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Original Article
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cDNA cloning and nucleotide sequencing of human connective tissue growth factor obtained from primary pulpal fibroblasts

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Abstract

Objective To clone human connective tissue growth factor (hCTGF) from pulpal fibroblasts.

Materials and methods Primary human pulpal fibroblasts were isolated from pulpal tissues. The reverse transcriptase-PCR assay was used to amplify the cDNA of hCTGF. The amplified PCR products were ligated into the TOPO® cloning vector and transformed into competent bacteria cells. The putative clones were bidirectionally sequenced to analyze nucleotide sequence and compare with hCTGF cDNA sequence references.

Results From RT-PCR reaction, expression of CTGF mRNA was detected in human pulpal fibroblast. Through bi-directional sequencing analysis, nucleotide sequence of our hCTGF has 100% homology to the hCTGF sequence reported.

Conclusion Human pulpal fibroblasts express CTGF mRNA. The hCTGF cDNA obtained from primary pulpal fibroblast has 100% homology to hCTGF sequence references.

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Key words*: cloning; connective tissue growth factor; pulpal fibroblasts*

Introduction

Connective tissue growth factor (CTGF) is a member of ctgf/cyr61/nov (CCN) family.[1-2] This growth factor contains four functional domains including an insulin-like growth factor binding domain, a von Willebrand factor type C repeat, a thrombospondin type I domain and a cystine knot domain, respectively.[2] The human connective tissue growth factor (hCTGF) gene is located on chromosome 6q23.1 and spans approximately 3 kb.[3]

CTGF has been implicated in several normal physiological activities such as embryonic development and differentiation, endochondral ossification and wound healing.[4-6] CTGF augments fibroblastic proliferation, chemotaxis, and extracellular matrix expression such as collagen and fibronectin.[7] In human osteoblastic cell lines, 10-50 ng/ml of CTGF stimulates cell proliferation and expression of collagen type I, osteocalcin, osteopontin, and alkaline phosphatase.[8] The *Ctgf*$^{-/-}$ knockout mice exhibits skeletal dysmorphism as a result of impaired chondrocyte function.[9] Daily subcutaneous injection of CTGF results in increasing connective tissue cell density and extracellular matrix synthesis.[10] Therefore, CTGF could potentially be a growth factor for oral tissue regeneration.

In this investigation, we report the expression of connective tissue growth factor in human pulpal fibroblast. hCTGF cDNA was cloned by RT-PCR based on homologous nucleotide sequence of 5' and 3' end of the gene. The putative PCR products were then cloned into TOPO® cloning vector. Through sequencing analysis, nucleotides of our hCTGF have 100% homology to the hCTGF which had previously been reported. Therefore, this hCTGF cDNA could be used as a template for protein production in suitable expression vector and host cells in the future.

Materials and methods

Cell cultures

Under a protocol approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. The procedures were done as previous described.[11] Briefly, primary pulpal fibroblasts were isolated from non-carious and non-periodontally involved permanent third molars from healthy patients of the Department of Oral Surgery, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. To isolate pulpal fibroblasts, each tooth was longitudinally divided and the pulp tissues removed aseptically. The tissues were rinsed several times with phosphate buffer saline solution, cut into small fragments of 1x1x1mm and placed in a 35 mm culture dish. Cells were grown in DMEM supplement with 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin, 2 mM L-glutamine and 10% fetal bovine serum. The medium was changed after 1 day and then every 48 hours. All cells were maintained in humidified 95% air 5% CO_2 atmosphere at 37°C. When cells reached confluence, they were subcultured using 0.05% trypsin with 0.01% EDTA in 60 mm plates, and these cells were considered the first-passage cells. The second- to third-passage cells were used for the experiments. All reagents were purchased from Invitrogen, USA.

RNA isolation and RT-PCR analysis

The procedures were done as previous described with some modifications.[12] Briefly, total cellular RNA was extracted from pulpal fibroblasts using TRIzol reagent. The cDNA of hCTGF was cloned by RT-PCR. Briefly, total RNA (5 μg) was converted to single stranded cDNA using Superscript III RNase

H Reverase transcriptase kit. The target cDNA was amplified using the primers homologous to 5' and 3' end of hCTGF cDNA. The sequence of primers was designed following the reference hCTGF mRNA (NM_001901, gi:4503122), National Centre for Biotechnology Information (http:/www.ncbi.nlm.nih.gov). The amplification cycles were 94°C for 1 minute, 5°C for 1 minute and 72°C for 1 minute. After 30 cycles, the reaction was extended at 72°C for an additional 5 minutes. To visualize and determine the length of the amplified fragments, the PCR product was electrophoresed on 1.5% agarose and compared with the 1kb DNA size markers ran in a neighboring lane (1050 bp for CTGF and 307 bp for GADPH as a positive control).

The putative CTGF cDNA was cloned into the TOPO® cloning vector and transformed into competent *E. coli* DH5α. Several individual colonies were selected and verified by confirming the predicted cDNA size. The clones were bidirectionally sequenced to analyze and confirm the data (Central Instrument Facility, Faculty of Science, Mahidol University, Bangkok, Thailand). The nucleotide sequence of putative CTGF cDNAs was analyzed by Chromas program version 1.45 (Conor McCarthy, Australia) and compared with human CTGF cDNA sequence reference.

Results

The RT-PCR product is hCTGF cDNA which has 100% homology to the reference cDNA

From RT-PCR reaction, we obtained fragments of PCR product about 1 kb in size (Fig. 1). The nucleotide sequence analysis revealed that our clones

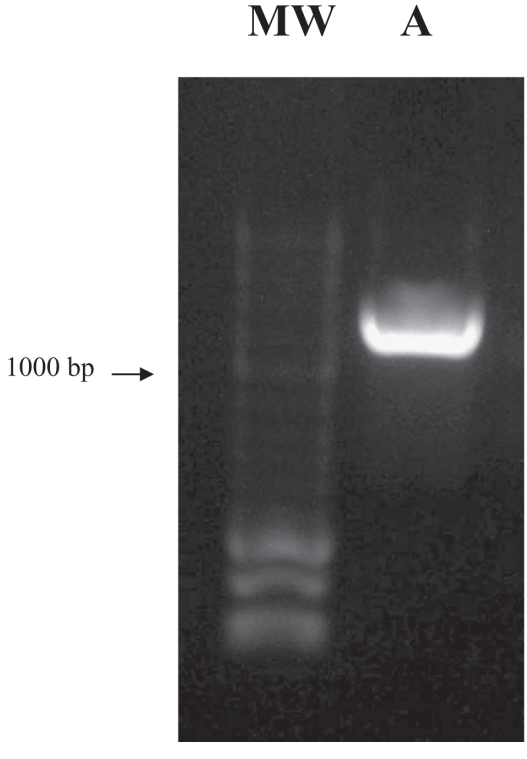

Fig. 1 The RT-PCR of putative hCTGF cDNA indicated that this gene is approximately 1 kb. The forward and reverse primers were designed using the nucletotide sequence from Genbank (NM_001901, gi:4503122). MW = DNA molecular weight marker; A = amplified product of RT-PCR reaction using the primers homologous to 5' and 3' end of hCTGF cDNA.

are hCTGF cDNA. The coding region of CTGF spans 1,050 bases from the start codon (ATG) to the stop codon (TGA). Comparison of our sequence cDNA data with the mRNA reference sequences revealed that our CTGF cDNA has 100% homology to the reference hCTGF cDNA (Fig. 2).

Genbank	1	atgaccgccg	ccagtatggg	ccccgtccgc	gtcgccttcg	tggtcctcct	cgccctctgc
Our clone	1	atgaccgccg	ccagtatggg	ccccgtccgc	gtcgccttcg	tggtcctcct	cgccctctgc
	61	agccggccgg	ccgtcggcca	gaactgcagc	gggccgtgcc	ggtgcccgga	cgagccggcg
	61	agccggccgg	ccgtcggcca	gaactgcagc	gggccgtgcc	ggtgcccgga	cgagccggcg
	121	ccgcgctgcc	cggcgggcgt	gagcctcgtg	ctggacggct	gcggctgctg	ccgcgtctgc
	121	ccgcgctgcc	cggcgggcgt	gagcctcgtg	ctggacggct	gcggctgctg	ccgcgtctgc
	181	gccaagcagc	tgggcgagct	gtgcaccgag	cgcgaccct	gcgacccgca	caagggcctc
	181	gccaagcagc	tgggcgagct	gtgcaccgag	cgcgaccct	gcgacccgca	caagggcctc
	241	ttctgtgact	tcggctcccc	ggccaaccgc	aagatcggcg	tgtgcaccgc	caaagatggt
	241	ttctgtgact	tcggctcccc	ggccaaccgc	aagatcggcg	tgtgcaccgc	caaagatggt
	301	gctccctgca	tcttcggtgg	tacggtgtac	cgcagcggag	agtccttcca	gagcagctgc
	301	gctccctgca	tcttcggtgg	tacggtgtac	cgcagcggag	agtccttcca	gagcagctgc
	361	aagtaccagt	gcacgtgcct	ggacggggcg	gtgggctgca	tgcccctgtg	cagcatggac
	361	aagtaccagt	gcacgtgcct	ggacggggcg	gtgggctgca	tgcccctgtg	cagcatggac
	421	gttcgtctgc	ccagccctga	ctgcccttc	ccgaggaggg	tcaagctgcc	cgggaaatgc
	421	gttcgtctgc	ccagccctga	ctgcccttc	ccgaggaggg	tcaagctgcc	cgggaaatgc
	481	tgcgaggagt	gggtgtgtga	cgagcccaag	gaccaaaccg	tggttgggcc	tgccctcgcg
	481	tgcgaggagt	gggtgtgtga	cgagcccaag	gaccaaaccg	tggttgggcc	tgccctcgcg
	541	gcttaccgac	tggaagacac	gtttggccca	gacccaacta	tgattagagc	caactgcctg
	541	gcttaccgac	tggaagacac	gtttggccca	gacccaacta	tgattagagc	caactgcctg
	601	gtccagacca	cagagtggag	cgcctgttcc	aagacctgtg	ggatgggcat	ctccacccgg
	601	gtccagacca	cagagtggag	cgcctgttcc	aagacctgtg	ggatgggcat	ctccacccgg
	661	gttaccaatg	acaacgcctc	ctgcaggcta	gagaagcaga	gccgcctgtg	catggtcagg
	661	gttaccaatg	acaacgcctc	ctgcaggcta	gagaagcaga	gccgcctgtg	catggtcagg
	721	ccttgcgaag	ctgacctgga	agagaacatt	aagaagggca	aaaagtgcat	ccgtactccc
	721	ccttgcgaag	ctgacctgga	agagaacatt	aagaagggca	aaaagtgcat	ccgtactccc
	781	aaaatctcca	agcctatcaa	gtttgagctt	tctggctgca	ccagcatgaa	gacataccga
	781	aaaatctcca	agcctatcaa	gtttgagctt	tctggctgca	ccagcatgaa	gacataccga
	841	gctaaattct	gtggagtatg	taccgacggc	cgatgctgca	ccccccacag	aaccaccacc
	841	gctaaattct	gtggagtatg	taccgacggc	cgatgctgca	ccccccacag	aaccaccacc
	901	ctgccggtgg	agttcaagtg	ccctgacggc	gaggtcatga	agaagaacat	gatgttcatc
	901	ctgccggtgg	agttcaagtg	ccctgacggc	gaggtcatga	agaagaacat	gatgttcatc
	961	aagacctgtg	cctgccatta	caactgtccc	ggagacaatg	acatctttga	atcgctgtac
	961	aagacctgtg	cctgccatta	caactgtccc	ggagacaatg	acatctttga	atcgctgtac
	1021	tacaggaaga	tgtacggaga	catggcatga			
	1021	tacaggaaga	tgtacggaga	catggcatga			

Fig. 2 Comparing nucleotide sequence of hCTGF between the ones from pulpal fibroblasts and reference nucle otide sequences [Genbank (accession no. NM_001901, gi:4503122 and gi:180923)].

Discussion

hCTGF is used as an excellent biological candidate gene for soft and hard tissue regeneration.[1,6,13] Without CTGF gene, mutant mice have defective chondroblast proliferation and extracellular matrix production.[9] Administering recombinant CTGF protein induces fibroblast activity and accelerates healing of burn-wounds in non-human primates.[6,14,15] Many researchers report the correlation between gingival fibrosis and connective tissue growth factor.[16-18] This is the first report regarding the expression of CTGF in human pulpal fibroblast. In this study, CTGF cDNA was cloned from Thai pulpal fibroblasts. Sequence analysis has confirmed that our clone has 100% homology to the previous reports.[19-21] Therefore, our hCTGF clone could be used as the template for future recombinant protein production. And hopefully, this protein will be utilized as local growth factor for medical and dental tissue engineering. However, the suitable vector and host cells will need to be further investigated.

Conclusion

The human pulpal fibroblasts express CTGF mRNA. The hCTGF cDNA obtained from primary pulpal fibroblast has 100% homology to hCTGF sequence references.

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การโคลนและการอ่านลำดับนิวคลีโอไทด์ของจีนคอนเน็กทีฟทิชชูโกรทแฟกเตอร์ของมนุษย์ที่ได้จากเซลล์สร้างเส้นใยโพรงฟัน

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ภาควิชากายวิภาคศาสตร์ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

บทคัดย่อ

วัตถุประสงค์ เพื่อโคลนจีนคอนเน็กทีฟทิชชูโกรทแฟกเตอร์ของมนุษย์จากเซลล์สร้างเส้นใยโพรงฟัน

วัสดุและวิธีการ เซลล์สร้างเส้นใยถูกแยกจากเนื้อเยื่อโพรงฟันของมนุษย์ จีนคอนเน็กทีฟทิชชูโกรทแฟกเตอร์ของมนุษย์ถูกขยายสัญญาณด้วยกระบวนการอาร์ที-พีซีอาร์ จากนั้นนำจีนคอนเน็กทีฟทิชชูโกรทแฟกเตอร์ที่ได้เชื่อมต่อกับเวกเตอร์และชักนำเข้าสู่เซลล์แบคทีเรีย จีนคอนเน็กทีฟทิชชูโกรทแฟกเตอร์ถูกแยกจากแบคทีเรียและตรวจลำดับนิวคลีโอไทด์แบบสองทางของจีน เปรียบเทียบกับลำดับนิวคลีโอไทด์ของจีนคอนเน็กทีฟฟิชชูโกรทแฟกเตอร์อ้างอิง

ผลการศึกษา จากกระบวนการอาร์ที-พีซีอาร์ ตรวจพบสัญญาณของอาร์เอ็นเอนำรหัสของจีนคอนเน็กทีฟทิชชูโกรทแฟกเตอร์ในเซลล์สร้างเส้นใยโพรงฟัน จากการอ่านลำดับนิวคลีโอไทด์แบบสองทางของจีนคอนเน็กทีฟทิชชูโกรทแฟกเตอร์ที่ได้ พบมีลำดับนิวคลีโอไทด์ที่เหมือนกับลำดับนิวคลีโอไทด์ของจีนคอนเน็กทีฟฟิชชูโกรทแฟกเตอร์อ้างอิง ร้อยละร้อย

สรุป เซลล์สร้างเส้นใยโพรงฟัน มีการแสดงออกของสายอาร์เอ็นเอนำรหัสของจีนคอนเน็กทีฟทิชชูโกรทแฟกเตอร์ จีนคอนเน็กทีฟทิชชูโกรทแฟกเตอร์ที่ได้มีลำดับนิวคลีโอไทด์ถูกต้องร้อยละร้อยเมื่อเปรียบเทียบกับจีนคอนเน็กทีฟทิชชูโกรทแฟกเตอร์อ้างอิง

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คำสำคัญ: *การโคลน; จีนคอนเน็กทีพทิชชูโกรทแฟกเตอร์; เซลล์สร้างเส้นใยโพรงฟัน*