เป็นหนังสือภาษาอังกฤษ

รายงานวิจัยฉบับสมบูรณ์

โครงการ
เอสโทรเจนยับยั้งการสร้างเซลล์สลายกระดูกซักนำโดยเซลล์ไฟโบรบลาสต์ของเอ็นยึดปริทันต์
Oestrogen inhibits osteoclast formation induced by periodontal ligament fibroblasts

โดย
ผศ.ดร.ทพญ.นัทธมน วัฒนอรุณวงศ์ และคณะ

พฤศจิกายน 2553

กิตติกรรมประกาศ

ผู้วิจัยขอขอบพระคุณ รศ.ดร.อุรีพร เล็กกัด ภาควิชาชีววิทยาช่องปากและระบบการบดเคี้ยว คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ และ ศาสตราจารย์ ดร.วินเซนต์ อีเวิรตส์ คณะทันตแพทยศาสตร์ มหาวิทยาลัยอัมเตอร์ดัม ประเทศเนเธอร์แลนด์ ผู้เป็นที่ปรึกษาโครงการวิจัยนี้ และขอขอบพระคุณกรรมการผู้ทรงคุณวุฒิผู้ประเมินโครงการวิจัยนี้ ทำให้ผู้วิจัยมีโอกาสได้ทำงานวิจัยนี้จนแล้วเสร็จ งานวิจัยนี้ได้รับทุนวิจัยจาก สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) และสำนักงานคณะกรรมการอุดมศึกษา (สกอ.) ซึ่งเป็นหน่วยงานที่ส่งเสริมและสนับสนุนการทำวิจัยในสาขาวิชาการด้านต่างๆ หลากหลายสาขา และผลงานวิจัยก่อให้เกิดประโยชน์ในการพัฒนาองค์ความรู้พื้นฐานและความรู้ประยุกต์แก่ประเทศชาติ ในท้ายสุดนี้ผู้วิจัยขอขอบพระคุณคณะผู้บริหารและเจ้าหน้าที่ของ สกว. และ สกอ. ทุกท่านที่ให้การสนับสนุนงานวิจัยนี้จนสำเร็จลุล่วงด้วยดี

Project Summary

Project Code : MRG5080025

(รหัสโครงการ)

Project Title : Oestrogen inhibits osteoclast formation induced by periodontal ligament fibroblasts

(ชื่อโครงการ): เอสโทรเจนยับยั้งการสร้างเซลล์สลายกระดูกชักนำโดยเซลล์ไฟโบรบลาสต์ของเอ็นยึดปริทันต์

Investigator :

(ชื่อนักวิจัยและสถาบัน)

1. Nutthamon Wattanaroonwong (Head)	Department of Oral Biology and Occlusion, Faculty of Dentistry, Prince of Songkla University
นัทธมน วัฒนอรุณวงศ์ (หัวหน้าโครงการ)	ภาควิชาชีววิทยาช่องปากและระบบการบดเคี้ยว คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์
2. Ton Schoenmaker	Department of Periodontology, University of Amsterdam and VU University Amsterdam, The Netherlands
3. Teun J de Vries	Department of Periodontology, University of Amsterdam and VU University Amsterdam The Netherlands
4. Vincent Everts	Department of Oral Cell Biology, University of Amsterdam and VU University Amsterdam, Research Institute MOVE The Netherlands

E-mail Address : nutthamon.w@psu.ac.th

Project Period : 3 years and 11 months (1 December 2006 – 10 November 2010)

(ระยะเวลาโครงการ)

เอสโทรเจนยับยั้งการสร้างเซลล์สลายกระดูกชักนำโดยเซลล์ไฟโบรบลาสต์ของเอ็นยึดปริทันต์

บทคัดย่อ

วัตถุประสงค์: เนื่องจากเซลล์ไฟโบรบลาสต์บริเวณฟันมีส่วนร่วมในการสร้างเซลล์สลายกระดูก และยังไม่เป็นที่ทราบว่าเอสโทรเจนมีผลต่อกระบวนการสร้างเซลล์สลายกระดูกบริเวณฟันอย่างไร ดังนั้นจึงทำการศึกษาผลของ 17 เบต้า-เอสทราไดออลต่อการสร้างเซลล์สลายกระดูกซึ่งชักนำโดยเซลล์ไฟโบรบลาสต์ของเอ็นยึดปริทันต์ในคนและเซลล์ไฟโบรบลาสต์ของเนื้อเหงือกในคน **วิธีการทดลอง:** นำเซลล์โมโนนิวเคลียร์จากเลือดของคนมาเลี้ยงบนเซลล์ไฟโบรบลาสต์ของเอ็นยึดปริทันต์ในคนและเซลล์ไฟโบรบลาสต์ของเนื้อเหงือกในคน และทำการเพาะเลี้ยงเซลล์ร่วมเป็นเวลา 14 วัน โดยมีหรือไม่มี 17 เบต้า-เอสทราไดออลในความเข้มข้นต่าง ๆ แล้วนับจำนวนของเซลล์ที่ย้อมติดทาร์เทรตรีซิสแทนเอซิดฟอสฟาเทส ซึ่งบ่งชี้ว่าเป็นเซลล์สลายกระดูก นอกจากนี้ทำการวิเคราะห์การแยกตัวกลับของเซลล์ไฟโบรบลาสต์ชักนำโดยเซลล์โมโนนิวเคลียร์จากเลือดของคน และทำการวิเคราะห์การแสดงออกของยีนตัวรับเอสโทรเจนชนิดแอลฟา (อีอาร์-แอลฟา) และ อีอาร์-เบต้า, รีเซปเตอร์แอกทิเวเตอร์นิวเคลียร์แฟกเตอร์แคปปาบีไลแกน (อาร์เอเอ็นเคแอล) และออสทิโอโปรทิเจริน (โอพีจี) ในเซลล์ไฟโบรบลาสต์ของเอ็นยึดปริทันต์ในคนและเซลล์ไฟโบรบลาสต์ของเนื้อเหงือกในคน **ผลการทดลอง:** เซลล์โมโนนิวเคลียร์จากเลือดของคนเมื่อนำมาเพาะเลี้ยงร่วมกับเซลล์ไฟโบรบลาสต์จะชักนำให้เกิดพื้นที่ที่ไม่มีเซลล์ไฟโบรบลาสต์ซึ่งพื้นที่นี้จะเป็นบริเวณที่สร้างเซลล์สลายกระดูก ในการเพาะเลี้ยงเซลล์ร่วมระหว่างเซลล์โมโนนิวเคลียร์จากเลือดของคนกับเซลล์ไฟโบรบลาสต์ของเอ็นยึดปริทันต์ในคนพบว่ามีจำนวนพื้นที่ที่ไม่มีเซลล์ไฟโบรบลาสต์มากกว่าและขนาดพื้นที่ใหญ่กว่าเมื่อเปรียบเทียบกับการเพาะเลี้ยงเซลล์ร่วมระหว่างเซลล์โมโนนิวเคลียร์จากเลือดของคนกับเซลล์ไฟโบรบลาสต์ของเนื้อเหงือกในคน นอกจากนี้พบว่าในการเพาะเลี้ยงเซลล์ร่วมระหว่างเซลล์โมโนนิวเคลียร์จากเลือดของคนร่วมกับเซลล์ไฟโบรบลาสต์ของเอ็นยึดปริทันต์ในคนมีจำนวนเซลล์สลายกระดูกมากกว่า อนึ่ง 17 เบต้า-เอสทราไดออลไม่เปลี่ยนแปลงการแสดงออกของยีนอาร์เอเอ็นเคแอล โอพีจี และอีอาร์-แอลฟาในกลุ่มประชากรของเซลล์ไฟโบรบลาสต์ทั้ง 2 กลุ่ม **สรุปผลการทดลอง:** ข้อมูลนี้บ่งชี้ว่าเซลล์ไฟโบรบลาสต์ของเอ็นยึดปริทันต์ในคนส่งเสริมการสร้างเซลล์สลายกระดูกมากกว่าเซลล์ไฟโบรบลาสต์ของเนื้อเหงือกในคน และพบว่า 17 เบต้า-เอสทราไดออลยับยั้งการสร้างเซลล์สลายกระดูกชักนำโดยเซลล์ไฟโบรบลาสต์ของเอ็นยึดปริทันต์ในคน ดังนั้นผลการยับยั้งการสร้างเซลล์สลายกระดูกของเอสโทรเจนขึ้นอยู่กับชนิดของเซลล์

คำสำคัญ: เอสโทรเจน เอ็นยึดปริทันต์ เซลล์สลายกระดูก เซลล์ไฟโบรบลาสต์ของเนื้อเหงือก เซลล์โมโนนิวเคลียร์จากเลือดของคน

Oestrogen inhibits osteoclast formation induced by periodontal ligament fibroblasts

Abstract

Objective: Since tooth-associated fibroblasts are taken to participate in the formation of osteoclasts and it is unknown whether estrogen affects this process, the effects of 17β-estradiol (17β-E_2) were studied on osteoclasto-genesis induced by human periodontal ligament (PLFs) and gingival fibro-blasts (GFs). ***Methods:*** Human peripheral blood mononuclear cells (PBMCs) were seeded on monolayers of PLFs and GFs and cocultured for 14 days in the presence or absence of various concentrations of 17β-E_2. The number of tartrate resistant acid phosphatase (TRACP)-positive osteoclast-like cells (OCs) was assessed. In addition, we analyzed the PBMC-induced withdrawal of the fibroblasts. mRNA expression was determined of estrogen receptor (ER)-α, ER-β, receptor activator nuclear factor kappa B ligand (RANKL), and osteoprotegerin (OPG) by PLFs and GFs. ***Results:*** PBMCs induced a higher number and larger fibroblast-free areas if cocultured with PLFs than with GFs. Concomitantly, the number of TRACP-positive OCs was significantly higher in PLF cocultures. 17β-E_2 inhibited the formation of OCs in PLF cocultures. 17β-E_2 did not alter the expression of RANKL, OPG, and ER-α mRNAs in either fibroblast cell population. ***Conclusions:*** Our data indicate that PLFs may promote osteoclastogenesis more strongly than GFs. 17β-E_2 inhibits the PLF-induced formation of osteoclast-like cells. Thus, the inhibitory effect of estrogen on osteoclast formation appears to be cell type dependent.

Key words: Estrogen, Periodontal ligament, Osteoclasts, Gingival fibroblasts, Human peripheral blood mononuclear cells

CONTENTS

Page

Acknowledgment iii

Project Summary iv

Abstract (Thai) v

Abstract (English) vi

Contents vii

Research Team viii

Executive Summary x

CHAPTER 1 INTRODUCTION 1

CHAPTER 2 METHODOLOGY 2

CHAPTER 3 RESULTS 5

CHAPTER 4 DISCUSSION 15

CHAPTER 5 CONCLUSION 17

CHAPTER 6 OUTPUTS 18

CHAPTER 7 REFERENCES 19

APPENDIX 23

รายชื่อคณะผู้วิจัยในโครงการ

คณะผู้วิจัย (RESEARCH TEAM) :

ชื่อหัวหน้าโครงการ

(ภาษาไทย) :	ผศ.ทพญ.ดร. นัทธมน วัฒนอรุณวงศ์
(ภาษาอังกฤษ) :	Asistant Prof.Dr. Nutthamon Wattanaroonwong
ตำแหน่งวิชาการ :	ผู้ช่วยศาสตราจารย์
สถานที่ติดต่อ :	
ที่ทำงาน	ภาควิชาชีววิทยาช่องปากและระบบการบดเคี้ยว คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อ.หาดใหญ่ จ.สงขลา 90112
โทรศัพท์:	074-429873
โทรสาร:	074-212922
E-mail:	nutthamon.w@psu.ac.th

ชื่อนักวิจัย 1

(ภาษาไทย) :	ทน ซูเอ็นเมกเกอร์
(ภาษาอังกฤษ) :	Ton Schoenmaker
สถานที่ติดต่อ :	
ที่ทำงาน	Department of Periodontology, University of Amsterdam and VU University Amsterdam, The Netherlands
โทรศัพท์:	31 20 5980882
โทรสาร:	31 20 5980882
E-mail:	t.schoenmaker@acta.nl

ชื่อนักวิจัย 2

(ภาษาไทย) :	เทิน เจ เดอร์ ฟายส์
(ภาษาอังกฤษ) :	Teun J de Vries
สถานที่ติดต่อ :	
ที่ทำงาน	Department of Periodontology, University of Amsterdam and VU University Amsterdam, The Netherlands
โทรศัพท์:	31 20 5980882
โทรสาร:	31 20 5980882
E-mail:	teun.devries@acta.nl

ชื่อนักวิจัยที่ปรึกษา 1

(ภาษาไทย) : รศ.ดร.อุรีพร เล็กกัต

(ภาษาอังกฤษ) : Assoc. Prof. Dr. Ureporn Leggat

สถานที่ติดต่อ :

ที่ทำงาน ภาควิชาชีววิทยาช่องปากและระบบการบดเคี้ยว
คณะทันตแพทยศาสตร์
มหาวิทยาลัยสงขลานครินทร์
อ.หาดใหญ่ จ.สงขลา 90112

โทรศัพท์: 074-429873

โทรสาร: 074-212922

E-mail: ureporn.k@psu.ac.th

ชื่อนักวิจัยที่ปรึกษา 2

(ภาษาไทย) : ศาสตราจารย์ ดร.วินเซนต์ อีเวิรตส์

(ภาษาอังกฤษ) : Prof. Dr. Vincent Everts

สถานที่ติดต่อ :

ที่ทำงาน Department of Oral Cell Biology, ACTA, University of Amsterdam
and VU University Amsterdam, Research Institute MOVE,
The Netherlands

E-mail: v.everts@acta.nl

โทรศัพท์: 31 20 5980882

โทรสาร: 31 20 5980882

E-mail: v.everts@acta.nl

หน้าสรุปโครงการ (Executive Summary)

1. ชื่อโครงการ

(ภาษาไทย) เอสโทรเจนยับยั้งการสร้างเซลล์สลายกระดูกชักนำโดยเซลล์ไฟโบรบลาสต์ของเอ็นยึดปริทันต์

(ภาษาอังกฤษ) Oestrogen inhibits osteoclast formation induced by periodontal ligament fibroblasts

2. ชื่อหัวหน้าโครงการ หน่วยงานที่สังกัด ที่อยู่ หมายเลขโทรศัพท์ โทรสาร และ e-mail

ชื่อหัวหน้าโครงการ ผศ.ดร.ทพญ.นัทธมน วัฒนอรุณวงศ์

หน่วยงานที่สังกัด ภาควิชาชีววิทยาช่องปากและระบบการบดเคี้ยว
คณะทันตแพทยศาสตร์
มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่
ตำบลคอหงส์ อำเภอหาดใหญ่
จังหวัด สงขลา 90112
โทรศัพท์ 074-429873
โทรสาร 074-429873
E-mail: nutthamon.w@psu.ac.th

3. สาขาวิชาที่ทำการวิจัย Cell Biology

4. งบประมาณทั้งโครงการ 480,875.- บาท

5. ระยะเวลาดำเนินงาน 3 ปี 11 เดือน

6. ความสำคัญและที่มาของปัญหา (IMPORTANCE AND MOTIVATION OF THE RESEARCH)

Estrogen deficiency has been found as one of the risk factors for periodontal disease in postmenopausal women. Estrogen-deficiency seems to enhance loss of the alveolar bone volume, leading to a more progressive form of periodontitis. Several studies show a relationship between tooth loss and alveolar ridge resorption on one hand and systemic bone loss in postmenopausal osteoporosis on the other. These data suggest that loss of alveolar bone is somehow related to more generalized bone loss which occurs in osteoporotic patients. Furthermore, radiographic studies show that estrogen deficiency in women is associated with a mean net loss in alveolar bone density. In ovariectomized rats a decrease in mandibular bone volume, an increase in periapical bone loss of first mandibular molars, an increase in cancellous bone loss in the mandibular condyle, an increase in residual ridge resorption, and an acceleration of tooth movement have been reported. It is recognized that estrogen deficiency stimulates bone resorption by increased osteoclast numbers due

to enhanced osteoclast formation and reduced osteoclast apoptosis as well as by increased osteoclast activity.

Osteoclasts are multinucleated cells derived from hematopoietic mononuclear cells. The osteoclasts attach to the bone surface and form a highly acidic environment at the site of attachment, thereby solubilizing bone minerals. Mature osteoclasts are characterized by markers such as tartrate resistant acid phosphatase (TRACP), cathepsin K, and receptors for calcitonin and vitronectin. Osteoclast differentiation and function is regulated by receptor activator nuclear factor kappa B ligand (RANKL)-RANK- osteoprotegerin (OPG) systems.

Bone resorbing osteoclasts have been shown to develop in cocultures of human fibroblasts associated with the tooth and human peripheral blood mononuclear cells (PBMCs). Tooth-associated fibroblasts are periodontal ligament fibroblasts (PLFs) and gingival fibroblasts (GFs). The morphology of these cells when cultured is similar; they are spindle-shaped and have an elongated appearance. Yet, a number of functional differences between PLFs and GFs have been reported such as a higher expression of alkaline phosphatase activity by PLFs and differences in the expression of fibronectin and collagen type I as well as integrin subunits. PLFs have been shown to induce the formation of osteoclast-like cells by up-regulation of RANKL and by a decrease of the anti-resorptive factor, OPG. In contrast with periodontal ligament fibroblasts gingival fibroblasts tend to inhibit osteoclast formation.

It has been shown that estrogen stimulates OPG expression by human osteoblasts by a mouse stromal cell line and by human PLFs. In this way the hormone could have an effect on bone metabolism via soft connective tissue cells in the periodontal domain. It was the aim of the present study to determine whether periodontal ligament fibroblasts and gingival fibroblasts do this to a comparable extent.

7. วัตถุประสงค์ (OBJECTIVES OF THE RESEARCH)

1) To analyze the formation of osteoclasts induced by human gingival fibroblasts and periodontal ligament fibroblasts and the effect of estrogen here upon.
2) To determine the mRNA expression of OPG, RANKL, estrogen receptor (ER)-α, and ER-β in human gingival fibroblasts and periodontal ligament fibroblasts in the presence or absence of estrogen.

8. ระเบียบวิธีวิจัย (METHODOLOGY)

Human peripheral blood mononuclear cells (PBMCs) were seeded on monolayers of human periodontal ligament (PLFs) and gingival fibroblasts (GFs) and cocultured for 14 days in the presence or absence of various concentrations of 17β-estradiol (17β-E_2). The number of tartrate resistant acid phosphatase (TRACP)-positive osteoclast-like cells (OCs) was assessed. In addition, we analyzed the PBMC-induced withdrawal of the fibroblasts. mRNA expression was determined of estrogen receptor (ER)-α, ER-β, receptor activator nuclear factor kappa B ligand (RANKL), and osteoprotegerin (OPG) by PLFs and GFs.

9. แผนงานวิจัย (RESEARCH PLAN)

Activities	Time (months)											
	4	8	12	16	20	24	28	32	36	40	44	48
1. primary cell (HGFs,HPLFs) cultures												
2. cocultures of HGFs/HPLFs and PBMCs												
3. TRACP analysis (for cocultures of HGFs/HPLFs and PBMCs)												
4. TRACP analysis (for only PBMCs cultures)												
5. RT-PCR												
6 Data analysis												
7. Writing reports and manuscript preparation												

10. Output:

Wattanaroonwong N, Schoenmaker T, de Vries TJ, Everts V. Oestrogen inhibits osteoclast formation induced by periodontal ligament fibroblasts. *Arch Oral Biol* doi:10.1016/j.archoralbio.2010.10.004 (impact factor, 2008 = 1.379)

CHAPTER 1 INTRODUCTION

Estrogen deficiency has been found as one of the risk factors for periodontal disease in postmenopausal women.[1,2] Estrogen-deficiency seems to enhance loss of the alveolar bone volume, leading to a more progressive form of periodontitis.[3,4] Several studies show a relationship between tooth loss and alveolar ridge resorption on one hand and systemic bone loss in postmenopausal osteoporosis on the other.[4-6] These data suggest that loss of alveolar bone is somehow related to more generalized bone loss which occurs in osteoporotic patients. Furthermore, radiographic studies show that estrogen deficiency in women is associated with a mean net loss in alveolar bone density.[7] In ovariectomized rats a decrease in mandibular bone volume,[8] an increase in periapical bone loss of first mandibular molars,[9] an increase in cancellous bone loss in the mandibular condyle,[10] an increase in residual ridge resorption,[11] and an acceleration of tooth movement [12] have been reported. It is recognized that estrogen deficiency stimulates bone resorption by increased osteoclast numbers due to enhanced osteoclast formation and reduced osteoclast apoptosis as well as by increased osteoclast activity.[13]

Osteoclasts are multinucleated cells derived from hematopoietic mononuclear cells. The osteoclasts attach to the bone surface and form a highly acidic environment at the site of attachment, thereby solubilizing bone minerals. Mature osteoclasts are characterized by markers such as tartrate resistant acid phosphatase (TRACP), cathepsin K, and receptors for calcitonin and vitronectin.[14] Osteoclast differentiation and function is regulated by receptor activator nuclear factor kappa B ligand (RANKL)-RANK- osteoprotegerin (OPG) systems.[14,15]

Bone resorbing osteoclasts have been shown to develop in cocultures of human fibroblasts associated with the tooth and human peripheral blood mononuclear cells (PBMCs).[16,17] Tooth-associated fibroblasts are periodontal ligament fibroblasts (PLFs) and gingival fibroblasts (GFs). The morphology of these cells when cultured is similar; they are spindle-shaped and have an elongated appearance.[18] Yet, a number of functional differences between PLFs and GFs have been reported such as a higher expression of alkaline phosphatase activity by PLFs and differences in the expression of fibronectin and collagen type I[19] as well as integrin subunits.[20] PLFs have been shown to induce the formation of osteoclast-like cells by up-regulation of RANKL and by a decrease of the anti-resorptive factor, OPG.[21,22] In contrast with periodontal ligament fibroblasts gingival fibroblasts tend to inhibit osteoclast formation.[17]

It has been shown that estrogen stimulates OPG expression by human osteoblasts,[23,24] by a mouse stromal cell line [25] and by human PLFs.[26] In this way the hormone could have an effect on bone metabolism via soft connective tissue cells in the periodontal domain. It was the aim of the present study to determine whether periodontal ligament fibroblasts and gingival fibroblasts do this to a comparable extent.

2.1 Fibroblast cell culture

Cell culture procedures were used as we have previously reported.[17] Briefly, PLFs and GFs were obtained from the periodontal ligament (PDL) and adjacent healthy gingiva of third molars from four male subjects aged 22-38 years who underwent tooth extraction. Prior to extraction, informed consent was obtained from all donors. The PDL was isolated from the middle third of the tooth roots to exclude the contamination of gingival and apical tissues. All the biopsies were cut into small pieces, placed in 6-well tissue culture plates, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, HyClone, Logan, UT) and 1% antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B [Antibiotic antimyotic solution, Sigma, St. Louis, MO]). Cultures were maintained at 37^{o}C in a humidified 5% CO_2 incubator. After cells were expanded and grown to 70-80% confluence, they were detached with 0.05% trypsin and 0.02% ethylene diaminetetra-acetic acid (EDTA) in phosphate buffered saline (PBS) and subcultured in culture flasks. Four pairs of gingival and PDL cultures were obtained. The two cell populations, PLFs and GFs were used at the same passage within one experiment. Between experiments, cells were used in either the fifth or the sixth passage.

2.2 Isolation and culture of human peripheral blood mononuclear cells (PBMCs)

To isolate PBMCs, buffy coats (Sanquin, Amsterdam, The Netherlands) were diluted 1:1 (v/v) in Hank's balanced salt solution (HBSS) containing 2% FCS. Twenty-five ml of diluted blood was carefully layered on 15 ml lymphoprep (Axisshield Po CAS, Oslo, Norway). This gradient was centrifuged at 1000 x g for 30 min without brake. After centrifugation, the interphase containing PBMCs were collected and washed two times in HBSS with 2% FCS, followed by centrifugation at 400 x g for 10 min. Finally, the cells were resuspended in DMEM supplemented with 10% FCS, 1% antibiotics, 10^{-8} M dexamethasone and 10^{-7} M vitamin D_3.

2.3 Osteoclastogenesis

The effects of estrogen on osteoclastogenesis in PLFs/GFs cocultured with PBMCs were investigated. PLFs and GFs were plated at a cell density of 1.5 x 10^4 cells/well in 24-well tissue culture plates. PBMCs were inoculated one day later at 1 x 10^6 cells/well onto fibroblast cell layers. The experimental cultures were maintained in DMEM supplemented with 10% FCS, 1% antibiotics, 10^{-8} M dexamethasone and 10^{-7} M vitamin D3 in the presence of 10^{-11}, 10^{-9}, and 10^{-7} M 17β-estradiol (17β-E_2; Sigma, Schnelldorf, Germany) for 14 days. Control cultures were maintained under the same conditions without 17β-E_2 but in the presence of the corresponding vehicle of ethanol. The levels of estrogens in the FCS used in the present studies were found to be very low: less than 10 pg/ml (manufacturer's data sheet). Therefore any possible effects are taken to be a result of the added estrogen. The medium was replaced every 3 days with agents described above throughout the experiments.

To examine the direct effect of estrogen on osteoclast precursors, PBMCs were cultured in the absence of fibroblasts under identical conditions. Cultures were performed for 14 days. Then, cells were stained for tartrate-resistant acid phosphatase (TRACP) as a marker enzyme of osteoclasts, using an acid phosphatase kit (Sigma, St Louis, MO). Nuclei were stained with 1 μg/ml diamidino-2-phenylindole dihydrochoride (DAPI). TRACP-positive cells containing three or more nuclei were counted as osteoclast-like cells (OCs) under a light microscope. The average size of 6 randomized OCs/well was measured using image analysis software.

Cell-free areas are the sites where osteoclastogenesis occurs.[27] These areas were quantified by assessing their size in three predetermined fields/well using image analysis software. In addition, total number of cell free areas per well was counted under a light microscope.

2.4 mRNA expression detection by quantitative real-time polymerase chain reaction (PCR)

The effect of estrogen on the expression of RANKL, OPG, estrogen receptor (ER)-α and ER-β by the fibroblasts was determined. Confluent layers of PLFs and GFs were cultured in 24-well tissue culture plates in DMEM supplemented with 10% FCS, 1% antibiotics, in the absence (as controls) or presence of 10^{-11}, 10^{-9}, and 10^{-7} M 17β-E_2 for 24 h. For a time course study, cells were cultured with either vehicle (ethanol) or with 10^{-7} M 17β-E_2. The cells were cultured for 3, 6, 24, and 48 h. Total RNA was extracted from the cultured PLFs and GFs using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration was determined with the RiboGreen Kit (Molecular Probes).

Reverse transcription (RT)-PCR was performed with 100 ng total RNA according to the MBI Fermentas cDNA synthesis kit (Vilnius, Lithuania). The specific primers, shown in Table 1, were designed using the Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on the ABI PRISM 7000 (Applied Biosystems). The reactions were performed with 5 ng cDNA in a total volume of 25 μl containing SYBR Green PCR Master Mix, consisting of SYBR Green I Dye, AmpliTaq Gold DNA polymerase, dNTPs with dUTP instead of dTTP, passive reference and buffer (Applied Biosystems) and 300 nM of each primer.

After an initial activation step of the AmpliTaq Gold DNA polymerase for 10 min at 94°C, 40 cycles were run of a two step PCR consisting of a denaturation step at 95°C for 30 s and annealing and extension step at 60°C for 1 min. Subsequently the PCR products were subjected to melting curve analysis to test if any unspecific PCR products were generated. The PCR reactions of the different amplicons had equal efficiencies. β2-Microglobulin was used as the housekeeping gene. Expression of this gene was not affected by estradiol treatment.

To test whether there was a difference in the expression of the genes between 17βE_2-treated and -untreated groups, mRNA levels were quantified using the comparative threshold-cycle (CT) method as follows. First, samples were normalized for the expression of β2-microglobulin by calculating the ΔCT as follows; ΔCT = CT, target - CT, β_2-microglobulin. Subsequently, the amount of targeted mRNA in the sample was compared by calculation of $2^{-(\Delta\Delta CT)}$, where $\Delta\Delta CT = \Delta C_{T,\ sample1} - \Delta C_{T,\ calibrator}$, setting the relative levels of mRNA expression in the untreated groups (calibrator) as 1.

The amount of mRNA expression in 17β-E_2-treated cells was therefore expressed as a relative ratio. Furthermore, the comparison of mRNA expression of the genes between PLFs and GFs was analyzed of which the amount of the gene expression is expressed as $2^{-(\Delta CT)}$.

2.5 Statistical analysis

All experiments were performed with the two dental fibroblast populations collected from four different patients. Each experiment was analyzed in duplicate or triplicate. Data were expressed as mean value ± standard deviation (SD). Comparisons of the numerical data between groups were performed by one-way analysis of variance (ANOVA), followed by a multiple-comparison Tukey test or the Kruskal-Wallis test. The Pearson's rank correlation coefficient was used for the correlations between the dose effect of estrogen and the number of TRACP-positive osteoclast-like cells. Results were considered statistically significant at $p < 0.05$.

3.1 Osteoclast-like cell formation

PBMCs seeded on the two different populations of fibroblasts resulted after 14 days in withdrawal of the latter cells and thus induced the formation of cell-free areas.[27] In these areas osteoclast precursors attached to the bottom and fused to form osteoclast-like cells. Osteoclast-like cells were identified as TRACP-positive cells with three or more nuclei, which could be visualized by DAPI staining (Fig. 1).

The cell-free areas of PLFs cultured with PBMCs were formed significantly more (Table 2) and were larger than those of the GF cocultures (Fig. 2). There were no significant changes in cell-free areas in PLFs or GFs cocultured with PBMCs in the presence of 17β-E_2 compared to the cultures without the hormone.

Coinciding with the results of the cell-free areas, the number of TRACP-positive osteoclast-like cells in cocultures of PLFs and PBMCs was significantly higher than in GF-PBMC cocultures (Fig. 3).The number of TRACP-positive osteoclast-like cells was significantly decreased in the 10^{-9} and 10^{-7} M 17β-E_2-treated PLF cocultures compared with controls. There was a significant negative correlation between the concentration of estrogen and the number of TRACP-positive osteoclast-like cells in the PLF cocultures (r = -0.466, $p < 0.01$). Such an inhibitory effect of 17β-E_2 on osteoclast-like cell formation was not seen in cocultures of GFs with PBMCs.

3.2 PBMC cultures

The effect of estrogen on osteoclast formation by osteoclast precursors was analyzed also by culturing human PBMC alone in the absence of fibroblasts but with the vitamin D_3 and dexamethasone. After 14 days in culture, TRACP-positive osteoclast-like cells were found. In general cells were round or oval and most cells contained one or two nuclei with only a few cells having three or more nuclei (Fig. 4). The number of TRACP-positive osteoclast-like cells was similar for cultures kept with or without 17β-E_2 (Fig. 5).

TRACP-positive osteoclast-like cells in the monocultures of PBMCs were 2.8 fold smaller compared with the cocultures with the two different tooth-associated fibroblast populations (Table 3) and they contained fewer nuclei. The maximum number of nuclei per cell in osteoclast-like cells in the PBMC cultures was 9 (Fig. 4) whereas more than 20 nuclei/cell were found in the cocultures.

3.3 mRNA expression

In an attempt to provide an explanation for the altered number of osteoclast-like cells in the cultures with 17β-E_2, the mRNA levels of RANKL, OPG, ER-β and ER-α in PLF and GF cultures were analyzed. The response of the different cell types to 10^{-7} M 17β-E_2 was analyzed also at various time points (3, 6, 24, and 48 h).

There were no significant differences in the mRNA expression of RANKL, OPG, ER-α by PLF and GF cultures that were cultured with or without 17β-E_2 (Fig. 6). Expression of ER-β could not be detected in either fibroblast population. Between the different fibroblast populations the mRNA expression of the different genes was comparable. Analysis of the different genes at different time points revealed no differences in expression between the two fibroblast populations. Also the addition of 17β-E_2 appeared without an effect (data not shown).

Table 1-Primers sequences and product size.

Target	Sequences	Product size (bp)
β_2-microglobulin forward	AAgATTCAggTTTACTCACgTC	294
β_2-microglobulin reverse	TgATgCTgCTTACATgTCTCg	
OPG forward	CTgCgCgCTCgTgTTTC	100
OPG reverse	ACAgCTgATgAgAggTTTCTTCgT	
RANKL forward	CATCCCATCTggTTCCCATAA	60
RANKL reverse	gCCCAACCCCgATCATg	
ER alpha forward	gTgCAgTgTgCAATgACTATgCT	100
ER alpha reverse	gTCgTTATgTCCTTgAATACTTCTCTTg	
ER beta forward	TCAAAAgAgAgTCCCTggTgTgAAg	125
ER beta reverse	CTCTTTgAACCTggACCAgTAACAg	

bp, base pair.

Table 2-The number of cell-free areas per well in the different cell cultures.

Type of cultures	Number of cell-free areas/well (mean ± SD)
Coculture of PLFs-PBMCs	9.9 ± 2.6a
Coculture of GFs-PBMCs	2.8 ± 1.0

Periodontal ligament fibroblasts (PLFs) or gingival fibroblasts (GFs) were cocultured with peripheral blood mononuclear cells (PBMCs) for 14 days. Then, the total number of cell-free areas per well was counted under a light microscope. Data were expressed as means ± standard deviation (SD) of four different cocultures of PLFs or GFs, and each analyzed in triplicate. a is significant vs. coculture of GFs-PBMCs, $p < 0.05$.

Table 3-Size of TRACP-positive osteoclast-like cells in the different cell cultures.

Type of cultures	Cell size (μm^2) (mean ± SD)
Coculture of PLFs-PBMCs	13,074 ± 3524a
Coculture of GFs-PBMCs	13,090 ± 1302 a
Monoculture of PBMCs	4607 ± 135

Periodontal ligament fibroblasts (PLFs) or gingival fibroblasts (GFs) were cocultured with peripheral blood mononuclear cells (PBMCs). After 14-day in culture, the average size of 6 randomized tartrate resistant acid phosphatase (TRACP)-positive osteoclast-like cells/well was measured using image analysis software. Data were expressed as means ± standard deviation (SD) of four different cocultures of PLFs/GFs and two different monocultures of PBMCs, and each analyzed in duplicate and triplicate, respectively. a is significant vs. monoculture of PBMCs, $p < 0.05$.

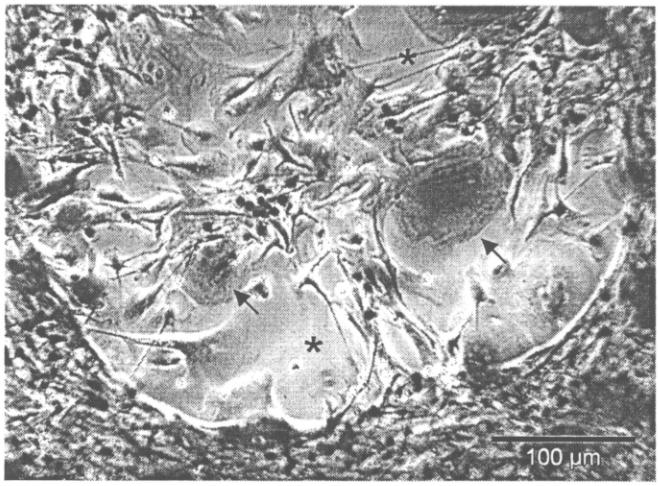

Fig.1 Tartrate-resistant acid phosphatase (TRACP)-positive osteoclast-like cells were seen as dark red cells (arrows) and were present in cell-free areas (asterisks).

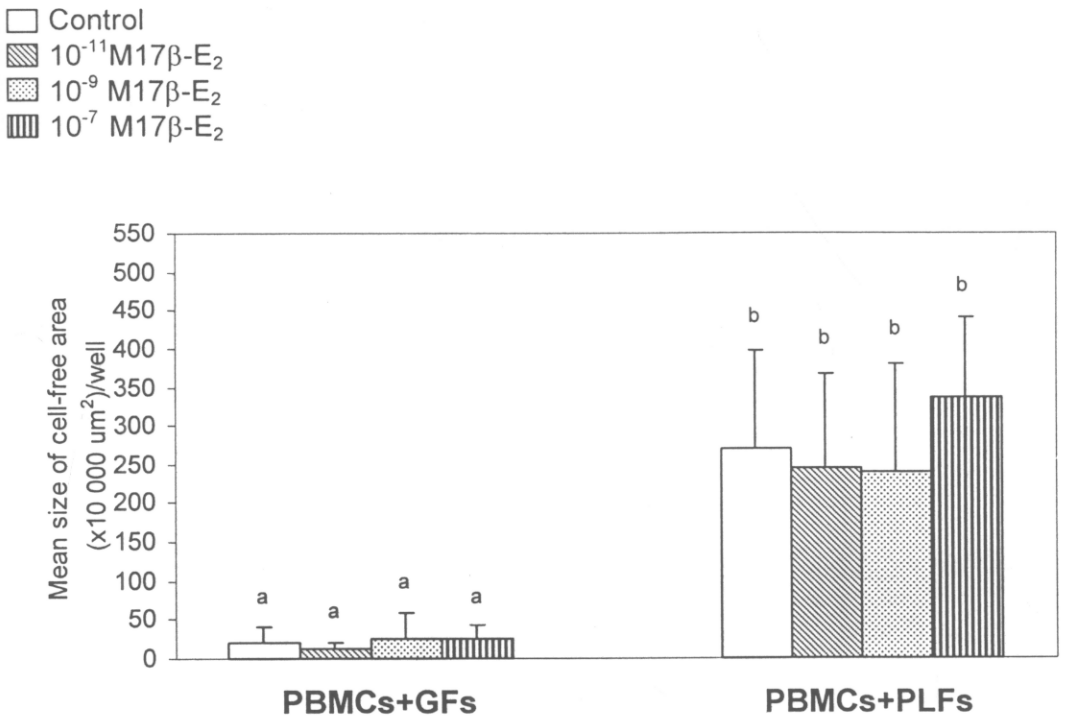

Fig. 2. Size of cell-free areas of 14-day cocultures of human peripheral blood mononuclear cells (PBMCs) and human periodontal ligament fibroblasts (PLFs)/gingival fibroblasts (GFs) in the presence or absence of different concentrations of 17β-E_2. Data were presented as means ± standard deviation (SD). Four different cocultures of PLFs or GFs, each consisting of three wells and three predetermined fields/well were analyzed. a is significant vs. b at $p < 0.05$.

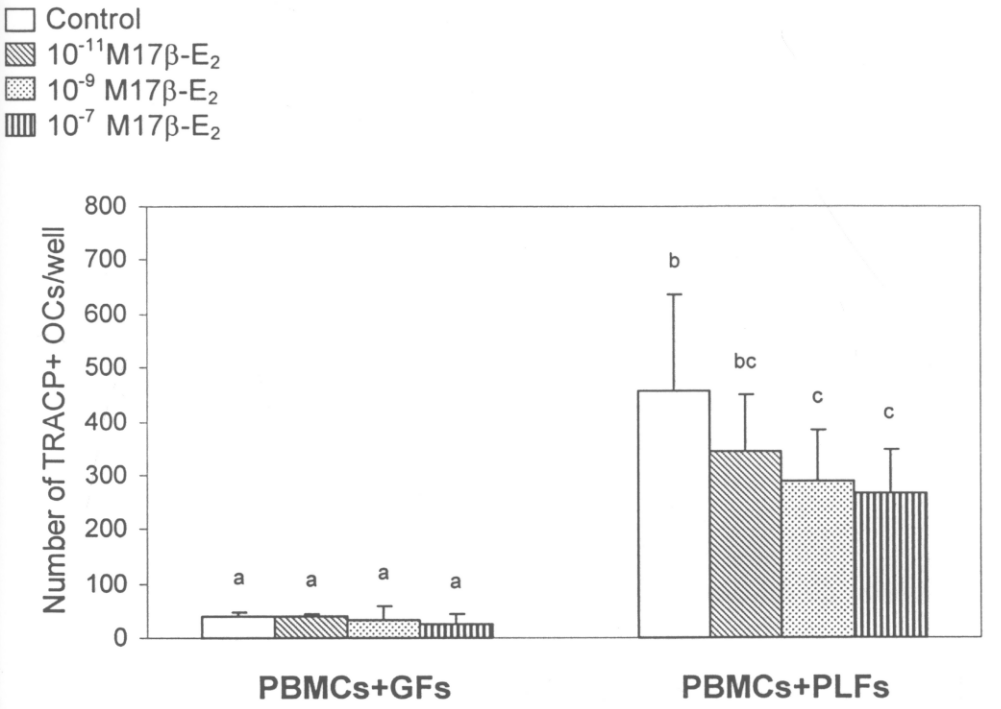

Fig. 3. The number of TRACP-positive osteoclast-like cells (OCs) per well in cocultures of human peripheral blood mononuclear cells (PBMCs) and human periodontal ligament fibroblasts (PLFs) in the presence or absence of different concentrations of 17β-E_2 was compared to that of cocultures with human gingival fibroblasts (GFs). Data were presented as means ± standard deviation (SD) from four different cocultures of PLFs or GFs and each analyzed in triplicate. a is significant vs. b and c; b is significant vs. a and c at $p < 0.05$.

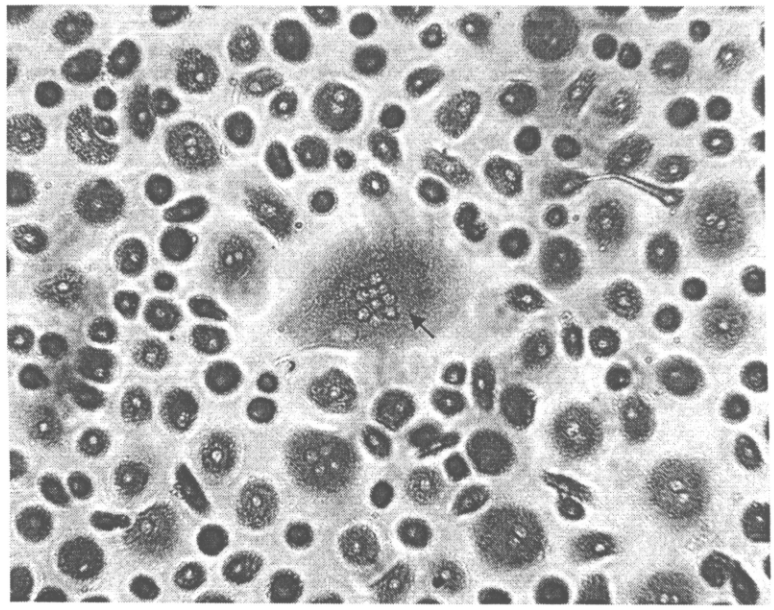

Fig. 4. TRACP-positive osteoclast-like cells (OCs) in a monoculture of human peripheral blood mononuclear cells. The micrograph shows a large TRACP-positive OCs with 9 nuclei (arrow) surrounded by small TRACP-positive cells with one or two nuclei.

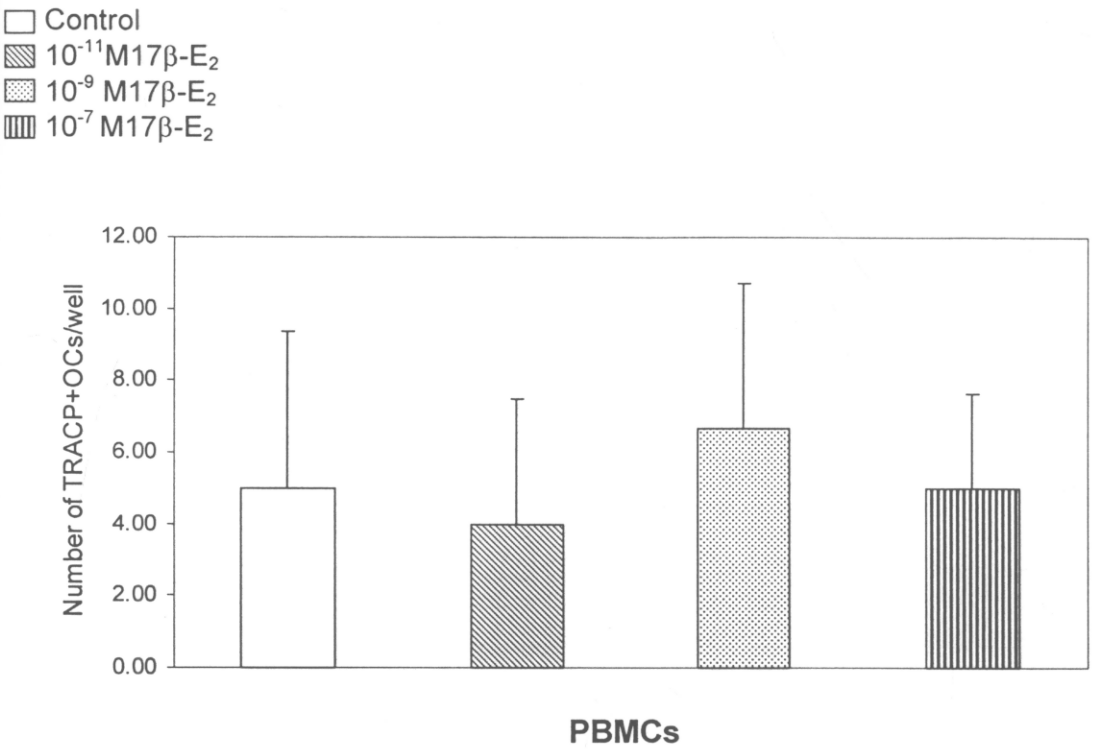

Fig. 5. The number of TRACP-positive osteoclast-like cells (OCs) per field in a monoculture of human peripheral blood mononuclear cells (PBMCs) in the presence or absence of different concentration of 17β-E_2. Data were presented as means ± standard deviation (SD) from three independent experiments.

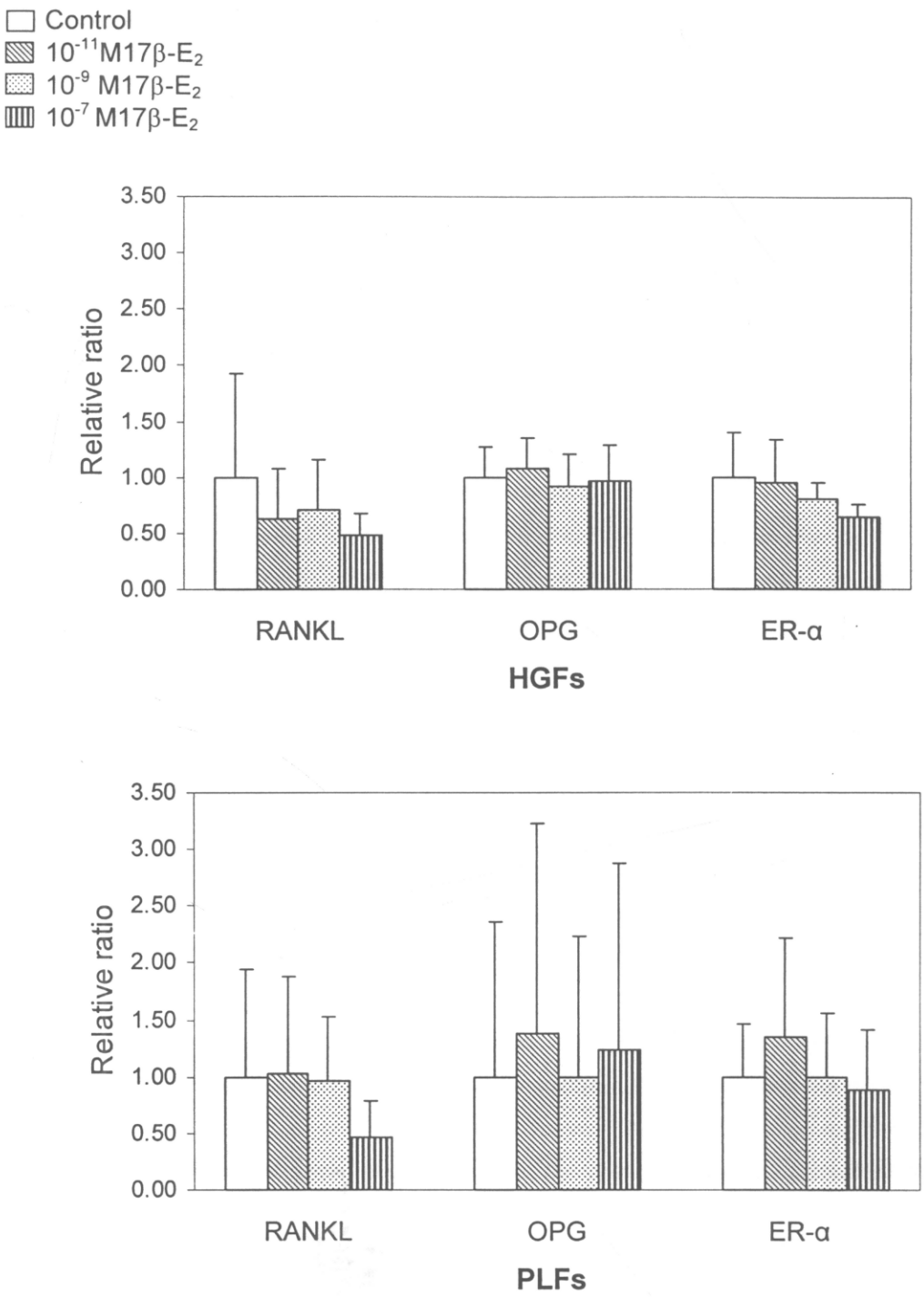

Fig. 6 mRNA expression of receptor activator nuclear factor kappa B ligand (RANKL), osteoprotegerin (OPG), and estrogen receptor (ER)-α by human periodontal ligament fibroblasts (PLFs) and gingival fibroblasts (GFs). The fibroblasts were cultured for 24 h in the presence or absence of different concentrations of 17β-E_2. Results were normalized to β_2-microglobulin. Results from untreated cells were set a value of 1. The mRNA expression by 17β-E_2-treated cells was therefore expressed as a relative ratio. Data were presented as means ± standard deviation (SD) from four different cultures of PLFs and GFs and each analyzed in triplicate.

In the present study we demonstrated that estrogen inhibited the formation of osteoclast-like cells in cocultures of PLFs and PBMCs. This inhibitory effect was not found in cocultures with GFs, where only a few osteoclast-like cells were formed. These observations suggest that GFs are not or less sensitive to estrogen; however, the underlying biological mechanisms are not clear. It is known that estrogen exerts its effects via intracellular estrogen receptors. Thus, the concentration of estrogen receptor is an important determinant of cellular responsiveness to estrogen.[28] The present data showed no difference in the mRNA expression level of ER-α expression between GFs and PLFs, suggesting that both cell types had the potency to respond similarly to estrogen. The difference in response can be explained by assuming that the interaction between the PBMCs and the fibroblasts resulted in an altered expression of the estrogen receptor.

An inhibitory effect of estrogen on the formation of osteoclast-like cells is in line with previous findings obtained from either osteoblast-induced osteoclastogenesis[29] or bone marrow cells employed as precursors.[30-32] In addition, in vivo studies using ovariectomized rats demonstrated that estrogen deficiency induced a higher number of osteoclasts in the rat periodontium.[33] These data together strongly suggest that estrogen may play an important role in modulating osteoclast formation.

Furthermore, an interesting finding was the higher number and larger size of cell-free areas in cocultures of PLFs with PBMCs compared to cocultures with GFs. Coinciding with an increased formation of cell free areas, a higher number of osteoclast-like cells were formed in PLF cocultures. These observations strongly suggest a difference in the interaction between different fibroblast populations and PBMCs. At present, we do not know the underlying mechanisms of this phenomenon.

It has been emphasized that a cross-talk is important between osteoblasts/fibroblasts and PBMCs during the formation of osteoclasts and the subsequent process of bone resorption. Not only precursors of osteoclasts receive signals from osteoblasts/fibroblasts essential for their differentiation and function, the reverse also proves to be true. The precursors activate osteoblasts or fibroblasts resulting in the retraction of these cells thus forming cell-free areas, the sites where osteoclastogenesis occurs.[27] The intercellular adhesion molecules (eg., ICAM-1) and vascular cell adhesion molecule (VCAM-1) have been proposed to play a role in osteoclast-like cell formation.[27,34] ICAM-1- expressing osteoblasts have been demonstrated to induce osteoclast differentiation.[35] Both PLFs and GFs express ICAM-1 and VCAM-1.[36,37] The present data showed that ligament fibroblasts responded more strongly to PBMCs than fibroblasts from the gingiva. Whether this difference in response is due to differences in expression of membrane-bound receptors (e.g. ICAM-1) known to be associated with these cell-cell interactions, needs to be investigated.

The present data showed that osteoclast-like cells were generated also from human peripheral blood mononuclear cell precursors cultured with vitamin D_3 and dexamethasone in the absence of stromal cells, albeit at severely reduced numbers and sizes compared to PBMC-fibroblast cocultures. An interesting finding is the difference in size of the osteoclast-like cells formed under

these conditions. The present data showed that the osteoclast-like cells generated in the presence of the fibroblasts were nearly 3 times larger than those formed without the mesenchymal cells. So it seems that the fibroblasts play a pivotal role in the formation of larger multinucleated osteoclasts. One of the first steps in the formation of osteoclasts is the attraction of precursors by the osteoblasts/fibroblasts and their subsequent attachment to these cells. To a single stromal cell, a relatively high number of precursors attaches[38] thus allowing a very effective fusion of precursors at one site. Such a "bringing-together" property of the stromal cells may eventually result in the formation of larger multinucleated cells.

In addition, we found no difference in the number of osteoclast-like cells formed by the monoculture of PBMCs in the presence or absence of estrogen. This is in agreement with other findings such as those of Matayoshi *et al.*[39] who showed that estrogen did not block osteoclast formation in cultures of purified blood-derived $CD34^+$ cells obtained from blood of healthy female donors and those of Kitazawa *et al.*[40] who investigated mouse bone marrow cultures. These findings, together with the lack of an estrogen effect in the cocultures with the gingival fibroblasts indicate that the PBMCs are not the target cells for the hormone. The present data seem to be in contrast to findings presented by others who showed that estrogens did attenuate osteoclastogenesis by PBMCs.[31,32,41,42] In these studies, however, the PBMCs were cultured in the presence of RANKL, whereas we added vitamin D_3 and dexamethasone. So it appears that depending on the stimulating compound present in the culture system estrogens have or have not an effect on osteoclastogenesis.

To further analyze the molecules that may be involved in the inhibitory effect of estrogen, we assessed the mRNA expression of RANKL, OPG, and estrogen receptors. We did not find any effect of estrogen on the expression of these genes in either tooth-associated fibroblast population, confirming previous data obtained from immortalized human marrow stromal cell lines.[43] Others, however, showed that estrogen altered the ratio of OPG to RANKL. This was not only shown with osteoblast cultures[24] but also for PLF cultures.[26] The present data suggested that the inhibitory effect of estrogen on osteoclast-like cell formation could be mediated independent of RANKL and OPG. Thus estrogen may exert its effect via expression of other regulating compounds like transforming growth factor (TGF)- β[44], tumor necrosis factor (TNF)-α,[45] interleukin (IL)-1,[45] IL-6,[46] IL-7,[47] and/or M-CSF.[48] Recent studies have demonstrated that estrogen deficiency results in an increased T cell mediated production of TNF-α and a concomitant increased osteoclast formation.[49-51] Factors that regulate T cell function during estrogen deficiency have been extensively reviewed by Pacifici.[52]

Furthermore, analysis of the expression of ERs revealed that PLF and GF cultures contained ER-α mRNA, not ER-β mRNA. However, the addition of estrogen did not alter ER-α mRNA levels in these cell types suggesting that ER-α did not mediate the osteoclast inhibition of estrogen. The data are in line with a recent study showing that estrogen treatment did not increase ER-α and ER-β expression in cultured human PLFs.[53] Thus their role in mediating estrogen action on osteoclastogenesis remains unclear. Further studies are therefore required.

CHAPTER 5 CONCLUSION

In conclusion, our present in vitro study shows an inhibitory effect of estrogen on osteoclast-like cell formation in cocultures of periodontal ligament fibroblasts and peripheral blood mononuclear cells. Estrogen exerts its inhibitory action probably not by interfering with the RANKL/OPG system. In addition, our data indicate that the two tooth-associated fibroblast populations differently modulate osteoclastogenesis. In the presence of gingival fibroblasts lower numbers of osteoclasts were formed. Estrogen may be an important modulator of osteoclast formation in the periodontal ligament under normal conditions. Our findings suggest that the hormone down-regulates this process tissue-site specifically. Yet, as soon as less or no estrogen is present anymore, higher numbers of osteoclasts are formed, thus resulting in osteoporosis.

CHAPTER 6 OUTPUTS

1. Publication

Wattanaroonwong N, Schoenmaker T, de Vries TJ, Everts V. Oestrogen inhibits osteoclast formation induced by periodontal ligament fibroblasts. *Arch Oral Biol* doi:10.1016/j.archoralbio.2010.10.004 (impact factor, 2008 = 1.379)

2. Public's benefit

The results of this research were given as a part of the lectures to the participants at the International Cell Culture Workshop at Department of Oral Biology and Occlusion, Faculty of Dentistry, Prince of Songkla University, 18-21 May, 2009.

3. Presentations

The studies were presented at the Thailand Research Fund (TRF) meetings at Ambassador City Jomtien, Chonburi, 11-13 October, 2007 and at Holiday Inn Resort Regent Beach Cha-Am, Petchaburi, 16-18 October, 2008.

1. Jeffcoat MK, Lewis CE, Reddy MS, Wang CY, Redford M. Post-menopausal bone loss and its relationship to oral bone loss. *Periodontol 2000* 2000;23:94-102.
2. Lerner UH. Inflammation-induced bone remodeling in periodontal disease and the influence of post-menopausal osteoporosis. *J Dent Res* 2006;85:596-607.
3. Haas AN, Rösing CK, Oppermann RV, Albandar JM, Susin C. Association among menopause, hormone replacement therapy, and periodontal attachment loss in southern Brazillian women. *J Periodontol* 2009;80:1380-7.
4. Krall EA, Dawson-Hughes B, Papas A, Garcia RI. Tooth loss and skeletal bone density in healthy postmenopausal women. *Osteoporos Int* 1994;4:104-9.
5. Krall EA, Garcia RI, Dawson-Hughes B. Increased risk of tooth loss is related to bone loss at the whole body, hip, and spine. *Calcif Tissue Int* 1996;59:433-7.
6. Tezal M, Wactawski-Wende J, Grossi SG, Ho AW, Dunford R, Genco RJ. The relationship between bone mineral density and periodontitis in postmenopausal women. *J Periodontol* 2000;71:1492-8.
7. Payne JB, Zachs NR, Reinhardt RA, Nummikoski PV, Patil K. The association between estrogen status and alveolar bone density changes in postmenopausal women with a history of periodontitis. *J Periodontol* 1997;68:24-31.
8. Elovic RP, Hipp JA, Hayes WC. Ovariectomy decreases the bone area fraction of the rat mandible. *Calcif Tissue Int* 1995;56:305-10.
9. Gilles JA, Carnes DL, Dallas MR, Holt SC, Bonewald LF. Oral bone loss is increased in ovariectomized rats. *J Endod* 1997;23:419-22.
10. Tanaka M, Ejiri S, Nakajima M, Kohno S, Ozawa H. Changes of cancellous bone mass in rat mandibular condyle following ovariectomy. *Bone* 1999;25:339-47.
11. Nishimura I, Hosokawa R, Kaplan ML, Atwood DA. Animal model for evaluating the effect of systemic estrogen deficiency on residual ridge resorption. *J Prosthet Dent* 1995;73:304-10.
12. Yamashiro T, Takano-Yamamoto T. Influences of ovariectomy on experimental tooth movement in the rat. *J Dent Res* 2001;80:1858-61.
13. Riggs BL. The mechanisms of estrogen regulation of bone resorption. *J Clin Invest* 2000;106:1203-4.
14. Sasaki T. Differentiation and functions of osteoclasts and odontoclasts in mineralized tissue resorption. *Microsc Res Tech* 2003;61:483-95.
15. Wada T, Nakashima T, Hiroshi N, Penninger JM. RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med* 2006;12:17-25.
16. Kanzaki H, Chiba M, Shimizu Y, Mitani H. Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. *J Dent Res* 2001;80:887-91.

17. de Vries TJ, Schoenmaker T, Wattanaroonwong N, van den Hoonaard M, Nieuwenhuijse A, Beertsen W, et al. Gingival fibroblasts are better at inhibiting osteoclast formation than periodontal ligament fibroblasts. *J Cell Biochem* 2006;98:370-82.

18. Somerman MJ, Archer SY, Imm GR, Foster RA. A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. *J Dent Res* 1988;67:66-70.

19. Kuru L, Parkar MH, Griffiths GS, Newman HN, Olsen I. Flow cytometry analysis of gingival and periodontal ligament cells. *J Dent Res* 1998;77:555-64.

20. Palaiologou AA, Yukna RA, Moses R, Lallier TE. Gingival, dermal, and periodontal ligament fibroblasts express different extracellular matrix receptors. *J Periodontol* 2001;72:798-807.

21. Hasegawa T, Kikuiri T, Takeyama S, Yoshimura Y, Mitome M, Oguchi H, et al. Human periodontal ligament cells derived from deciduous teeth induce osteoclastogenesis in vitro. *Tissue Cell* 2002;34:44-51.

22. Zhang D, Yang YQ, Li XT, Fu MK. The expression of osteoprotegerin and the receptor activator of nuclear factor kappa B ligand in human periodontal ligament cells cultured with and without 1α, 25-dihydroxyvitamin D_3. *Arch Oral Biol* 2004;49:71-6.

23. Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Spelsberg TC, Riggs BL. Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. *Endrocrinology* 1999;140:4367-70.

24. Bord S, Ireland DC, Beavan SR, Compston JE. The effects of estrogen on osteoprotegerin, RANKL, and estrogen receptor expression in human osteoblasts. *Bone* 2003;32:136-41.

25. Saika M, Inoue D, Kido S, Matsumoto T. 17β-Estradiol stimulates expression of osteoprotegerin by a mouse stromal cell line, ST-2, via estrogen receptor-α. *Endocrinology* 2001;142:2205-12.

26. Shu L, Guan SM, Fu SM, Guo T, Cao M, Ding Y. Estrogen modulates cytokine expression in human periodontal ligament cells. *J Dent Res* 2008;87:142-7.

27. Perez-Amodio S, Beertsen W, Everts V. (Pre-)Osteoclasts induce retraction of osteoblasts before their fusion to osteoclasts. *J Bone Miner Res* 2004;19:1722-31.

28. Santagati S, Gianazza E, Agrati P, Vegeto E, Patrone C, Pollio G, et al. Oligonucleotide squelching reveals the mechanism of estrogen receptor autologous down-regulation. *Mol Endocrinol* 1997;11:938-49.

29. Krum SA, Miranda-Carboni GA, Hauschka PV, Carroll JS, Lane TF, Freedman LP, et al. Estrogen protects bone by inducing Fas ligand in osteoblasts to regulate osteoclast survival. *EMBO J* 2008;27:535-45.

30. Schiller C, Gruber R, Redlich K, Ho GM, Katzgraber F, Willheim M, et al. 17β-Estradiol antagonizes effects of 1α, 25-dihydroxyvitamin D_3 on interleukin-6 production and osteoclast-like cell formation in mouse bone marrow primary cultures. *Endrocrinology* 1997;138: 4567-71.

31. Sarma U, Edwards M, Motoyoshi K, Flanagan AM. Inhibition of bone resorption by 17β-estradiol in human bone marrow cultures. *J Cell Physiol* 1998;175:99-108.

32. Srivastava S, Toraldo G, Weitzmann MN, Cenci S, Ross FP, Pacifici R. Estrogen decreases osteoclast formation by down-regulating receptor activator of NF-kB ligand (RANKL)-induced JNK activation. *J Biol Chem* 2001;276:8836-40.

33. Kawamoto S, Ejiri S, Nagaoka E, Ozawa H. Effects of oestrogen deficiency on osteoclastogenesis in the rat periodontium. *Arch Oral Biol* 2002;47:67-73.

34. Bloemen V, de Vries TJ, Schoenmaker T, Everts V. Intercellular adhesion molecule-1 clusters during osteoclastogenesis. *Biochem Biophys Res Commun* 2009;385:640-5.

35. Tanaka Y, Maruo A, Fujii K, Nomi M, Nakamura T, Eto S, et al. Intercellular adhesion molecule 1 discriminates functionally different populations of human osteoblasts: characteristic involvement of cell cycle regulators. *J Bone Miner Res* 2000;15:1912-23.

36. Joe BH, Borke JL, Keskintepe M, Hanes PJ, Mailhot JM, Singh BB. Interleukin-1β regulation of adhesion molecules on human gingival and periodontal ligament fibroblasts. *J Periodontol* 2001;72:865-70.

37. Hosokawa Y, Hosokawa I, Ozaki K, Nakae H, Matsuo T. Cytokines differentially regulate ICAM-1 and VCAM-1 expression on human gingival fibroblasts. *Clin Exp Immunol* 2006;144:494-502.

38. de Vries TJ, Mullender MG, van Duin MA, Semeins CM, James N, Green TP, et al. The Src inhibitor AZD0530 reversibly inhibits the formation and activity of human osteoclasts. *Mol Cancer Res* 2009;7:476-88.

39. Matayoshi A, Brown C, DiPersio JF, Haug J, Abu-Amer Y, Liapis H, et al. Human blood-mobilized hematopoietic precursors differentiate into osteoclasts in the absence of stromal cells. *Proc Natl Acad Sci USA* 1996;93:10785-90.

40. Kitazawa R, Kimble RB, Vannice JL, Kung VT, Pacifici R. Interleukin-1 receptor antagonist and tumor necrosis factor binding protein decrease osteoclast formation and bone resorption in ovariectomized mice. *J Clin Invest* 1994;94:2397-406.

41. Shevde NK, Bendixen AC, Dienger KM, Pike JW. Estrogens suppress RANK ligand-induced osteoclast differentiation via a stromal cell independent mechanism involving c-Jun repression. *Proc Natl Acad Sci USA* 2000;97:7829-34.

42. Sørensen MG, Henriksen K, Dziegiel MH, Tankó LB, Karsdal MA. Estrogen directly attenuates human osteoclastogenesis, but has no effect on resorption by mature osteoclasts. *DNA Cell Biol* 2006;25:475-83.

43. Ramalho AC, Jullienne A, Couttet Ph, Graulet AM, Morieux C, de Vernejoul MC, et al. Effect of oestradiol on cytokine production in immortalized human marrow stromal cell lines. *Cytokine* 2001;16:126-30.

44. Gao Y, Qian WP, Dark K, Toraldo G, Lin AS, Guldberg RE, et al. Estrogen prevents bone loss through transforming growth factor β signaling in T cells. *Proc Natl Acad Sci USA* 2004;101:16618-23.

45. Kimble RB, Srivastava S, Ross FP, Matayoshi A, Pacifici R. Estrogen deficiency increases the ability of stromal cells to support murine osteoclastogenesis via an interleukin-1 and tumor necrosis factor-mediated stimulation of macrophage colony-stimulating factor production. *J Biol Chem* 1996;271:28890-7.

46. Girasole G, Jilka RL, Passeri G, Boswell S, Boder G, Williams DC, et al. 17β-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens. *J Clin Invest* 1992;89:883-91.

47. Sato T, Watanabe K, Masuhara M, Hada N, Hakeda Y. Production of IL-7 is increased in ovariectomized mice, but not RANKL mRNA expression by osteoblasts/stromal cells in bone, and IL-7 enhances generation of osteoclast precursors in vitro. *J Bone Miner Metab* 2007;25:19-27.

48. Srivastava S, Weitzmann MN, Kimble RB, Rizzo M, Zahner M, Milbrandt J, et al. Estrogen blocks M-CSF gene expression and osteoclast formation by regulating phosphorylation of Egr-1 and its interaction with Sp-1. *J Clin Invest* 1998;102:1850-9.

49. Cenci S, Weitzmann MN, Roggia C, Namba N, Novack D, Woodring J, et al. Estrogen deficiency induces bone loss by enhancing T-cell production of TNF-α. *J Clin Invest* 2000;106:1229-37.

50. D'Amelio P, Grimaldi A, Di Bella S, Brianza SZ, Cristofaro MA, Tamone C, et al. Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: a key mechanism in osteoporosis. *Bone* 2008;43:92-100.

51. Rifas L, Weitzmann MN. A novel T cell cytokine, secreted osteoclastogenesis factor of activated T cells, induces osteoclast formation in a RANKL-independent manner. *Arthritis Rheum* 2009;60:3324-35.

52. Pacifici R. T cells: critical bone regulators in health and disease. *Bone* 2010;47:461-71.

53. Cao M, Shu L, Li J, Su J, Zhang W, Wang Q, et al. The expression of estrogen receptors and the effects of estrogen on human periodontal ligament cells. *Methods Find Exp Clin Pharmacol* 2007;29:329-35.

APPENDIX

(ภาคผนวก)

ARCHIVES OF ORAL BIOLOGY XXX (2010) XXX–XXX

available at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/aob

Oestrogen inhibits osteoclast formation induced by periodontal ligament fibroblasts

Nutthamon Wattanaroonwong [a,*], *Ton Schoenmaker* [b,c], *Teun J. de Vries* [b,c], *Vincent Everts* [b]

[a] *Department of Oral Biology and Occlusion, Faculty of Dentistry, Prince of Songkla University (PSU), 15 Karnjanavanit Road, Haad Yai, Songkhla 90112, Thailand*

[b] *Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and VU University Amsterdam, Research Institute MOVE, Gustav Mahlerlaaan 3004, 1081 LA Amsterdam, The Netherlands*

[c] *Department of Periodontology, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and VU University Amsterdam, Gustav Mahlerlaan 3004, 1081 LA Amsterdam, The Netherlands*

ARTICLE INFO

Article history:
Accepted 1 October 2010

Keywords:
Oestrogen
Periodontal ligament
Osteoclasts
Gingival fibroblasts
Human peripheral blood mononuclear cells

ABSTRACT

Objective: Since tooth-associated fibroblasts are taken to participate in the formation of osteoclasts and it is unknown whether oestrogen affects this process, the effects of 17β-estradiol (17β-E_2) were studied on osteoclastogenesis induced by human periodontal ligament fibroblasts (PLFs) and gingival fibroblasts (GFs).

Methods: Human peripheral blood mononuclear cells (PBMCs) were seeded on monolayers of PLFs and GFs and cocultured for 14 days in the presence or absence of various concentrations of 17β-E_2. The number of tartrate resistant acid phosphatase (TRACP)-positive osteoclast-like cells (OCs) was assessed. In addition, we analysed the PBMC-induced withdrawal of the fibroblasts. mRNA expression was determined of oestrogen receptor (ER)-α, ER-β, receptor activator nuclear factor kappa B ligand (RANKL), and osteoprotegerin (OPG) by PLFs and GFs.

Results: PBMCs induced a higher number and larger fibroblast-free areas if cocultured with PLFs than with GFs. Concomitantly, the number of TRACP-positive OCs was significantly higher in PLF cocultures. 17β-E_2 inhibited the formation of OCs in PLF cocultures. 17β-E_2 did not alter the expression of RANKL, OPG, and ER-α mRNAs in either fibroblast cell population.

Conclusion: Our data indicate that PLFs may promote osteoclastogenesis more strongly than GFs. 17β-E_2 inhibits the PLF-induced formation of osteoclast-like cells. Thus, the inhibitory effect of oestrogen on osteoclast formation appears to be cell type dependent.

1. Introduction

Oestrogen deficiency has been found as one of the risk factors for periodontal disease in postmenopausal women.[1,2] Oestrogen-deficiency seems to enhance loss of the alveolar bone volume, leading to a more progressive form of periodontitis.[3,4] Several studies show a relationship between tooth loss and alveolar ridge resorption on one hand and systemic bone loss in postmenopausal osteoporosis on the other.[4–6] These data suggest that loss of alveolar bone is somehow related to more generalized bone loss which occurs in osteoporotic patients. Furthermore, radiographic studies show that oestrogen deficiency in women is associated with a mean net loss in alveolar bone density.[7] In ovariectomized rats a decrease in

* *Corresponding author.* Tel.: +66 74 429 873; fax: +66 74 212 922.
E-mail addresses: nutthamon.w@psu.ac.th, pnutthamon@gmail.com (N. Wattanaroonwong).

0003-9969/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.archoralbio.2010.10.004

mandibular bone volume,[8] an increase in periapical bone loss of first mandibular molars,[9] an increase in cancellous bone loss in the mandibular condyle,[10] an increase in residual ridge resorption,[11] and an acceleration of tooth movement[12] have been reported. It is recognized that oestrogen deficiency stimulates bone resorption by increased osteoclast numbers due to enhanced osteoclast formation and reduced osteoclast apoptosis as well as by increased osteoclast activity.[13]

Osteoclasts are multinucleated cells derived from haematopoietic mononuclear cells. The osteoclasts attach to the bone surface and form a highly acidic environment at the site of attachment, thereby solubilizing bone minerals. Mature osteoclasts are characterized by markers such as tartrate resistant acid phosphatase (TRACP), cathepsin K, and receptors for calcitonin and vitronectin.[14] Osteoclast differentiation and function is regulated by receptor activator nuclear factor kappa B ligand (RANKL)-RANK-osteoprotegerin (OPG) systems.[14,15]

Bone resorbing osteoclasts have been shown to develop in cocultures of human fibroblasts associated with the tooth and human peripheral blood mononuclear cells (PBMCs).[16,17] Tooth-associated fibroblasts are periodontal ligament fibroblasts (PLFs) and gingival fibroblasts (GFs). The morphology of these cells when cultured is similar; they are spindle-shaped and have an elongated appearance.[18] Yet, a number of functional differences between PLFs and GFs have been reported such as a higher expression of alkaline phosphatase activity by PLFs and differences in the expression of fibronectin and collagen type I[19] as well as integrin subunits.[20] PLFs have been shown to induce the formation of osteoclast-like cells by up-regulation of RANKL and by a decrease of the anti-resorptive factor, OPG.[21,22] In contrast with periodontal ligament fibroblasts gingival fibroblasts tend to inhibit osteoclast formation.[17]

It has been shown that oestrogen stimulates OPG expression by human osteoblasts,[23,24] by a mouse stromal cell line[25] and by human PLFs.[26] In this way the hormone could have an effect on bone metabolism via soft connective tissue cells in the periodontal domain. It was the aim of the present study to determine whether periodontal ligament fibroblasts and gingival fibroblasts do this to a comparable extent.

2. Materials and methods

2.1. *Fibroblast cell culture*

Cell culture procedures were used as we have previously reported.[17] Briefly, PLFs and GFs were obtained from the periodontal ligament (PDL) and adjacent healthy gingiva of third molars, respectively from four male subjects aged 22–38 years who underwent tooth extraction. Prior to extraction, informed consent was obtained from all donors. The PDL was isolated from the middle third of the tooth roots to exclude the contamination of gingival and apical tissues. All the biopsies were cut into small pieces, placed in 6-well tissue culture plates, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS, HyClone, Logan, UT) and 1% antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B [Antibiotic antimyotic solution, Sigma, St. Louis, MO]). Cultures were maintained at 37 °C in a humidified 5% CO_2 incubator. After cells were expanded and grown to 70–80% confluence, they were detached with 0.05% trypsin and 0.02% ethylene diaminetetra-acetic acid (EDTA) in phosphate buffered saline (PBS) and subcultured in culture flasks. Four pairs of gingival and PDL cultures were obtained. The two cell populations, PLFs and GFs, were used at the same passage within one experiment. Between experiments, cells were used in either the fifth or the sixth passage.

2.2. *Isolation and culture of human peripheral blood mononuclear cells (PBMCs)*

To isolate PBMCs, buffy coats (Sanquin, Amsterdam, The Netherlands) were diluted 1:1 (v/v) in Hank's balanced salt solution (HBSS) containing 2% FCS. Twenty-five millilitres of diluted blood was carefully layered on 15 ml lymphoprep (Axisshield Po CAS, Oslo, Norway). This gradient was centrifuged at 1000 × *g* for 30 min without brake. After centrifugation, the interphase containing PBMCs were collected and washed two times in HBSS with 2% FCS, followed by centrifugation at 400 × *g* for 10 min. Finally, the cells were resuspended in DMEM supplemented with 10% FCS, 1% antibiotics, 10^{-8} M dexamethasone and 10^{-7} M vitamin D_3.

2.3. *Osteoclastogenesis*

The effects of oestrogen on osteoclastogenesis in PLFs/GFs cocultured with PBMCs were investigated. PLFs and GFs were plated at a cell density of 1.5×10^4 cells/well in 24-well tissue culture plates. PBMCs were inoculated one day later at 1×10^6 cells/well onto fibroblast cell layers. The experimental cultures were maintained in DMEM supplemented with 10% FCS, 1% antibiotics, 10^{-8} M dexamethasone and 10^{-7} M vitamin D_3 in the presence of 10^{-11}, 10^{-9}, and 10^{-7} M 17β-estradiol (17β-E_2; Sigma, Schnelldorf, Germany) for 14 days. Control cultures were maintained under the same conditions without 17β-E_2 but in the presence of the corresponding vehicle of ethanol. The levels of oestrogen in the FCS used in the present studies were found to be very low: less than 10 pg/ml (manufacturer's data sheet). Therefore any possible effects are taken to be a result of the added oestrogen. The medium was replaced every 3 days with agents described above throughout the experiments.

To examine the direct effect of oestrogen on osteoclast precursors, PBMCs were cultured in the absence of fibroblasts under identical conditions. Cultures were performed for 14 days. Then, cells were stained for tartrate-resistant acid phosphatase (TRACP) as a marker enzyme of osteoclasts, using an acid phosphatase kit (Sigma, St. Louis, MO). Nuclei were stained with 1 μg/ml diamidino-2-phenylindole dihydrochloride (DAPI). TRACP-positive cells containing three or more nuclei were counted as osteoclast-like cells (OCs) under a light microscope. The average size of 6 randomized OCs/well was measured using image analysis software.

Cell-free areas are the sites where osteoclastogenesis occurs.[27] These areas were quantified by assessing their size in three predetermined fields/well using image analysis

Please cite this article in press as: Wattanaroonwong N, et al. Oestrogen inhibits osteoclast formation induced by periodontal ligament fibroblasts. *Archives of Oral Biology* (2010), doi:10.1016/j.archoralbio.2010.10.004

Table 1 – Primers sequences and product size.

Target	Sequences	Product size (bp)
β2-Microglobulin forward	AAgATTCAggTTTACTCACgTC	294
β2-Microglobulin reverse	TgATgCTgCTTACATgTCTCg	
OPG forward	CTgCgCgCTCgTgTTTC	100
OPG reverse	ACAgCTgATgAgAggTTTCTTCgT	
RANKL forward	CATCCCATCTggTTCCCATAA	60
RANKL reverse	gCCCAACCCCgATCATg	
ER alpha forward	gTgCAgTgTgCAATgACTATgCT	100
ER alpha reverse	gTCgTTATgTCCTTgAATACTTCTCTTg	
ER beta forward	TCAAAAgAgAgTCCCTggTgTgAAg	125
ER beta reverse	CTCTTTgAACCTggACCAgTAACAg	

bp, base pair.

software. In addition, total number of cell free areas per well was counted under a light microscope.

2.4. *mRNA expression detection by quantitative real-time polymerase chain reaction (PCR)*

The effect of oestrogen on the expression of RANKL, OPG, oestrogen receptor (ER)-α and ER-β by the fibroblasts was determined. Confluent layers of PLFs and GFs were cultured in 24-well tissue culture plates in DMEM supplemented with 10% FCS, 1% antibiotics, in the absence (as controls) or presence of 10^{-11}, 10^{-9}, and 10^{-7} M 17β-E_2 for 24 h. For a time course study, cells were cultured with either vehicle (ethanol) or with 10^{-7} M 17β-E_2. The cells were cultured for 3, 6, 24, and 48 h. Total RNA was extracted from the cultured PLFs and GFs using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration was determined with the RiboGreen Kit (Molecular Probes).

Reverse transcription (RT)-PCR was performed with 100 ng total RNA according to the MBI Fermentas cDNA synthesis kit (Vilnius, Lithuania). The specific primers, shown in Table 1, were designed using the primer express software, version 2.0 (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on the ABI PRISM 7000 (Applied Biosystems). The reactions were performed with 5 ng cDNA in a total volume of 25 μl containing SYBR Green PCR Master Mix, consisting of SYBR Green I Dye, AmpliTaq Gold DNA polymerase, dNTPs with dUTP instead of dTTP, passive reference and buffer (Applied Biosystems) and 300 nM of each primer.

After an initial activation step of the AmpliTaq Gold DNA polymerase for 10 min at 94 °C, 40 cycles were run of a two step PCR consisting of a denaturation step at 95 °C for 30 s and annealing and extension step at 60 °C for 1 min. Subsequently the PCR products were subjected to melting curve analysis to test if any unspecific PCR products were generated. The PCR reactions of the different amplicons had equal efficiencies. β2-Microglobulin was used as the housekeeping gene. Expression of this gene was not affected by estradiol treatment.

To test whether there was a difference in the expression of the genes between 17βE_2-treated and -untreated groups, mRNA levels were quantified using the comparative threshold-cycle (C_T) method as follows. First, samples were normalized for the expression of β_2-microglobulin by calculating the ΔC_T as follows; $\Delta C_T = C_{T,target} - C_{T,\beta2\text{-microglobulin}}$. Subsequently, the amount of targeted mRNA in the sample was compared by calculation of $2^{-(\Delta\Delta C_T)}$, where $\Delta\Delta C_T = \Delta C_{T,sample1} - \Delta C_{T,calibrator}$, setting the relative levels of mRNA expression in the untreated groups (calibrator) as 1. The amount of mRNA expression in 17β-E_2-treated cells was therefore expressed as a relative ratio. Furthermore, the comparison of mRNA expression of the genes between PLFs and GFs was analysed of which the amount of the gene expression is expressed as $2^{-(\Delta C_T)}$.

2.5. *Statistical analysis*

All experiments were performed with the two dental fibroblast populations collected from four different patients. Each experiment was analysed in duplicate or triplicate. Data were expressed as mean value ± standard deviation (SD). Comparisons of the numerical data between groups were performed by one-way analysis of variance (ANOVA), followed by a multiple-comparison Tukey's test or the Kruskal–Wallis test. The Pearson rank correlation coefficient was used for the correlations between the dose effect of oestrogen and the number of TRACP-positive osteoclast-like cells. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. *Osteoclast-like cell formation*

PBMCs seeded on the two different populations of fibroblasts resulted after 14 days in withdrawal of the latter cells and thus induced the formation of cell-free areas.[27] In these areas osteoclast precursors attached to the bottom and fused to form osteoclast-like cells. Osteoclast-like cells were identified as TRACP-positive cells with three or more nuclei, which could be visualized by DAPI staining (Fig. 1).

The cell-free areas of PLFs cultured with PBMCs were formed significantly more (Table 2) and were larger than those of the GF cocultures (Fig. 2). There were no significant changes in cell-free areas in PLFs or GFs cocultured with PBMCs in the presence of 17β-E_2 compared to the cultures without the hormone.

Coinciding with the results of the cell-free areas, the number of TRACP-positive osteoclast-like cells in cocultures of PLFs and PBMCs was significantly higher than in GF-PBMC cocultures (Fig. 3). The number of TRACP-positive osteoclast-like cells was significantly decreased in the 10^{-9} and 10^{-7} M

Please cite this article in press as: Wattanaroonwong N, et al. Oestrogen inhibits osteoclast formation induced by periodontal ligament fibroblasts. *Archives of Oral Biology* (2010), doi:10.1016/j.archoralbio.2010.10.004

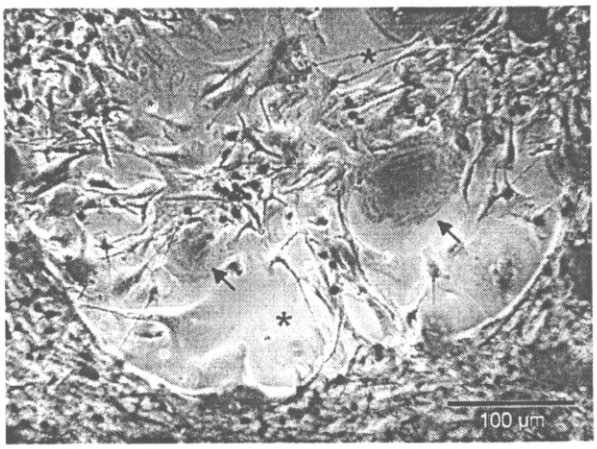

Fig. 1 – Tartrate-resistant acid phosphatase (TRACP)-positive osteoclast-like cells were seen as dark red cells (arrows) and were present in cell-free areas (asterisks).

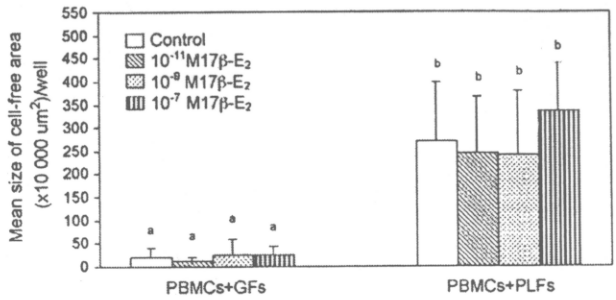

Fig. 2 – Size of cell-free areas of 14-day cocultures of human peripheral blood mononuclear cells (PBMCs) and human periodontal ligament fibroblasts (PLFs)/gingival fibroblasts (GFs) in the presence or absence of different concentrations of 17β-E_2. Data were presented as means ± standard deviation (SD). Four different cocultures of PLFs or GFs, each consisting of three wells and three predetermined fields/well were analysed. a is significant vs. b at $p < 0.05$.

17β-E_2-treated PLF cocultures compared with controls. There was a significant negative correlation between the concentration of oestrogen and the number of TRACP-positive osteoclast-like cells in the PLF cocultures ($r = -0.466$, $p < 0.01$). Such an inhibitory effect of 17β-E_2 on osteoclast-like cell formation was not seen in cocultures of GFs with PBMCs.

3.2. *PBMC cultures*

The effect of oestrogen on osteoclast formation by osteoclast precursors was analysed also by culturing human PBMC alone in the absence of fibroblasts but with the vitamin D_3 and dexamethasone. After 14 days in culture, TRACP-positive multinucleated cells were found. In general cells were round or oval and most cells contained one or two nuclei with only a few cells having three or more nuclei (Fig. 4). The number of TRACP-positive osteoclast-like cells was similar for cultures kept with or without 17β-E_2 (Fig. 5).

TRACP-positive osteoclast-like cells in the monocultures of PBMCs were 2.8-fold smaller compared with the cocultures with the two different tooth-associated fibroblast populations (Table 3) and they contained fewer nuclei. The maximum number of nuclei per cell in osteoclast-like cells in the PBMC cultures was 9 (Fig. 4) whereas more than 20 nuclei/cell were found in the cocultures.

3.3. *mRNA expression*

In an attempt to provide an explanation for the altered number of osteoclast-like cells in the cultures with 17β-E_2, the mRNA levels of RANKL, OPG, ER-β and ER-α in PLF and GF cultures were analysed. The response of the different cell types to 10^{-7} M 17β-E_2 was analysed also at various time points (3, 6, 24, and 48 h).

There were no significant differences in the mRNA expression of RANKL, OPG, ER-α by PLF and GF cultures that were cultured with or without 17β-E_2 (Fig. 6). Expression of ER-

Table 2 – The number of cell-free areas per well in the different cell cultures.

Type of cultures	Number of cell-free areas/well (mean ± SD)
coculture of PLFs-PBMCs	9.9 ± 2.6[a]
coculture of GFs-PBMCs	2.8 ± 1.0

Periodontal ligament fibroblasts (PLFs) or gingival fibroblasts (GFs) were cocultured with peripheral blood mononuclear cells (PBMCs) for 14 days. Then, the total number of cell-free areas per well was counted under a light microscope. Data were expressed as means ± standard deviation (SD) of four different cocultures of PLFs or GFs, and each analysed in triplicate.
[a] Significant vs. coculture of GFs-PBMCs, $p < 0.05$.

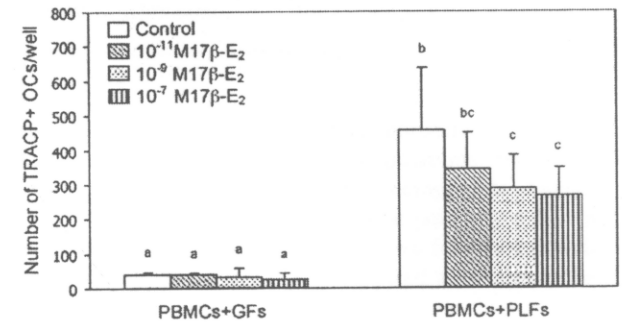

Fig. 3 – The number of TRACP-positive osteoclast-like cells (OCs) per well in cocultures of human peripheral blood mononuclear cells (PBMCs) and human periodontal ligament fibroblasts (PLFs) in the presence or absence of different concentrations of 17β-E_2 was compared to that of cocultures with human gingival fibroblasts (GFs). Data were presented as means ± standard deviation (SD) from four different cocultures of PLFs or GFs and each analysed in triplicate. a is significant vs. b and c; b is significant vs. a and c at $p < 0.05$.

Please cite this article in press as: Wattanaroonwong N, et al. Oestrogen inhibits osteoclast formation induced by periodontal ligament fibroblasts. *Archives of Oral Biology* (2010), doi:10.1016/j.archoralbio.2010.10.004

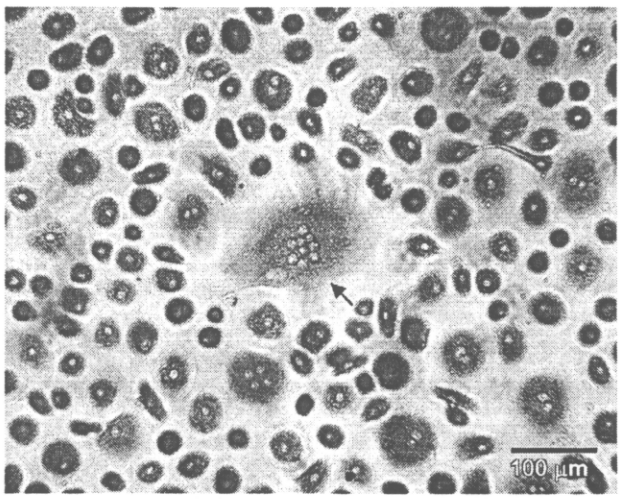

Fig. 4 – TRACP-positive osteoclast-like cells (OCs) in a monoculture of human peripheral blood mononuclear cells. The micrograph shows a large TRACP-positive OCs with 9 nuclei (arrow) surrounded by small TRACP-positive cells with one or two nuclei.

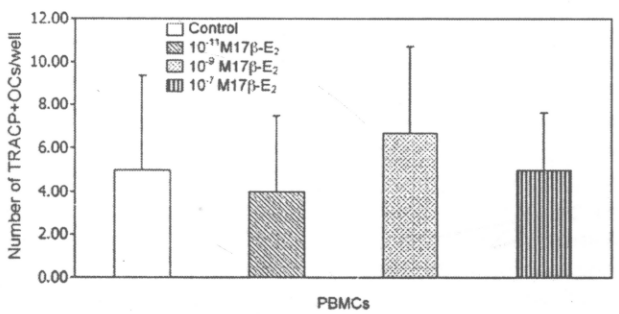

Fig. 5 – The number of TRACP-positive osteoclast-like cells (OCs) per field in a monoculture of human peripheral blood mononuclear cells (PBMCs) in the presence or absence of different concentration of 17β-E_2. Data were presented as means ± standard deviation (SD) from three independent experiments.

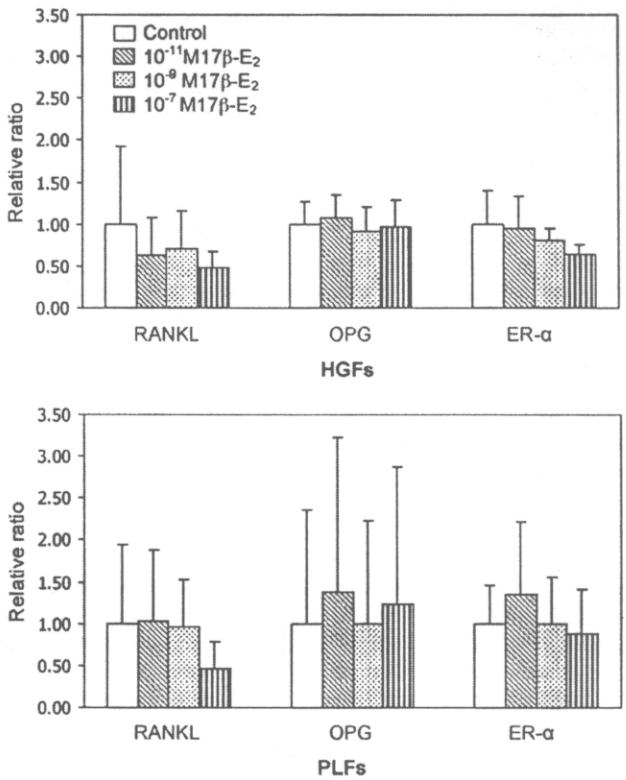

Fig. 6 – mRNA expression of receptor activator nuclear factor kappa B ligand (RANKL), osteoprotegerin (OPG), and oestrogen receptor (ER)-α by human periodontal ligament fibroblasts (PLFs) and gingival fibroblasts (GFs). The fibroblasts were cultured for 24 h in the presence or absence of different concentrations of 17β-E_2. Results were normalized to β_2-microglobulin. Results from untreated cells were set a value of 1. The mRNA expression by 17β-E_2-treated cells was therefore expressed as a relative ratio. Data were presented as means ± standard deviation (SD) from four different cultures of PLFs and GFs and each analysed in triplicate.

Table 3 – Size of TRACP-positive osteoclast-like cells in the different cell cultures.

Type of cultures	Cell size (μm^2) (mean ± SD)
Coculture of PLFs-PBMCs	13,074 ± 3524[a]
Coculture of GFs-PBMCs	13,090 ± 1302[a]
Monoculture of PBMCs	4607 ± 135

Periodontal ligament fibroblasts (PLFs) or gingival fibroblasts (GFs) were cocultured with peripheral blood mononuclear cells (PBMCs). After 14-day in culture, the average size of 6 randomized tartrate resistant acid phosphatase (TRACP)-positive osteoclast-like cells/well was measured using image analysis software. Data were expressed as means ± standard deviation (SD) of four different cocultures of PLFs/GFs and two different monocultures of PBMCs, and each analysed in duplicate and triplicate, respectively.
[a] Significant vs. monoculture of PBMCs, $p < 0.05$.

β could not be detected in either fibroblast population. Between the different fibroblast populations the mRNA expression of the different genes was comparable. Analysis of the different genes at different time points revealed no differences in expression between the two fibroblast populations. Also the addition of 17β-E_2 appeared without an effect (data not shown).

4. Discussion

In the present study we demonstrated that oestrogen inhibited the formation of osteoclast-like cells in cocultures of PLFs and PBMCs. This inhibitory effect was not found in cocultures with GFs, where only a few osteoclast-like cells were formed. These observations suggest that GFs are not or less sensitive to oestrogen; however, the underlying biological mechanisms are not clear. It is known that oestrogen exerts its effects via

intracellular oestrogen receptors. Thus the concentration of oestrogen receptor is an important determinant of cellular responsiveness to oestrogen.[28] The present data showed no difference in the mRNA expression level of ER-α expression between GFs and PLFs, suggesting that both cell types had the potency to respond similarly to oestrogen. The difference in response can be explained by assuming that the interaction between the PBMCs and the fibroblasts resulted in an altered expression of the oestrogen receptor.

An inhibitory effect of oestrogen on the formation of osteoclast-like cells is in line with previous findings obtained from either osteoblast-induced osteoclastogenesis[29] or bone marrow cells employed as precursors.[30–32] In addition, in vivo studies using ovariectomized rats demonstrated that oestrogen deficiency induced a higher number of osteoclasts in the rat periodontium.[33] These data together strongly suggest that oestrogen may play an important role in modulating osteoclast formation.

Furthermore, an interesting finding was the higher number and larger size of cell-free areas in cocultures of PLFs with PBMCs compared to cocultures with GFs. Coinciding with an increased formation of cell free areas, a higher number of osteoclast-like cells were formed in PLF cocultures. These observations strongly suggest a difference in the interaction between different fibroblast populations and PBMCs. At present, we do not know the underlying mechanisms of this phenomenon.

It has been emphasized that a cross-talk is important between osteoblasts/fibroblasts and PBMCs during the formation of osteoclasts and the subsequent process of bone resorption. Not only precursors of osteoclasts receive signals from osteoblasts/fibroblasts essential for their differentiation and function, the reverse also proves to be true. The precursors activate osteoblasts or fibroblasts resulting in the retraction of these cells thus forming cell-free areas, the sites where osteoclastogenesis occurs.[27] The intercellular adhesion molecules (e.g., ICAM-1) and vascular cell adhesion molecule (VCAM-1) have been proposed to play a role in osteoclast-like cell formation.[27,34] ICAM-1-expressing osteoblasts have been demonstrated to induce osteoclast differentiation.[35] Both PLFs and GFs express ICAM-1 and VCAM-1.[36,37] The present data showed that ligament fibroblasts responded more strongly to PBMCs than fibroblasts from the gingiva. Whether this difference in response is due to differences in expression of membrane-bound receptors (e.g., ICAM-1) known to be associated with these cell–cell interactions, needs to be investigated.

The present data showed that osteoclast-like cells were generated also from human peripheral blood mononuclear cell precursors cultured with vitamin D_3 and dexamethasone in the absence of stromal cells, albeit at severely reduced numbers and sizes compared to PBMC-fibroblast cocultures. An interesting finding is the difference in size of the osteoclast-like cells formed under these conditions. The present data showed that the osteoclast-like cells generated in the presence of the fibroblasts were nearly three times larger than those formed without the mesenchymal cells. So it seems that the fibroblasts play a pivotal role in the formation of larger multinucleated osteoclasts. One of the first steps in the formation of osteoclasts is the attraction of precursors by the osteoblasts/fibroblasts and their subsequent attachment to these cells. To a single stromal cell, a relatively high number of precursors attaches[38] thus allowing a very effective fusion of precursors at one site. Such a "bringing-together" property of the stromal cells may eventually result in the formation of larger multinucleated cells.

In addition, we found no difference in the number of osteoclast-like cells formed by the monoculture of PBMCs in the presence or absence of oestrogen. This is in agreement with other findings such as those of Matayoshi et al.[39] who showed that oestrogen did not block osteoclast formation in cultures of purified blood-derived $CD34^+$ cells obtained from blood of healthy female donors and those of Kitazawa et al.[40] who investigated mouse bone marrow cultures. These findings, together with the lack of an oestrogen effect in the cocultures with the gingival fibroblasts indicate that the PBMCs are not the target cells for the hormone. The present data seem to be in contrast to findings presented by others who showed that oestrogens did attenuate osteoclastogenesis by PBMCs.[31,32,41,42] In these studies, however, the PBMCs were cultured in the presence of RANKL, whereas we added vitamin D_3 and dexamethasone. So it appears that depending on the stimulating compound present in the culture system oestrogens have or have not an effect on osteoclastogenesis.

To further analyse the molecules that may be involved in the inhibitory effect of oestrogen, we assessed the mRNA expression of RANKL, OPG, and oestrogen receptors. We did not find any effect of oestrogen on the expression of these genes in either tooth-associated fibroblast population, confirming previous data obtained from immortalized human marrow stromal cell lines.[43] Others, however, showed that oestrogen altered the ratio of OPG to RANKL. This was not only shown with osteoblast cultures[24] but also for PLF cultures.[26] The present data suggested that the inhibitory effect of oestrogen on osteoclast-like cell formation could be mediated independent of RANKL and OPG. Thus oestrogen may exert its effect via expression of other regulating compounds like transforming growth factor (TGF)-β,[44] tumour necrosis factor (TNF)-α,[45] interleukin (IL)-1,[45] IL-6,[46] IL-7,[47] and/or macrophage colony-stimulating factor (M-CSF).[48] Recent studies have demonstrated that oestrogen deficiency results in an increased T cell mediated production of TNF-α and a concomitant increased osteoclast formation.[49–51] Factors that regulate T cell function during oestrogen deficiency have been extensively reviewed by Pacifici.[52]

Furthermore, analysis of the expression of ERs revealed that PLF and GF cultures contained ER-α mRNA, not ER-β mRNA. However, the addition of oestrogen did not alter ER-α mRNA levels in these cell types suggesting that ER-α did not mediate the osteoclast inhibition of oestrogen. The data are in line with a recent study showing that oestrogen treatment did not increase ER-α and ER-β expression in cultured human PLFs.[53] Thus their role in mediating oestrogen action on osteoclastogenesis remains unclear. Further studies are therefore required.

In conclusion, our present in vitro study shows an inhibitory effect of oestrogen on osteoclast-like cell formation in cocultures of periodontal ligament fibroblasts and peripheral blood mononuclear cells. Oestrogen exerts its inhibitory action probably not by interfering with the RANKL/OPG system. In addition, our data indicate that the two tooth-

Please cite this article in press as: Wattanaroonwong N, et al. Oestrogen inhibits ostcoclast formation induced by periodontal ligament fibroblasts. *Archives of Oral Biology* (2010), doi:10.1016/j.archoralbio.2010.10.004

associated fibroblast populations differently modulate osteoclastogenesis. In the presence of gingival fibroblasts lower numbers of osteoclasts were formed. Oestrogen may be an important modulator of osteoclast formation in the periodontal ligament under normal conditions. Our findings suggest that the hormone down-regulates this process tissue-site specifically. Yet, as soon as less or no oestrogen is present anymore, higher numbers of osteoclasts are formed, thus resulting in osteoporosis.

Funding

This study was supported by PSU Collaborative Research Fund (DEN46368), and the Thailand Research Fund and the Office of the Higher Education Commission (MRG5080025).

Competing interests

None declared.

Ethical approval

The scanned official letter stating that the informed consent was obtained is attached separately.

Acknowledgements

The authors wish to thank Dr. Ureporn Leggat (Department of Oral Biology and Occlusion, Faculty of Dentistry, PSU) for valuable advice on statistical analysis.

REFERENCES

1. Jeffcoat MK, Lewis CE, Reddy MS, Wang CY, Redford M. Postmenopausal bone loss and its relationship to oral bone loss. *Periodontology* 2000;**23**:94–102.
2. Lerner UH. Inflammation-induced bone remodeling in periodontal disease and the influence of post-menopausal osteoporosis. *J Dent Res* 2006;**85**:596–607.
3. Haas AN, Rösing CK, Oppermann RV, Albandar JM, Susin C. Association among menopause, hormone replacement therapy, and periodontal attachment loss in southern Brazilian women. *J Periodontol* 2009;**80**:1380–7.
4. Krall EA, Dawson-Hughes B, Papas A, Garcia RI. Tooth loss and skeletal bone density in healthy postmenopausal women. *Osteoporos Int* 1994;**4**:104–9.
5. Krall EA, Garcia RI, Dawson-Hughes B. Increased risk of tooth loss is related to bone loss at the whole body, hip, and spine. *Calcif Tissue Int* 1996;**59**:433–7.
6. Tezal M, Wactawski-Wende J, Grossi SG, Ho AW, Dunford R, Genco RJ. The relationship between bone mineral density and periodontitis in postmenopausal women. *J Periodontol* 2000;**71**:1492–8.
7. Payne JB, Zachs NR, Reinhardt RA, Nummikoski PV, Patil K. The association between estrogen status and alveolar bone density changes in postmenopausal women with a history of periodontitis. *J Periodontol* 1997;**68**:24–31.
8. Elovic RP, Hipp JA, Hayes WC. Ovariectomy decreases the bone area fraction of the rat mandible. *Calcif Tissue Int* 1995;**56**:305–10.
9. Gilles JA, Carnes DL, Dallas MR, Holt SC, Bonewald LF. Oral bone loss is increased in ovariectomized rats. *J Endod* 1997;**23**:419–22.
10. Tanaka M, Ejiri S, Nakajima M, Kohno S, Ozawa H. Changes of cancellous bone mass in rat mandibular condyle following ovariectomy. *Bone* 1999;**25**:339–47.
11. Nishimura I, Hosokawa R, Kaplan ML, Atwood DA. Animal model for evaluating the effect of systemic estrogen deficiency on residual ridge resorption. *J Prosthet Dent* 1995;**73**:304–10.
12. Yamashiro T, Takano-Yamamoto T. Influences of ovariectomy on experimental tooth movement in the rat. *J Dent Res* 2001;**80**:1858–61.
13. Riggs BL. The mechanisms of estrogen regulation of bone resorption. *J Clin Invest* 2000;**106**:1203–4.
14. Sasaki T. Differentiation and functions of osteoclasts and odontoclasts in mineralized tissue resorption. *Microsc Res Tech* 2003;**61**:483–95.
15. Wada T, Nakashima T, Hiroshi N, Penninger JM. RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med* 2006;**12**:17–25.
16. Kanzaki H, Chiba M, Shimizu Y, Mitani H. Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. *J Dent Res* 2001;**80**:887–91.
17. de Vries TJ, Schoenmaker T, Wattanaroonwong N, van den Hoonaard M, Nieuwenhuijse A, Beertsen W, et al. Gingival fibroblasts are better at inhibiting osteoclast formation than periodontal ligament fibroblasts. *J Cell Biochem* 2006;**98**: 370–82.
18. Somerman MJ, Archer SY, Imm GR, Foster RA. A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. *J Dent Res* 1988;**67**:66–70.
19. Kuru L, Parkar MH, Griffiths GS, Newman HN, Olsen I. Flow cytometry analysis of gingival and periodontal ligament cells. *J Dent Res* 1998;**77**:555–64.
20. Palaiologou AA, Yukna RA, Moses R, Lallier TE. Gingival, dermal, and periodontal ligament fibroblasts express different extracellular matrix receptors. *J Periodontol* 2001;**72**:798–807.
21. Hasegawa T, Kikuiri T, Takeyama S, Yoshimura Y, Mitome M, Oguchi H, et al. Human periodontal ligament cells derived from deciduous teeth induce osteoclastogenesis in vitro. *Tissue Cell* 2002;**34**:44–51.
22. Zhang D, Yang YQ, Li XT, Fu MK. The expression of osteoprotegerin and the receptor activator of nuclear factor kappa B ligand in human periodontal ligament cells cultured with and without 1α,25-dihydroxyvitamin D_3. *Arch Oral Biol* 2004;**49**:71–6.
23. Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Spelsberg TC, Riggs BL. Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. *Endrocrinology* 1999;**140**:4367–70.
24. Bord S, Ireland DC, Beavan SR, Compston JE. The effects of estrogen on osteoprotegerin, RANKL, and estrogen receptor expression in human osteoblasts. *Bone* 2003;**32**:136–41.
25. Saika M, Inoue D, Kido S, Matsumoto T. 17β-Estradiol stimulates expression of osteoprotegerin by a mouse stromal cell line ST-2, via estrogen receptor-α. *Endocrinology* 2001;**142**:2205–12.
26. Shu L, Guan SM, Fu SM, Guo T, Cao M, Ding Y. Estrogen modulates cytokine expression in human periodontal ligament cells. *J Dent Res* 2008;**87**:142–7.
27. Perez-Amodio S, Beertsen W, Everts V. (Pre-)Osteoclasts induce retraction of osteoblasts before their fusion to osteoclasts. *J Bone Miner Res* 2004;**19**:1722–31.

Please cite this article in press as: Wattanaroonwong N, et al. Oestrogen inhibits osteoclast formation induced by periodontal ligament fibroblasts. *Archives of Oral Biology* (2010), doi:10.1016/j.archoralbio.2010.10.004

28. Santagati S, Gianazza E, Agrati P, Vegeto E, Patrone C, Pollio G, et al. Oligonucleotide squelching reveals the mechanism of estrogen receptor autologous down-regulation. *Mol Endocrinol* 1997;**11**:938–49.
29. Krum SA, Miranda-Carboni GA, Hauschka PV, Carroll JS, Lane TF, Freedman LP, et al. Estrogen protects bone by inducing Fas ligand in osteoblasts to regulate osteoclast survival. *EMBO J* 2008;**27**:535–45.
30. Schiller C, Gruber R, Redlich K, Ho GM, Katzgraber F, Willheim M, et al. 17β-Estradiol antagonizes effects of 1α, 25-dihydroxyvitamin D_3 on interleukin-6 production and osteoclast-like cell formation in mouse bone marrow primary cultures. *Endrocrinology* 1997;**138**: 4567–71.
31. Sarma U, Edwards M, Motoyoshi K, Flanagan AM. Inhibition of bone resorption by 17β-estradiol in human bone marrow cultures. *J Cell Physiol* 1998;**175**:99–108.
32. Srivastava S, Toraldo G, Weitzmann MN, Cenci S, Ross FP, Pacifici R. Estrogen decreases osteoclast formation by down-regulating receptor activator of NF-κB ligand (RANKL)-induced JNK activation. *J Biol Chem* 2001;**276**:8836–40.
33. Kawamoto S, Ejiri S, Nagaoka E, Ozawa H. Effects of oestrogen deficiency on osteoclastogenesis in the rat periodontium. *Arch Oral Biol* 2002;**47**:67–73.
34. Bloemen V, de Vries TJ, Schoenmaker T, Everts V. Intercellular adhesion molecule-1 clusters during osteoclastogenesis. *Biochem Biophys Res Commun* 2009;**385**:640–5.
35. Tanaka Y, Maruo A, Fujii K, Nomi M, Nakamura T, Eto S, et al. Intercellular adhesion molecule 1 discriminates functionally different populations of human osteoblasts: characteristic involvement of cell cycle regulators. *J Bone Miner Res* 2000;**15**:1912–23.
36. Joe BH, Borke JL, Keskintepe M, Hanes PJ, Mailhot JM, Singh BB. Interleukin-1β regulation of adhesion molecules on human gingival and periodontal ligament fibroblasts. *J Periodontol* 2001;**72**:865–70.
37. Hosokawa Y, Hosokawa I, Ozaki K, Nakae H, Matsuo T. Cytokines differentially regulate ICAM-1 and VCAM-1 expression on human gingival fibroblasts. *Clin Exp Immunol* 2006;**144**:494–502.
38. de Vries TJ, Mullender MG, van Duin MA, Semeins CM, James N, Green TP, et al. The Src inhibitor AZD0530 reversibly inhibits the formation and activity of human osteoclasts. *Mol Cancer Res* 2009;**7**:476–88.
39. Matayoshi A, Brown C, DiPersio JF, Haug J, Abu-Amer Y, Liapis H, et al. Human blood-mobilized hematopoietic precursors differentiate into osteoclasts in the absence of stromal cells. *Proc Natl Acad Sci USA* 1996;**93**: 10785–90.
40. Kitazawa R, Kimble RB, Vannice JL, Kung VT, Pacifici R. Interleukin-1 receptor antagonist and tumor necrosis factor binding protein decrease osteoclast formation and bone resorption in ovariectomized mice. *J Clin Invest* 1994;**94**:2397–406.
41. Shevde NK, Bendixen AC, Dienger KM, Pike JW. Estrogens suppress RANK ligand-induced osteoclast differentiation via a stromal cell independent mechanism involving c-Jun repression. *Proc Natl Acad Sci USA* 2000;**97**:7829–34.
42. Sørensen MG, Henriksen K, Dziegiel MH, Tankó LB, Karsdal MA. Estrogen directly attenuates human osteoclastogenesis, but has no effect on resorption by mature osteoclasts. *DNA Cell Biol* 2006;**25**:475–83.
43. Ramalho AC, Jullienne A, Couttet Ph. Graulet AM, Morieux C, de Vernejoul MC, et al. Effect of oestradiol on cytokine production in immortalized human marrow stromal cell lines. *Cytokine* 2001;**16**:126–30.
44. Gao Y, Qian WP, Dark K, Toraldo G, Lin AS, Guldberg RE, et al. Estrogen prevents bone loss through transforming growth factor β signaling in T cells. *Proc Natl Acad Sci USA* 2004;**101**:16618–23.
45. Kimble RB, Srivastava S, Ross FP, Matayoshi A, Pacifici R. Estrogen deficiency increases the ability of stromal cells to support murine osteoclastogenesis via an interleukin-1 and tumor necrosis factor-mediated stimulation of macrophage colony-stimulating factor production. *J Biol Chem* 1996;**271**:28890–7.
46. Girasole G, Jilka RL, Passeri G, Boswell S, Boder G, Williams DC, et al. 17β-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens. *J Clin Invest* 1992;**89**:883–91.
47. Sato T, Watanabe K, Masuhara M, Hada N, Hakeda Y. Production of IL-7 is increased in ovariectomized mice, but not RANKL mRNA expression by osteoblasts/stromal cells in bone, and IL-7 enhances generation of osteoclast precursors in vitro. *J Bone Miner Metab* 2007;**25**:19–27.
48. Srivastava S, Weitzmann MN, Kimble RB, Rizzo M, Zahner M, Milbrandt J, et al. Estrogen blocks M-CSF gene expression and osteoclast formation by regulating phosphorylation of Egr-1 and its interaction with Sp-1. *J Clin Invest* 1998;**102**:1850–9.
49. Cenci S, Weitzmann MN, Roggia C, Namba N, Novack D, Woodring J, et al. Estrogen deficiency induces bone loss by enhancing T-cell production of TNF-α. *J Clin Invest* 2000;**106**:1229–37.
50. D'Amelio P, Grimaldi A, Di Bella S, Brianza SZ, Cristofaro MA, Tamone C, et al. Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: a key mechanism in osteoporosis. *Bone* 2008;**43**:92–100.
51. Rifas L, Weitzmann MN. A novel T cell cytokine, secreted osteoclastogenesis factor of activated T cells, induces osteoclast formation in a RANKL-independent manner. *Arthritis Rheum* 2009;**60**:3324–35.
52. Pacifici R. T cells: critical bone regulators in health and disease. *Bone* 2010;**47**:461–71.
53. Cao M, Shu L, Li J, Su J, Zhang W, Wang Q, et al. The expression of estrogen receptors and the effects of estrogen on human periodontal ligament cells. *Methods Find Exp Clin Pharmacol* 2007;**29**:329–35.

Please cite this article in press as: Wattanaroonwong N, et al. Oestrogen inhibits osteoclast formation induced by periodontal ligament fibroblasts. *Archives of Oral Biology* (2010), doi:10.1016/j.archoralbio.2010.10.004