Bull Tokyo Dent Coll (2007) 48(3): 143–146

Short Communication

Age-related Differences in Expression of Vascular Endothelial Growth Factor by Periodontal Ligament Cells *In Vitro*

Kenichi Matsuzaka, Morito Tsuruoka, Eitoyo Kokubu, Akira Katakura, Takayuki Endo, Yoshihiro Shibukawa, Masuro Shintani, Masakazu Tazaki, Kazuyuki Ishihara, Sadamitsu Hashimoto, Masao Yoshinari and Takashi Inoue

Oral Health Science Center HRC7, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan

Received 28 May, 2007/Accepted for publication 20 July, 2007

Abstract

The purpose of this study was to evaluate age-related differences in expression of vascular endothelial growth factor (VEGF) by periodontal ligament (PDL) cells. PDL cells were obtained from Wistar male rats weighing approximately 150g each in the young group and 350g each in the old group. PDL cells derived from upper and lower incisors were seeded in 35-mm culture dishes after primary culture. For cell proliferation assays, cells were detached and counted at 1, 3, 5, 7, 11 and 14 days after culture. VEGF mRNA expression was analyzed with TaqMan®. The number of cells in both groups increased day by day, but the rate of increase in the young group was higher than that in the old group. VEGF mRNA expression in the young group increased from 3 to 14 days, but in the old group increased only slightly over the same time period. Expression ratios in the young group were higher than those in the old group, and there were significant differences between the young and old groups at 7 and 14 days of culture. In conclusion, the data revealed that PDL cells varied with age, and suggest that in view of such changes in cell proliferation and VEGF mRNA expression, age should be taken into consideration in periodontal treatment.

Key words: Aging—VEGF—Periodontal ligament—*In vitro*

Introduction

Periodontal ligament (PDL) cells are heterogeneous cell populations containing fibroblasts and progenitor cells[3]. Furthermore, PDL cells are multifunctional cells that can differentiate into osteoblasts, cementoblasts or fibroblasts during wound healing in PDL. Vascular endothelial growth factor (VEGF) has been demonstrated to have a remarkable potency to induce specific proliferation of endothelial cells[4]. Periodontal regeneration requires a coordinated series of events that includes not only the recruitment of PDL-specific cells, but vascular cells as well[9]. VEGF is an angiogenic growth factor that elicits cellular responses to

injury[1]. Cell proliferation and VEGF expression play extremely important roles in the early stage of wound healing. Cellular activity is down-regulated with age, and the capacity for healing of wounds decreases with age. Angiogenesis plays an important role in homeostasis, wound healing and resistance to disease in PDL. Few studies have investigated age-related differences in characteristics of periodontal ligament cells[5,8]. The purpose of this study was to investigate age-related differences in cell proliferation and expression of VEGF mRNA in PDL cells.

Materials and Methods

All animal studies were conducted in compliance with the Guidelines for the Treatment of Experimental Animals at Tokyo Dental College. PDL cells were obtained from Wistar male rats weighing approximately 150 g each (5 weeks old) in the young group and 350 g each (15 weeks old) in the old group. Five animals were sacrificed with an overdose of thiopental (RAVONAL®; Tanabe, Japan). Both the upper and lower incisors were extracted and washed in α-minimal essential medium (α-MEM; GIBCO, Carlsbad, CA, USA) containing 10% gentamycin and 1.2% fungizone for 5 min. The pulp was then removed mechanically. Each tooth, with ameloblasts and tooth germ at the apical area removed, was placed with the PDL-side of the lingual aspect facing down in a 35-mm culture dish and cultured using α-MEM containing 10% fetal bovine serum (FBS, inactivated at 56°C for 35 min, GIBCO), ascorbic acid (50 μl/ml, Wako, Japan) and antibiotic (gentamycin, SIGMA, St. Louis, MO, USA). The cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at a temperature of 37°C. The culture medium was changed every 2 days. Fourteen days after primary culture, the cells were detached using trypsin/EDTA solution (0.1% trypsin, 0.02% EDTA, pH 7.2) and subcultured. Cells from the 4th subculture were then used in the following experiments.

For cell proliferation assays, approximately 2×10^3 PDL cells were seeded in 24-well dishes and cultured. The culture medium was changed every 2 days. Cells were detached at days 1, 3, 5, 7, 11 and 14 using trypsin/EDTA solution, and counted with a Coulter counter (Coulter Z-1, Tokyo, Japan) at each time point.

For quantitative RT-PCR, total RNA was extracted from each sample using the acid guanidium thiocyanate/phenol-chloroform method as follows. The cells were homogenized in Trisol® Reagent (Invitrogen, Carlsbad, CA, USA) after 3, 7 or 14 days of incubation after rinsing with PBS. Each solution was transferred to a tube containing chloroform and mixed. The solutions were centrifuged at 14,000 rpm at 4°C for 20 min, after which the supernatants were placed in tubes containing 70% isopropanol at −80°C for 1 h. After centrifugation, the remaining mRNA pellets were washed with 70% cold ethanol. Finally, the mRNA pellets were dissolved in RNAase-free (DEPC-treated) water. Total RNAs were reverse transcribed and amplified using an RT-PCR kit (Takara Biomedicals, Shiga, Japan). RT-PCR products were analyzed by quantitative real-time RT-PCR in TaqMan® Gene Expression Assays for two target genes, VEGF (Rn 00582935-m1) and β-actin (4352340E; as an endogenous control) (Applied Biosystems; Foster City, CA, USA), to determine variations in amounts of each RNA. All PCR reactions were performed using the real time PCR 7500 Fast System (Applied Biosystems). Gene expression quantitation using TaqMan® Gene Expression Assays was performed as the second step in a two-step RT-PCR. Assays were performed in singleplex reactions containing TaqMan® Fast Universal PCR Master Mix, TaqMan® Gene Expression Assays, distilled water and cDNA according to the manufacturer's instructions (Applied Biosystems). Reaction conditions consisted of primary denaturation at 95°C for 20 sec, and cycling for 40 cycles at 95°C for 3 sec and 62°C for 30 sec. PCR data were measured in a relative manner as folds of the corresponding 3 days incubation in the young group with the threshold cycle.

Three experimental runs were conducted,

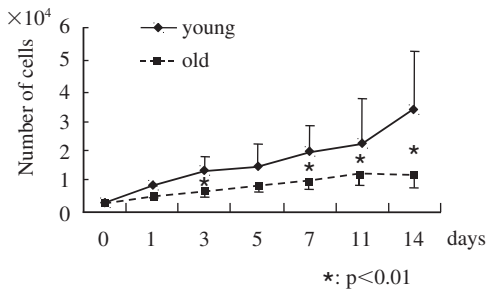

Fig. 1 Cell proliferation assay
The rate of increase in the young group is higher than in the old group.

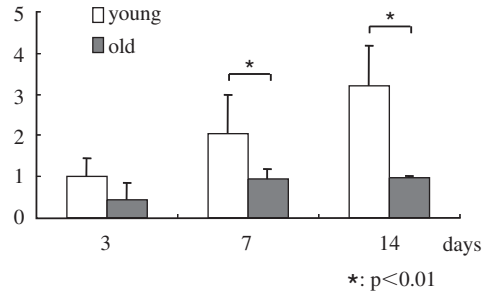

Fig. 2 Expression of VEGF mRNA
Although expression of VEGF by the old group increased slightly, expression of VEGF by the young group increased day by day. Data are shown as folds of the corresponding 3 days of incubation in the young group.

and the data were analyzed using the Student *t*-test ($p<0.05$).

Results

The number of cells in both groups increased day by day, but the rate of increase in the young group was higher than that in the old group. There were significant differences between the young and old groups at 3, 11 and 14 days of culture (Fig. 1).

VEGF mRNA expression in the young group increased from 3 to 14 days of incubation, but increased only slightly in the old group. Expression ratios in the young group were higher than those in the old group, and there were significant differences between the young and old groups at 7 and 14 days of culture (Fig. 2).

Discussion

Angiogenesis plays a role in maintaining homeostasis in and restoring PDL after chronic inflammatory periodontal disease. Generally, wound healing commences with proliferation of blood vessels and cell proliferation. Ohta *et al.* concluded that stem cells were not involved in the regeneration of periodontium, although cells which migrated from residual PDL regenerated at an early stage[6]. This indicates that proliferation of PDL cells is absolutely imperative for wound healing in PDL. The results of this study indicate that a decline in cell proliferation in older people delays wound healing. Angiogenesis is essential in organ development and wound healing[10]. Booth *et al.* demonstrated that VEGF was involved in angiogenic processes in healthy as well as in diseased periodontal tissue[2]. Many studies have investigated angiogenesis in PDL, gradually clarifying its function been clarified. However, age-related differences in angiogenesis are less well understood. VEGF is a crucial regulator of vascular development during embryogenesis (vasculogenesis), as well as in blood-vessel formation (angiogenesis) in adults[7]. Yoshino *et al.* reported that mechanical stress stimulated production of VEGF[10]. In this study, VEGF mRNA expression in the older rats was lower than that in the younger rats. The PDL in older animals is a disadvantage for orthodontic tooth moment. PDL cells play an important role in periodontal regeneration following treatment, including flap operation, guided tissue regeneration and emdogain application. PDL cells in older rats proliferate slowly compared to in younger rats, which suggests that some sort of growth factor is necessary during periodontal treatment in older patients.

In conclusion, our data revealed that PDL cells varied with age and suggest that the age

of the patient needs to be taken into consideration during periodontal treatment.

Acknowledgements

The authors would like to thank Ms. Saori Takano for her technical assistance, and associate professor Jeremy Williams for his assistance with the writing of the manuscript. This research was supported by Oral Health Science Center Grant HRC7 from the Tokyo Dental College by a "High-Tech Research Center" Project for Private Universities and matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan and 2007-2010 (No. 19592414) respectively.

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Reprint requests to:
Dr. Kenichi Matsuzaka
Department of Clinical Pathophysiology,
Tokyo Dental College,
1-2-2 Masago, Mihama-ku,
Chiba 261-8502, Japan
Tel: +81-43-270-3581
Fax: +81-43-270-3583
E-mail: matsuzak@tdc.ac.jp