ENHANCEMENT OF MOUSE CDC46 GENE EXPRESSION IN THE OSTEOBLAST BY LASER IRRADIATION

Kazutoshi Tamura, Shiki Hosoya, Koichi Hiratsuka and Yoshimitsu Abiko

Department of Biochemistry, Nihon University School of Dentistry at Matsudo, Chiba, Japan

To identify any gene whose expression is enhanced by irradiation with low incident levels of laser energy, we previously constructed the cDNA library of the osteoblastic cell line MC3T3-E1, using a stepwise subtraction procedure between cells with and without laser irradiation. In the present study, we focused on one cDNA clone, designated as MCL-60. Our preliminary sequencing experiment indicated that this clone exhibited high homology with the gene coding for mouse CDC46 (cell division cycle 46 gene which is involved in the initiation of DNA replication of eukaryotic cells. To confirm the gene product, the DNA sequence of the cDNA clone MCL-60 was determined and assessed in the GenBank and EMBL nucleic acid databases. The transcription level of the cDNA clone MCL-60 was examined by Northern blot analysis. The DNA sequence of clone MCL-60 exhibited 99.6% homology with that of CDC46 /MCM5 (minichromosome maintenance 5), and the deduced amino acid sequence exhibited 100% homology. Higher CDC46 mRNA levels were observed in laser irradiated cells compared to the levels in non-irradiated cells. Furthermore, DNA synthesis was increased by laser irradiation. These findings suggest that low incident levels of laser irradiation may play a principal role in stimulating proliferation of osteoblasts with enhancement of the gene expression of CDC46.

Key words: GaAlAs diode laser, gene expression, DNA sequence, MC3T3-E1 cell, CDC46

Introduction

Several reports have indicated that irradiation with low incident levels of laser energy stimulated wound healing,⁽¹⁻³⁾ fibroblast proliferation,^(4,5) collagen synthesis⁽⁶⁻¹⁰⁾ and bone regeneration.^(11,12) In bone metabolism, some *in vivo* experimental models using biochemical and quantitative histomorphometrical experiments revealed that low incident laser irradiation (LILI) significantly stimulated alkaline phosphatase (ALP) activity, calcium accumulation, and rapid accumulation of reparative new bone in an artificial defect in the rat tibia.^(13,14) Furthermore, cell growth, DNA synthesis, calcification,^(15,16) and bone nodule formation⁽¹⁷⁾ were significantly stimulated by LILI. However, the regulatory mechanisms leading to these findings are not yet understood. Therefore, it is neces-

sary to establish a molecular biological approach to comprehensively study the effects of LILI on bone cells. To accomplish this, we previously constructed the cDNA library of MC3T3-E1, a clonal osteoblast cell line, using a stepwise subtraction procedure between cells with and without laser irradiation.⁽¹⁸⁾ Among subtractive genes, we focused on one cDNA clone designated as MCL-60 which exhibited high homology with the mouse CDC46 gene.⁽¹⁸⁾ In eukaryotic cells the replication of the whole genome can occur only once in each cell cycle. No DNA is re-replicated until passage through mitosis into the next S-phase. CDC46 has been identified as the licensing factor and involved in the initiation of replication.⁽²²⁾

In the present study, we further characterized this clone by partial nucleotide sequencing and analysis, with the addition of Northern blot analysis to understand the mechanism of biostimulatory effects of LILI.

Addressee for Correspondence:
Yoshimitsu Abiko DDS PhD,
Department of Biochemistry, Nihon University School of Dentistry
at Matsudo, 2-870-1, Sakaecho-nishi, Matsudo, Chiba 271-8587,,
Japan

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Materials and Methods

Cell culture procedure

MC3T3-E1 cells, established from newborn mouse calvaria by Kodama et al.,⁽¹⁹⁾ were cultured in minimal essential medium (a-MEM; GIBCO BRL) containing 10% fetal calf serum and antibiotics comprising 100 μ g/ml penicillin G (Sigma Chemical Co.) and 50 μ g/ml gentamicin sulfate (Sigma) in multiwell plates.

Construction of plasmid DNA

The subtracted cDNA library of MC3T3-E1, using a stepwise subtraction procedure between cells with and without laser irradiation, was previously constructed.⁽¹⁸⁾ The construction of plasmid DNA isolated from the subtracted cDNA library is as follows. The cDNA fragments were synthesized from Poly (A)⁺ RNA by reacting with reverse transcriptase (GIBCO BRL. SuperscriptTM II) and 1.6 μ g of oligo (dT) primer carrying a *Not*I site. Each end was blunt-ended with T4 DNA polymerase and ligated to a *Bgl*II-*Sma*I adapter. After digestion with *Not*I, the cDNA fragments were directionally inserted between the *Not*I and *Bgl*II sites of vector pAP3*neo* (H. Nojima unpublished) in which the multi-cloning-sites were flanked by the T7 and T3 promoter regions.

Restriction Endonuclease Analysis

Plasmid DNA from MCL-60 clone was isolated and digested with *Not*I, *Pvu*II and *Xmn*I. The inserted DNA was mapped by using various restriction endonucleases

Fig 1: A. (above) Restriction analysis of MCL-60. Lane 1, cDNA clone MCL-60 digested with *Not*I, Lane 2, digested with *Pvu*II, and Lane 3, digested with *Xmn*I. The pAP3*neo* vector (5373 bp) contains one each *Not*I and *Xmn*I site, and two *Pvu*II sites. B. (below) Restriction endonuclease map of the CDC46 (upper column), and the cDNA inserted in clone MCL-60 (lower column). The open box shows the coding regions of CDC46. Sequenced regions of MCL-60 are shown by the solid box.

Laser irradiation and total RNA extraction

As the low-power laser source, a GaAlAs diode laser device (Matsushima Industrial Equipment Inc., Osaka, Japan model: Panalas® 1000) was used. The technical specification of this laser device was as follows: wavelength: 830 nm; output power: 100~700 mW, variable. Laser irradiation was performed at a distance of 550 mm (area of spot size: 78.5 cm²) from the probe to the cell layer. Continuous wave at 500 mW output power for 20 min (energy density: 7.64 J/cm²) was used. Total RNAs were harvested from cells with or without GaAlAs diode laser irradiation.

DNA sequencing

Dideoxy-chain termination sequencing⁽²⁰⁾ was performed with fluorescent dye-labeled T7 universal primers (Aloka, Tokyo, Japan) and Sequi Therm™ Long-Read™ cycle sequencing kits for Li-Cor® Sequencing (Epicentre Technologies, USA). The reaction products were analyzed by a 4000LS Long ReadIR™ DNA sequencing system (LI-COR, USA).

Northern blot analysis

The total RNAs were isolated from laser irradiated MC3T3-E1 cells. RNA samples containing 2.2 M formaldehyde were subjected to electrophoresis in 1.0% agarose gel containing 0.74 M formaldehyde.⁽²¹⁾ The RNA was transferred to a nitrocellulose filter in 20 x SSC. To prepare the hybridization probe, DNA insert was gel-purified from *NotI-SmaI* digested clone MCL-60 and labeled with ³²P using a BcaBest random primer kit (Takara, Japan). Hybridization was performed in a solution containing 4x SSC, 5x Denhardt's reagent, 0.2% SDS, 12 µg/ml denatured salmon sperm DNA and 50% formamide at 65°C for 16 h. Filters were washed twice in 0.1% SSC and 0.1% SDS at 55°C. The signals of the bands were detected by radio-autoradiograph using X-ray film.

Assay of DNA synthesis

DNA synthesis was determined by incorporation of ³H-thymidine (47 Ci/mmol, Amersham). Following laser irradiation, the cells were further cultured for 7 hrs and labeled with 0.5 µCi/ml ³H-thymidine for 2 hrs. At the end of the period, the cultured medium was removed and the cells were washed twice with PBS and ice-cold 10% (w/v) TCA solution and subsequently with ethanol-ether (3:1, v/v). The cells were dissolved in 0.5N NaOH-10% SDS solution. The radioactivity was measured in a scintillation counter (Aloka).

Results

Restriction endonucleases analysis

The plasmid was purified from MCL-60 clone and the inserted DNA fragment was isolated, then digested with restriction enzymes as shown in Figure 1A. The restriction map was constructed from digestion patterns and compared to the map from CDC46 gene data base.

As shown in Figure 1B, the inserted DNA revealed a restriction map which was identical with the patterns of CDC46 gene.

Sequencing of cDNA

Figure 2 shows partial DNA sequence and the deduced amino acid sequence data of inserted DNA from MCL-60. The sequence data exhibited high homology with CDC46.⁽¹⁸⁾

Northern blot hybridization

To confirm whether the transcription level of the CDC46 gene in clone MCL-60 was enhanced by low-power laser irradiation, the total RNAs were isolated from both laser irradiated and non-irradiated MC3T3-E1 cells, and examined by Northern blot hybridization.

As shown in Figure 3, it is apparent that the CDC46 gene expression was higher in laser-irradiated cells compared to that in non-irradiated cells at 6 hrs.

DNA synthesis

Figure 4 shows the effects of laser irradiation on DNA synthesis in MC3T3-E1 cells. ³H-thymidine incorporation was markedly stimulated by laser-irradiation.

Discussion

Recent clinical and experimental studies indicated the importance of the biostimulatory effects of low-power laser irradiation. In bone metabolism, some *in vivo* studies reported that low-power laser irradiation promoted bone regeneration in damaged bone tissue: e.g. bone fracture,⁽¹¹⁾ tooth-extraction⁽¹²⁾ and experimental hole-like injuries.⁽¹³⁾ These findings indicate that laser irradiation may play a principal role in stimulating proliferation and differentiation of osteoblasts. However, the regulatory mechanism leading to these observations is not yet understood in detail. Therefore, we had constructed a cDNA library by a stepwise subtraction procedure prepared from low-power laser irradiated mouse osteoblastic cell line (MC3T3-E1), and isolated the clone MCL-60.⁽¹⁸⁾

The restriction map of inserted DNA from MCL-60 was identical with that of mouse CDC46 gene data base. Furthermore, The 789 bp cDNA insert in clone MCL-60 was highly homologous with mouse CDC46 gene by DNA sequencing. Northern blot hybridiza-

	1	IleValLysAspGluHisAsnGluGluArgAspMetMetLeuAla	15
MCL-60	1	ATCGTCAAAGATGAGCACAATGAGGAGAGGGACATGATGCTAGCC	45
mCDC46	1636	ATCGTCAAAGATGAGCACAATGAGGAGAGGGACATGATGCTAGCC	1680
	518	IleValLysAspGluHisAsnGluGluArgAspMetMetLeuAla	532
	16	LysHisValMetThrLeuHisValSerAlaLeuThrGlnThrGlnAlaValGluGlyGlu	35
MCL-60	46	AAACATGTGATGACTCTGCATGTGAGTGCACACAGACACAGGCTGTGGAGGGTGAG	105
mCDC46	1681	AAACATGTGATGACTCTGCATGTGAGTGCACACAGACACAGGCTGTGGAGGGTGAG	1740
	533	LysHisValMetThrLeuHisValSerAlaLeuThrGlnThrGlnAlaValGluGlyGlu	552
	36	IleAspLeuAlaLysMetLysLysPheIleAlaTyrCysArgAlaArgCysGlyProArg	55
MCL-60	106	ATCGACCTGGCCAAAGATGAAGAAGTTTCATAGCCTACTGCCGAGCGAGGTGTGGACCTCGG	165
mCDC46	1741	ATCGACCTGGCCAAAGATGAAGAAGTTTCATAGCCTACTGCCGAGCGAGGTGTGGACCTCGG	1800
	553	IleAspLeuAlaLysMetLysLysPheIleAlaTyrCysArgAlaArgCysGlyProArg	572
	56	LeuSerAlaGluAlaAlaGluLysLeuLysAsnArgTyrIleIleMetArgSerGlyAla	75
MCL-60	166	CTATCAGCAGAGGCGAGCAGAGAAGCTGAAAAACCGCTACATCATCATGCGGAGTGGGGCT	225
mCDC46	1801	CTATCAGCAGAGGCGAGCAGAGAAGCTGAAAAACCGCTACATCATCATGCGGAGTGGGGCT	1860
	573	LeuSerAlaGluAlaAlaGluLysLeuLysAsnArgTyrIleIleMetArgSerGlyAla	592
	76	ArgGlnHisGluArgAspSerAspArgArgSerSerIleProIleThrValArgGlnLeu	95
MCL-60	226	CGTCAGCATGAGAGGGACAGTGACCGGCGTTCCAGCATCCCCATCACTGTGCGGAGCTG	285
mCDC46	1861	CGTCAGCATGAGAGGGACAGTGACCGGCGTTCCAGCATCCCCATCACTGTGCGGAGCTG	1920
	593	ArgGlnHisGluArgAspSerAspArgArgSerSerIleProIleThrValArgGlnLeu	612
	96	GluAlaIleValArgIleAlaGluAlaLeuSerLysMetLysLeuGlnProPheAlaThr	115
MCL-60	286	GAGGCTATTGTGCGCATTGCTGAGGCGCTCAGTAAGATGAAACTGCAGCCCTTTGCCACT	345
mCDC46	1921	GAGGCTATTGTGCGCATTGCTGAGGCGCTCAGTAAGATGAAACTGCAGCCCTTTGCCACT	1980
	613	GluAlaIleValArgIleAlaGluAlaLeuSerLysMetLysLeuGlnProPheAlaThr	632
	116	GluAlaAspValGluGluAlaLeuArgLeuPheGlnValSerThrLeuAspAlaAlaLeu	135
MCL-60	346	GAGGCTGATGTAGAGGAGGCATTGAGACTGTTCCAGGTGTCCACACTGGATGCTGCTTTG	405
mCDC46	1981	GAGGCTGATGTAGAGGAGGCATTGAGACTGTTCCAGGTGTCCACACTGGATGCTGCTTTG	2040
	633	GluAlaAspValGluGluAlaLeuArgLeuPheGlnValSerThrLeuAspAlaAlaLeu	652
	136	SerGlyAsnLeuSerGlyValGluGlyPheThrThrGlnGluAspGlnGluMetLeuSer	155
MCL-60	406	TCTGGCAATCTGTGCGGGGTGGAGGGCTTCACTACCCAGGAGGACAGGAGATGCTGAGC	465
mCDC46	2041	TCTGGCAATCTGTGCGGGGTGGAGGGCTTCACTACCCAGGAGGACAGGAGATGCTGAGC	2100
	653	SerGlyAsnLeuSerGlyValGluGlyPheThrThrGlnGluAspGlnGluMetLeuSer	672
	156	ArgIleGluLysGlnLeuLysArgArgPheAlaIleGlySerGlnValSerGluHisSer	175
MCL-60	466	CGCATTGAGAGCAACTCAAGCGCCGTTTTCGCTTGGCTCTCAGGTGTCTGAACACAGC	525
mCDC46	2101	CGCATTGAGAGCAACTCAAGCGCCGTTTTCGCTTGGCTCTCAGGTGTCTGAACACAGC	2160
	673	ArgIleGluLysGlnLeuLysArgArgPheAlaIleGlySerGlnValSerGluHisSer	692
	176	IleValGlnAspPheThrLysGlnLysTyrProGluHisAlaIleArgLysValLeuGln	195
MCL-60	526	ATTGTCCAGGACTTCACCAACAGAAATATCCAGAGCACGCTATCCGAAAGGTGCTGCAG	585
mCDC46	2161	ATTGTCCAGGACTTCACCAACAGAAATATCCAGAGCACGCTATCCGAAAGGTGCTGCAG	2220
	693	IleValGlnAspPheThrLysGlnLysTyrProGluHisAlaIleArgLysValLeuGln	712
	196	LeuMetLeuArgArgGlyGluIleGlnHisArgIleGlnArgLysValLeuTyrArgLeu	215
MCL-60	586	CTCATGCTACGCGAGGGGTGAGATCCAACACCGTATGCAGCGCAAGGTGCTCTATGCGCTC	645
mCDC46	2221	CTCATGCTACGCGAGGGGTGAGATCCAACACCGTATGCAGCGCAAGGTGCTCTATGCGCTC	2280
	713	LeuMetLeuArgArgGlyGluIleGlnHisArgIleGlnArgLysValLeuTyrArgLeu	732
	216	Lys	
MCL-60	646	AASTGAGCCCATTTGCCATCAACCCCTCAAGCCTGAAATGCTGCCACCAACCTATCTCCCA	705
mCDC46	2281	AASTGAGCCCATTTGCCATCAACCCCTCAAGCCTGAAATGCTGCCACCAACCTATCTCCCA	2340
	733	Lys	
	706	GTCAGTGTCTCAAACCTCCTTTTGCCTGCTCTCCACCTCAGACTGCACTGTGCAAC	765
MCL-60	706	GTCAGTGTCTCAAACCTCCTTTTGCCTGCTCTCCACCTCAGACTGCACTGTGCAAC	765
mCDC46	2341	GTCAGTGTCTCAAACCTCCTTTTGCCTGCTCTCCACCTCAGACTGCACTGTGCAAC	2400
	766	ATCTGCAGCCCTTGAAATGTACT	789
MCL-60	766	ATCTGCAGCCCTTGAAATGTACT	789
mCDC46	2401	ATCTGCAGCCCTTGAAATGTACT	2424

Fig 2: The nucleotide and deduced amino acid sequences of the cDNA inserted in clone MCL-60, respectively. Sequence similarity was examined by using the GenBank and EMBL nucleic acid databases and compared with CDC46. Conserved bases and residues are shadowed. The DNA sequence of clone \mcl-60 exhibited 99.6 homology with that of CDC46 and the deduced amino acid sequence exhibited 100% homology.

Fig 3: Northern blot analysis of MCL-60 (CDC46). Each lane contained 4 μ g of total RNA (control: non- laser irradiated MC3T3-E1 cells). The probes used were MCL-60 (CDC46) (upper column) and β -actin genes (lower column). The arrow heads indicate the size of the transcripts.

Fig 4: Effect of laser irradiation on DNA synthesis of MC3T3-E1 cells. Procedures of laser irradiation and 3 H-thymidine incorporation were described in MATERIALS AND METHODS. Values are mean $\pm$ S.D. for 4 cultures. Significant difference from non-irradiation control was (* P < 0.001).

tion confirmed that this gene was highly expressed in laser irradiated cells when compared with that of non-irradiated cells. Therefore it is likely that mouse CDC46 gene expression was enhanced by low-power laser irradiation.

Low intensity laser irradiation has several biostimulatory effects, the principal of which is promotion of cell proliferation.^(4,5,13,15) Several studies on osteoblasts showed that DNA synthesis was stimulated by low-power laser irradiation.^(15,16) Yamada⁽¹⁵⁾ reported that cell proliferation and DNA synthesis of MC3T3-E1 were significantly increased after laser irradiation. They carried out a thymidine-incorporation experiment for 24 hrs after laser irradiation. Since CDC46 gene expression was enhanced at 6 hrs after laser irradiation (Figure 3), we confirmed the enhancement of DNA synthesis by 3 H-thymidine incorporation experiment from 7 to 9 hrs after laser irradiation. The study present here also showed the enhancement of DNA synthesis in MC3T3-E1 cells by laser irradiation (Figure 4).

The gene expression of CDC46 gene isolated from laser irradiated MC3T3-E1 cDNA library is cell-cycle specific at the late G1 to S phase.⁽²²⁾ The CDC(MCM) gene family is conserved from yeast^(23,24) to mammals^(22,25,26) and consists of at least six members.⁽²²⁾ This gene family is known as a "licensing factor",⁽²⁷⁾ and plays important roles in the once-per-cell-cycle DNA replica-

tion. The microinjection of a specific antibody against a licensing factor P1MCM3 inhibited DNA replication in mouse 3T3 cells.⁽²⁶⁾ The CDC46 interacted with the P1MCM3 in a similarly very tightly regulated manner and/or functioned as a complex throughout the cell cycle.⁽²²⁾ These findings suggested that MCM proteins had an important role in the regulation of DNA replication during the S phase. Furthermore, Karu *et al.*⁽²⁸⁾ reported that low power laser irradiation promoted the shift of the cell cycle from G1 to the S phase. As a result, the proportion of S phase cells occurring in whole cells increased. These findings suggest that CDC46 is involved in promoting a new cell cycle as a licensing factor at certain stages of the cell cycle. In conclusion, laser irradiation may play an important role in stimulating cell proliferation of osteoblasts through enhancement of CDC46 gene expression.

Since MCM proteins form a large complex for the initiation of DNA replication, it would be of interest to study the effect of laser irradiation on the gene expression of other MCM related genes. At this point, it is still unclear what kind of signal transduction system and transducer culminating in transient expression of transcription factors are expressed by the stimuli of laser irradiation. It is necessary to clarify these changes in the signal transduction system levels through laser irradiation at molecular levels.

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