

above, together suggesting critical roles of CCN2/CTGF in the development and remodeling of dental tissues.

Conclusion

In this study, expression of CCN2/CTGF in MPL cells was confirmed *in vitro*. Utilizing this system, we uncovered the potential of CCN2/CTGF to promote the growth and differentiation of MPL cells. These findings indicate the critical roles of CCN2/CTGF in periodontal tissue development and strongly suggest the utility of CCN2/CTGF in periodontal tissue regeneration, as already proven in articular cartilage.

Methods

Cells and Cell culture

The mouse periodontal ligament cell line (MPL) was established from periodontal ligament explants from extracted mandibular molars of a BALB/c mouse [27] and maintained as described [28]. The cells were grown in alpha-modified Eagle's medium (α -MEM; Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), and kanamycin (100 μ g/ml) at 37°C in a humidified atmosphere of 5% CO₂ in air.

Production of recombinant connective tissue growth factor (rCCN2/CTGF)

Human *cnn2/ctgf* cDNA containing the entire coding region was inserted into the plasmid expression vector pcDNA3.1(-) (Invitrogen, Carlsbad, CA, USA), and the vector was used to transform HeLa cells. The conditioned medium of the HeLa cells was concentrated and purified by heparin-affinity column and anti-CTGF/Hcs24 affinity-column chromatography. Purity was determined by immunoblotting and silver staining of SDS-PAGE gels, and highly purified fractions were used for experiments. The biological activity of rCCN2/CTGF was almost the same as that of the factor purified from a human chondrosarcoma cell line (HCS-2/8), as determined by their biological activity toward chondrocytes.

Estimation of DNA synthesis

The mitogenic effect of rCCN2/CTGF was assessed by measuring [³H] thymidine (Amersham Bioscience Corp., Piscataway, NJ; Specific activity 74 TBq/nmol) incorporation into MPL cells [29,30]. The cells were labeled with [³H] thymidine (4.8 MBq/ml in DMEM) for 4 hrs, then washed with phosphate-buffered saline (PBS), and collected in 100 μ l of PBS containing 0.25% trypsin (w/v) and 0.02% EDTA (w/v). They were next harvested onto glass-fiber paper filters (WALLAC, Turku, Finland) with a semi-automatic microharvester (TOMTEC, Yamaguchi, Japan), washed with PBS, and treated subsequently with 5% trichloroacetic acid (TCA). The radioactivity was determined with a Micro Beta PLUS (WALLAC).

Estimation of cell proliferation

The effect of rCCN2/CTGF on cell proliferation was assessed by using Tetracolor-One (Seikagaku Corp., Tokyo, Japan) as instructed by the manufacturer.

Northern blotting

Ten micrograms of total RNA was electrophoresed in a formaldehyde agarose gel and subsequently blotted onto a nylon membrane. For hybridization probes, CCN2/CTGF and CCN1/Cyr61 cDNA fragments were prepared by RT-PCR with pairs of specific primers. Primers specific for CCN2/CTGF [27] and CCN1/Cyr61 [28] were described previously. These PCR products were radiolabeled and used for hybridization as described earlier [20].

Reverse transcription-polymerase chain reaction (RT-PCR)

By using ISOGEN (Nippon Gene, Tokyo, Japan)[31], we extracted total cellular RNA from MPL cell cultures treated with rCCN2/CTGF (100 ng/ml) or vehicle; and before RT-PCR, the RNA was treated with RNase-free DNase I. Total RNA (1 μ g) was reverse-transcribed by using an RNA PCR kit (Perkin Elmer, Branchburg, NJ), and the cDNA was used as a template to amplify fibroblast or osteoblast-related marker genes and murine CCN2/CTGF and GAPDH genes by PCR using Taq GOLD polymerase (Perkin-Elmer). Primer sequences employed and PCR conditions were as follow:

murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH), sense 5'-CACCATGGAGAAGGCCGGG-3' and antisense 5'-GACGGACACATTGGGGTAG-3'(418 bp); type I collagen, sense 5'-TCTCCACTCTCTAG-GTCCT-3' and antisense 5'-TTGGGTCAATTCCACATGC-3'(250 bp); osteopontin, sense 5'-ACACTTTCACTC-CAATCGTCC-3' and antisense 5'-TGCCCTTCCGTTGTT-GTCC-3'(239 bp); osteocalcin, sense 5'-TCTGACAAACCITCATGTCC-3' and antisense 5'-AAAT-AGTGTACCGTAGATGCG-3'(198 bp); periostin, sense 5'-TGTTCCCTCCTGCCCTTA-3' and antisense 5'-ACCATGCCGTITCAGGTC-3'(556 bp); ALPase, sense 5'-GCCCTCTCCAAGACATATA-3' and antisense 5'-CCAT-GATCACGTCGATATCC-3'(372 bp); Osf-2/Cbfa-1, sense 5'-GAGGGCACAAAGTCTATCTGGA-3' and antisense 5'-GGTGGTCCCGCGATGATGTC-3'(385 bp); murine CCN2/CTGF, sense 5'-GGTAAGGTCCGATTCTACCAGG-3' and antisense 5'-CTAGAAAGGTGCAAACATGTAAC-3'(120 bp).

After pre-denaturation for 7 min at 94°C, the chain reaction was repeated for 25 cycles to maintain exponential conditions for amplifications (30 cycles for murine osteopontin). Each cycle consisted of denaturation at 94°C for 1 min, with annealing and polymerization at 60°C for 2 min. Ten-micro liter samples were analyzed on 2%