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Regulation of matrix metalloproteinase-9 expression between gingival fibroblast cells from old and young rats

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ABSTRACT

Gingival fibroblast cells (rGF) from aged rats have an age-related decline in proliferative capacity compared with young rats. We investigated G1 phase cell cycle regulation and MMP-9 expression in both young and aged rGF. G1 cell cycle protein levels and activity were significantly reduced in response to interleukin-1β (IL-1β) stimulation with increasing *in vitro* age. Tumor necrosis factor-α (TNF-α)-induced matrix metalloproteinase-9 (MMP-9) expression was also decreased in aged rGF in comparison with young rGF. Mutational analysis and gel shift assays demonstrated that the lower MMP-9 expression in aged rGF is associated with lower activities of transcription factors NF-κB and AP-1. These results suggest that cell cycle dysregulation and down-regulation of MMP-9 expression in rGF may play a role in gingival remodeling during *in vitro* aging.

It is well known that aging is a major risk factor for inflammation [1]. Previous studies aimed at determining the effects of aging on gingival fibroblast cells proliferation have indicated that gingival fibroblast cells (rGF) from aged rats have impaired proliferative capacity compared with those derived from young rats. Aged rGF also have increased levels of elevated reactive oxygen species (ROS) levels. It is suggested that in GF mitochondrial DNA damage, in particular, leads to further ROS generation and impaired cellular ATP production (Park et al., unpublished results). Therefore, we postulated that this chronic cellular damage and dysfunction might also contribute to the periodontal phenotype of aged GF, in synergy with their decreased proliferative capacity. It has been shown that in aged GF, impaired proliferative capacity is associated with dysregulation of G1 phase cell cycle-associated proteins such as cyclin D1. However, the issue of how cell cycle machinery is changed during cellular aging remains unclear.

A critical outcome of periodontal diseases is degradation of the collagenous structure of periodontal tissues and matrix metalloproteinases (MMPs) has been implicated in the progression of this disease [2,3]. As the periodontal lesion develops, the junctional epithelium migrates apically leading to loss of attachment. This process requires not only cell proliferation, but also migration of the cells over the tooth and connective tissue substratum that has been modified by the inflammatory process [4]. Previous reports have demonstrated that gingival epithelial cells express several MMPs in inflamed periodontal tissues, including MMP-2, -3, -8, and -13 [5–8]. MMP-9 is expressed by gingival keratinocytes *in vitro* [9,10] and this enzyme is expressed during re-epithelialization of palatal wounds [11]. Considering that MMPs expressed by gingival epithelial cells might have a prominent role in cell migration and wound healing, it is postulated that the basal levels of MMP-9 are usually low, and that its expression can be induced by treatment of periodontal cells with tumor necrosis factor-α (TNF-α). Recent results from our laboratory demonstrated that TNF-α induces MMP-9 expression through NF-κB and AP-1 *cis*-elements in vascular smooth muscle cells [12,13].

In this study, we report that the expression of G1 cell cycle-associated proteins such as cyclin D1, cyclin E, CDK2, and CDK4 and kinase activities associated with CDK2 and CDK4 were significantly diminished with cellular aging, using primary culture GF derived from the explants of gingival tissue removed from the young (6 month old) and aged (16 month old) rats. We also find an age-associated decrease in TNF-α-induced MMP-9 expression that is regulated at the protein and transcriptional level. We further identified the NF-κB and AP-1 motifs as the *cis*-elements that are involved in cellular age-associated MMP-9 transcriptional regulation.

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Materials and methods

Materials. TNF-α was obtained from R&D systems. Polyclonal antibodies to cyclin D1, cyclin E, CDK2, and CDK4, were obtained from New England Biolabs. Polyclonal MMP-9 antibody was obtained from Chemicon.

Experimental design with old animals. Male Wistar rats were maintained in a controlled environment consisting of fluorescent lighting on a 12-h cycle and an air temperature of approximately 23 °C. Six-week-old (five rats) and 16 month old (five rats) rats were chosen as young and old animals, respectively. The protocol was approved by the Institutional Animal Guideline Board of the Department of Orthodontics.

rGF cell separation and culture. rGF cells were established by cellular outgrowth from explants of gingival tissue removed from the young and old rats, as described previously [13]. The gingival tissue was dissected into approximately 1-mm cubes and transferred to 35-mm tissue-culture dishes containing: modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 100 μg/ml penicillin G (Sigma, St. Louis, MO), 100 μg/ml streptomycin, 50 μg/ml gentamicin sulphate (Sigma), 0.3 μg/ml amphotericin B (Flow Laboratories, McLean, VA) and 10% fetal calf serum (FCS; Wellgene Co., Korea). We used early passage rat gingival fibroblasts (4th–6th passage).

Immunoblotting, immunoprecipitation and immune complex kinase assays. Growth-arrested rGF were treated with 10% FBS for the specified time periods at 37 °C. Cell lysates were prepared, and immunoblotting was performed as described previously [12]. The kinase assay was performed with either 1 μg of glutathione *S*-transferase (GST)–pRb C-terminal (pRb amino acids 769–921) fusion protein (Santa Cruz Biotechnology) or 5 μg of histone H_1 (Life Technologies Inc.), 20 μM/L ATP, and 5 μCi of [γ-32P]ATP (4500 Ci/mmol; ICN) and incubated for 20 min at 30 °C with occasional mixing. Proteins were resolved on 10% or 12.5% SDS–polyacrylamide gels.

MMP zymography, transfection and promoter analyses. We used the gelatinolytic assay as described and the cells were transfected by lipofectamine reagent (Life Technologies, Grand Island, NY) as described previously [12]. To assess promoter activity, cells were collected and disrupted by sonication in lysis buffer (25 mM tris–phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using the luciferase assay system (Promega, Madison, WI). Firefly luciferase activities were standardized for β-galactosidase activity with the MMP-9 promoter constructs [12]. In addition, the introduction of a double-point mutation into the NF-κB-site (GGAATTCCCCC to GGAATTGGCCC) to generate pGL2-MMP-9mNF-κB was performed, using the following (forward) primer: 5′-GGGTTGCCCCGTGGAATTGGCCCAAATCCTGC-3′ (corresponding to the region from −572 to −541). The creation of a double transition within the AP-1 binding site (CTGAGTCA to CTGAGTTG) to generate pGL2-MMP-9mAP-1 was performed using the following (forward) primer: 5′-CACACACCCTGAGTTGGCGT AAGCCTGGAGGG-3′ (corresponding to the region from −98 to −65). The mutants were generated using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Nuclear extracts and electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared essentially as previously described [12]. Binding reactions (20 μl) containing 2 μg poly(dI–dC), 10 mM HEPES, pH 7.9, 50 mM NaCl, 0.5 mM DTT, 2.5 mM EDTA, 7 mM $MgCl_2$, 4% glycerol, and 4–6 μg nuclear extract were incubated on ice for 30 min with 20,000 cpm of ^{32}P-labelled oligonucleotide corresponding to the MMP-9 NF-κB element (5′-CAGTGGAATTC CCCAGCC-3′) or the proximal AP-1 element (5′-CTGACCCCTGAGTC AGCACTT-3′). Complexes were separated on 6% non-denaturing polyacrylamide gels.

Results

Impaired proliferation is associated with diminished cell cycle proteins expression in aged rGF

Recently, we found that basal cell growth and thymidine uptake were greater in young rGF compared with aged rGF. Then, we examined the expression of cell cycle regulatory molecules using western blot and kinase assays. The expression of cyclin D1, Cyclin E, CDK2, and CDK4, all G1-associated factors, was down-regulated in aged rGF after IL-1β treatment (Fig. 1A). We assessed the kinase activities associated with CDK2 and CDK4 in both young and aged rGF. CDK complexes were immunoprecipitated using specific anti-CDK antibodies, and the levels of CDK-associated kinase activity were measured against Rb protein or histone H1 as the substrate. CDK2 and CDK4 kinase activities were decreased by 50% and 30%, respectively, in aged rGF compared with young cells (Fig. 1B). These specific changes in the G1 cell cycle machinery in aged rGF are consistent and likely account for the observed decreased proliferative capacity.

Decreased MMP-9 expression and promoter activity in aged rGF stimulated by TNF-α

It was known that MMP-9 is important for epithelial proliferation and migration of the cells over the tooth and connective tissue substratum [4]. Then, we tested the relationship between MMP-9 expression and cellular aging. We measured MMP-9 expression in response to TNF-α in young and aged rGF. The gelatinases secreted by young and aged rGF cultures were identified by zymographic detection of gelatinolytic activity after TNF-α treatment (Fig. 2). The conditioned media from aged rGF showed a lower MMP-9 gelatinolytic activity, compared to young rGF. Similar results were found in cell lysates and immunoblot analysis (Fig. 2). However, in the presence of TNF-α, the expression levels of another matrix metalloproteinase, MMP-2, remained essentially un-

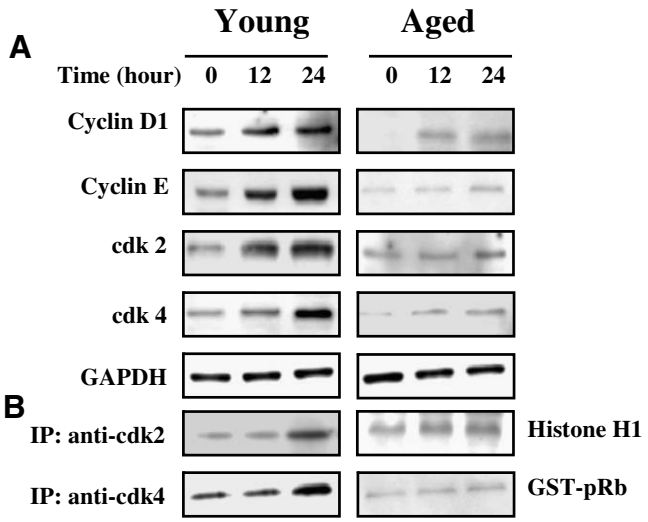

Fig. 1. G1 cell cycle dysregulation in rGF from aged rats. (A) rGF were treated with IL-1β (1 U/ml) for the indicated times, and Western blot analysis was performed with antibodies specific for cyclin D1, cyclin E, CDK2 and CDK4. Results from representative experiments were normalized to GAPDH expression by densitometry. (B) rGF were stimulated with thrombin for the indicated times, and then harvested. Total cell lysates were then immunoprecipitated with anti-CDK2 and anti-CDK4 antibodies. The kinase reaction was performed using histone H1 (for CDK2) or GST-Rb (for CDK4) as substrate. The results represent three independent experiments.