sigma) and incubated for 10min. After the PFA was removed, each dish was washed with 1ml of 0.1M cacodylate buffer. Each dish was added to 2ml of 0.1M cacodylate buffer and was incubated for 30min. And each dish was added to 1ml of 3% silver nitrate solution(Sigma). All the procedures were performed on the ice. And each dish was incubated for 30min under the sun light and washed tapwater for 10min. After the procedure was performed, each dish was counter stained with toludine blue and stained calcified materials were observed microscopically.

8. Statistical Analysis

Sample mean and standard error of the means were determined by Student's *t* test.

Ⅲ. Result

1. Cytotoxic effect of the total saponin in cultured PDL cell

After 0.01, 0.1, 1, 10, 100 and 1000μg/ml of total saponin were added in the DMEM and the PDL cells were cultured for 7 days. The cytotoxic effect of total saponin on the PDL cells were increased significantly by 1 mg/ml concentration($P<0.05$)(Table 1).

2. Effect of total saponin on the cell viability of PDL cell

After 0.01, 0.1, 1 and 10μg/ml of total saponin was added in the DMEM and the PDL cells were cultured 7 days. The cell viability was increased significantly in all concentrations(Table 2).

Table 1. Effect of total saponin on cytotoxicity of cultured PDL cell

TS(μg/ml)	Survival cell rate(%) Mean± SE
Control	94.78± 2.19
0.01	93.82± 1.10
0.1	97.32± 0.60
1	96.22± 0.96
10	95.42± 0.53
100	93.05± 1.37
1000	87.49± 1.37*

TS : Total saponin, *P : <0.05

Table 2. Effect of total saponin on cell viability of cultured PDL cell

TS(μg/ml)	Cell viability(%) Mean± SE
Control	92.06± 0.92
0.01	97.48± 0.32**
0.1	98.53± 0.08**
1	98.41± 0.36**
10	96.25± 2.30*

TS : Total saponin, **P : <0.01, *P : <0.05

3. Effect of total saponin on the proliferation of PDL cell

1) Cell proliferation by various concentration

Table 3 shows the number of PDL cells that were cultured for 7 days after 0.1, 1, 10 and 100μg/ml of total saponin was added in the DMEM. 0.1, 1 and 10μg/ml of total saponin increased the proliferation of PDL cells significantly. However, after the test group was cultured in DMEM containing 100μg/ml of total saponin, the proliferation of PDL cells were lower than the control.

2) Time course of cell proliferation

The effect of total saponin on proliferation of PDL cells culturing in 1μg/ml of total saponin for 1 to 9 days was increased significantly, as the time span increased(Table 4).

4. Effect of total saponin on ALP activity of PDL cell

In case of 0.1, 1 and 10μg/ml of total saponin adding to the DMEM, 10μg/ml of total saponin increased the activity of alkaline phosphatase activity significantly($P<0.01$)(Table 5).

5. Effect of total saponin on ALP activity of PDL cell by histochemistry

Fig. 1, 2 and 3 showes that ALP positive cells were seen in cultured PDL cells by histochemistry.

Table 3. Effect of total saponin on the proliferation of PDL cell

TS(μg/ml)	Cell number($\times 10^4$) Mean± SE
Control	7.50± 0.50
0.1	10.50± 0.50*
1	11.50± 0.50**
10	11.00± 0.58*
100	5.50± 0.50*

TS : Total saponin, **P : <0.01, *P : <0.05

Table 4. Effect of total saponin on the proliferation of cultured PDL cell

Day		Cell number($\times 10^4$) Mean± SE
1	C	3.50± 0.50
	TS	4.50± 0.50
3	C	5.50± 0.50
	TS	7.50± 0.50*
5	C	6.50± 0.50
	TS	10.50± 0.50**
7	C	7.50± 0.50
	TS	11.50± 0.50**
9	C	8.50± 0.50
	TS	12.50± 0.50**

C : Control, TS : Total saponin(1μg/ml), *P : <0.05

Table 5. Effect of total saponin on ALP activity of PDL cell

TS(μg/ml)	*Alkaline phosphatase activity* (n mol PNP cleaved/h/mg protein) Mean± SE
Control	149.18± 8.92
0.1	155.90± 5.23
1	151.50± 5.06
10	217.53± 10.29**

6. Effect of total saponin *in vitro* bone formation of PDL cell

Fig. 4 and 5 showes that the bone nodule formation was seen in cultured PDL cells by von Kossa's staining.

Ⅳ. Discussion

The present study demonstrates that the total saponin have several biologic properties on the growth and differentiation of PDL cells.

The cytotoxic effect of total saponin on PDL cells was shown at 1mg/ml concentration. This finding is similar to the reports by other investigators. The ginseng saponin has no tumoricidal effect but it increases the tumoricidal activity of K 562 tumor cells through the tumoricidal activity of the macrophage[11] and inhibit the development of sarcoma 180 and ginseng fraction stimulates the release of TNF from macrophage[14]. As the other study, combined administration of ginseng extract and vit. C demonstrates a synergistic inhibition of cancer cell growth in mouse[11].

In the effect of total saponin on cell viability of PDL cells, after 0.01, 0.1, 1 and 10μg/ml of total saponin was added in the medium and the cells were cultured for 7 days, cell viability was increased significantly in all concentration. 0.1 and 1μg/ml of total saponin was the similar result of cell viability. Therefore, these concentrations may have protective effect on the PDL cells. Hahn and Kim[13] studied preliminary investigation of membrane modifying effects of ginseng components. They suggested the preventive effect of ginseng component on the degranulation of mast cells in the guinea pig mesentery by compound 48.80 and venom toxin.

In the effect of total saponin on cell proliferation of PDL cells, 0.1 1, 10 and 100μg/ml of total saponin were added in the medium and the cells were cultured 7 days, the cell proliferation was increased in 0.1, 1 and 10 μg/ml of total saponin. As the similar studies about the proliferation of PDL cells, Limeback [15] and Ten Cate[16] described that PDL cell is capable of collagen synthesis. Terranova[17,18] reported that fibronectin, the extracellular matrix protein, have been shown to selectively enhance PDL cell adhesion, migration and proliferation. Somerman et al.[19] studied PDL cells and gingival fibroblasts responded differently to attachment factors *in vitro*. They suggested that fibronectin enhanced the attachment and the spread of PDL cells and gingival

fibroblasts. In this study, total saponin may have the proliferative effect on PDL cells. However, the cell proliferation from cultures incubated with 10μg total saponin/ml medium were retarded compared to the control. This result indicates that the low concentration of total saponin on cell proliferation is more effective than the high concentration of total saponin. Thus, the high concentration(100 and 1000μg/ml) of total saponin is rather cytotoxic effect than proliferation.

The proliferation of PDL cells was noted at regular interval and cultures were counted at 1, 3, 5, 7 and 9 days after the addition of 1μg/ml of total spaonin. As the time sapn increased, the cell proliferation was increased significantly.

PDL cells in culture are assayed for ALP activity, as a marker enzyme for bone cells[20]. PDL cells resembles that present in osteoblasts, human PDL cells may function as osteoblastic fibroblasts[21]. In our study on the effect of total saponin on ALP activity of PDL cells, 10μg/ml of total saponin in creased the ALP activity significantly. AS the similar studies, many authors described that PDL cells have high ALP acitivity significantly[22] and phenotypes typical of osteoblasts include intense ALP activity[23]. Melcher[4] suggested that the cells of the periodontal ligament are responsible not only for osteogenesis and osteoclasis, but also for fibrogenesis and fibroclasis in the ligament itself, and cementogensis. Aukhil et al.[24] suggested that the progenitor cells from the PDL would differentiate into cementoblasts when they come in contact with root dentin. In this experiment, after PDL cells were incubated in media containing 0.1, 1 and 10μg/ml of total saponin for 4 days. ALP activity was increased by the addition fo 10μg/ml of total saponin. Thus, it could be concluded that total saponin may stimulate the differentiation of PDL cells.

This study of human PDL cells demonstrates that the total saponin has the cytotoxic effect on the PDL cells and influence on the growth and differentiation of the PDL cells. Our results suggest that the effect of total saponin and these reactions on growth and differentiation of PDL cells are very interesting and the total saponin may act as a growth factor on the PDL cells. Futher investigation must be performed to elucidate the action of total saponin on the PDL cells.

V. Conclusion

This study was performed to define the cytotoxic effect of total saponin, major compartment of Korean red ginseng, on cultured PDL cells and knowing the effect of the total saponin on growth and differentiation of the PDL cells *in vitro*. The results are as follows

1. After the various concentrations of total saponin were added in the medium, the cytotoxic effect of total saponin on the PDL cells were shown at 1mg/ml of total saponin.
2. Seven days culture after addition of 0.01, 0.1, 1 and 10μg/ml total saponin, the cell viabilities were increased significantly in all concentration.
3. Seven days culture after addition of 0.1, 1, 10 and 100μg/ml of total saponin, the cell proliferation was increased significantly in 0.1, 1, and 10μg/ml of total saponin.
4. 1μg/ml of total saponin increased the proliferation of PDL cells, as the time span increased.
5. 10μg/ml of total saponin increased the ALP activity significantly.
6. ALP positive cells were seen in cultured PDL cells by histochemistry.
7. The bone nodule formation was seen in

cultured PDL cells by von Kossa's staining.

On the basis of above results, it is concluded that total saponin may be possible to affect the proliferation and differentiation of PDL cells in culture.

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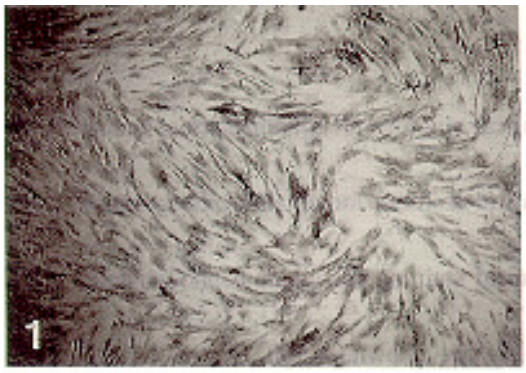
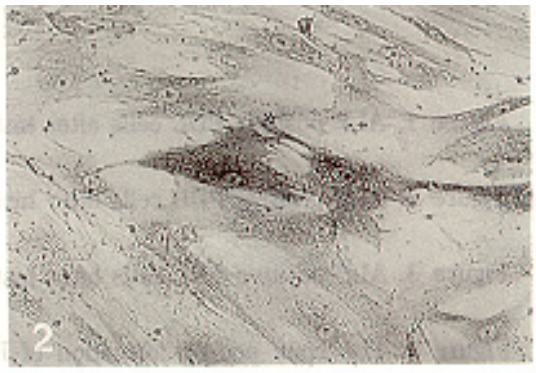
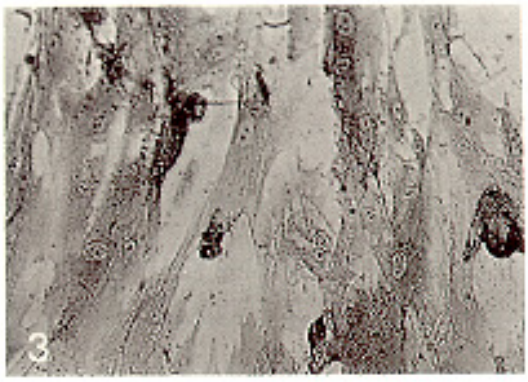
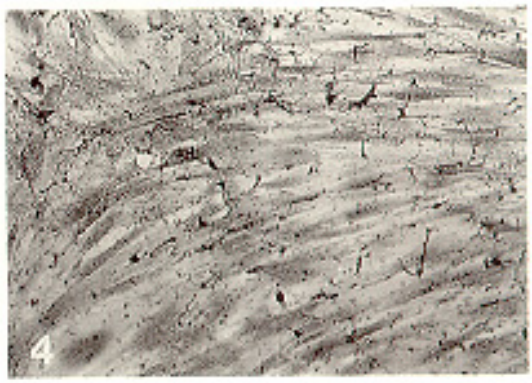
LIST OF FIGURES

논문사진부도









한국 홍삼 사포닌이 배양중인 치주인대 세포의 성장 및 분화에 미치는 영향

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치주질환의 치유에 있어서 치주인대 세포의 증식과 분화는 매우 중요하다. 몇몇 학자들에 의해 치주인대 세포의 증식과 분화에 영향을 주는 platelet derived growth factor나 fibronectin과 같은 growth factor에 대한 연구가 있었다. 이 연구는 홍삼 총사포닌이 치주인대 세포에 미치는 세포독성과 세포의 성장 및 분화에 미치는 영향을 규명하고자 사람의 치주인대 세포를 분리, 배양하여 실험하였다.

총사포닌이 치주인대 세포에 미치는 세포독성을 특정하기 위해 여러가지 농도의 총사포닌을 세포배양액에 첨가하여 1주일 배양후의 결과와 단일 농도(1μg/ml)하에서의 세포 성장을 혈구계산반을 사용하여 관찰하였다. 치주인대 세포가 조골세포양세포로의 분화과정에 사포닌이 영향을 미치는 것을 관찰하기 위해 개개의 총사포닌 농도)0.1, 1, 10μg/ml)를 세포배양액에 첨가하여 배양하였다. 치주인대 세포가 조골세포의 표현형으로 분화되는데 미치는 총사포닌의 영향을 알아보기 위하여 총사포닌의 단일 농도(1μg/ml)하에서 50μg/ml ascorbic acid와 10mM β-glycerophosphate를 배양액에 첨가하여 배양후 von Kossa's staining을 시행하여 생성된 골결절을 관찰하였다.

이상의 실험에서 얻어진 결과는 아래와 같다.

1. 각각의 농도를 투여한 결과, 1μg/ml의 총사포닌에 의해서 세포독성이 유의성있게 증가하였다.
2. 0.01, 0.1, 1, 10μg/ml의 총사포닌을 세포배양액에 첨가한 다음 7일 후의 관찰시 cell viability가 실험농도 모두에서 유의성있게 증가하였다.
3. 0.1, 1, 10, 100μg/ml의 총사포닌을 세포배양액에 첨가한 다음 7일 후의 관찰시, 0.1, 1, 10μg/ml의 농도에서 유의성있는 세포 증식이 있었다.
4. 1μg/ml의 총사포닌을 세포배양액에 첨가한 다음 1, 3, 5, 7, 9일의 관찰시 시간경과에 따라 유의성있는 세포 증식이 있었다.
5. 10μg/ml의 총사포닌을 세포배양액에 첨가시 ALP activity가 대조군에 비해 유의성있게 증가하였다.
6. 1μg/ml의 총사포닌으로 배양된 치주인대 세포내에서 ALP positive cell이 관찰되었다.
7. 1μg/ml의 총사포닌으로 배양된 치주인대 세포내에서 골결절 형성이 관찰되었다.