研究課題名：レーザー照射の生物学的効果の細胞学的解明
- 培養細胞の遺伝子発現プロファイリングとパスウエイ解析 -

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【研究目的】

レーザー照射は創傷、難治性潰瘍に治癒効果があると報告されて以来、炎症、疼痛に対する抑制作用や骨形成の促進効果が証明されている。しかしながら、一方ではレーザー療法に懐疑的な見解も多く、レーザー照射の効果を心理的なプラセボ効果が大きいとする考えやレーザー治療は、臨床応用が試行錯誤的に実施されて効果のあった事象だけが誇張されているという意見もある。また、生物学的効果は単なる温度上昇効果によるものであろうとする議論やレーザー照射の副作用について十分検討されていないという指摘もされている。また、レーザー波長の違いによる生物学的効果についても不明な点が多い。このような背景からレーザー照射の生物学的効果の作用機序の解明は未だ不十分であり、積極的な臨床応用が期待されているなかで、レーザー医療をさらに推進、発展させるためには、有用性の高いレーザー照射の機種、照射法を開発するとともに、生物学的効果を実証科学的に解明していく必要があると思われる。

本研究では、歯肉線維芽細胞、関節リュウマチの膝滑膜細胞、骨芽細胞の細胞培養系を用いてレーザー照射による遺伝子発現変化をプロファイリングし、生物学的効果の機序解明を試みた。

【研究概要】

成人の８０％以上が罹患する歯周病は歯の喪失原因となる。高齢者における歯喪失の最大原因は歯周病であり、加齢により歯周病の病態が進展すると信じられている。しかし、高齢者の歯周病は老化という因子だけが引き金となって発症はしないと考えられている。高齢者の歯周病といえども歯肉炎・歯周炎の発症には細菌感染が重要な原因であることは間違いない。

インターロイキン-1β (interleukin-1β;1L-1β)は、遺伝子の転写、翻訳後は前駆体として生合成され、変換酵素(1L-1β converting enzyme; ICE)によって活性型になる。1L-1βは炎症性サイトカインとして炎症の進展に関与するとともに、破骨細胞の分化、活性化因子として働き、発痛物質としても知られている。プロスタグランジン E_2 (prostaglandin E_2; PGE_2)は、ホスホリパーゼ A2 によって細胞膜のリン脂質から遊離されるアラキドン酸を基質に律速酵素シクロオキシゲナーゼによってプロスタグランジン G_2/H_2 が合成され、ついで PGE_2 合成酵素によって合成される。PGE_2 血管透過性促進因子として炎症の進展に関与するとともに、破骨細胞の分化、活性化因子として働き、発痛の促進物質としも知られている。

そこで、細菌感染に対する防御組織としての観点から歯周病原細菌内毒素(LPS)で人歯肉線維芽細胞を刺激する培養細胞系で PGE_2、IL-1βの炎症メディエーター産生が誘導される実験モデル系を設定した。この培養実験系を応用して、レーザー照射の影響を調べた。その結果、LPS 刺激で増大した PGE_2、IL-1β産生量はいずれもレーザー照射によって減少していた。この効果は照射量に依存しており、産生抑制は COX2 と IL-1βの遺伝子発現の抑制により起こっていることが明らかとなっ

た。また、関節リュウマチの膝滑膜細胞を IL-1βで刺激したモデル実験系へのレーザー照射は、キモカイン群の遺伝子発現レベルを抑制することを明らかにした。

レーザー照射による骨折や骨欠損部の骨形成促進作用が報告されているが、その作用機序は解明されていない。我々は、骨芽細胞へのレーザー照射により骨結節数、総面積が増大し、培養早期のレーザー照射がより強い骨形成促進効果をすることから、レーザー照射は未分化間葉系細胞の増殖と骨芽細胞への分化を促進することにより引き起こされていることを見出しているが、レーザーの生物学的効果に関与するメカニズムはもっと多様で且つ複雑であり、未知の生命現象が関わっている。そこで、レーザー照射した細胞から mRNA を分離して cDNA 遺伝子バンクを作成し、非レーザー照射細胞から回収した mRNA を用いて同一遺伝子を差し引く差分化遺伝子クローニングを行い、差分化遺伝子ライブラリーの各遺伝子クローンの塩基配列を解読してゲノムデータベースの遺伝子配列とホモロジー検索することによって当該遺伝子を同定した。

MCM (minichromosome maintenance) 遺伝子ファミリーは細胞の細胞分裂時に細胞周期 S 期で DNA 複製が起こることを保証するライセンス因子で真核細胞の DNA 複製に必須であることが明らかになっている。 Northern blot 分析を行ったところ、レーザー照射後レーザー非照射細胞に比較して MCM mRNA レベルの増加が認められ、DNA 合成能を放射性チミジン取り込み量により検討した結果、レーザー非照射群に比較して増加が認められた。このことからレーザー照射の細胞増殖促進作用は MCM 遺伝子発現介して DNA 複製を進行させることが関与することが示唆された。また、骨芽細胞の骨形成作用に関連する遺伝子として Annexin III と MIF(macrophage migration inhibitory factor) 遺伝子を同定し、RT-PCR 法によって確認してした。Annexin III は、細胞カルシウムの調節に関与するといわれており、MIF は、最近、骨芽細胞にも産生されており、骨芽細胞の増殖や骨改造を調節する機能をもつことが示唆されている。

ゲノムデータベースを応用して、短時間に遺伝子発現度を解析できる DNA マイクロアレイ法の開発が進んでいる。約 3900 遺伝子の cDNA マイクロアレイを応用して骨芽細胞 MC3T3-E1 に低出力レーザー照射を行い、遺伝子発現が変動する遺伝子をモニタリングした。その結果、osteoglycin mRNA レベルが低出力レーザー照射によって増大することが見出し、Real time PCR 法によって確認した。Osteogl y cin は、骨誘導因子として精製された標品の構成成分でとして見出されたプロテオグリカンで骨形成因子の保持、機能発現に関与することが報告されていることから、レーザー照射による骨芽細胞の osteoglysin 産生促進は骨形成能の促進に関与していると考えられる。

オリゴヌクレオチドをガラス基板に直接合成し、RNA サンプルを低分子化してハイブリダイズさせることで信頼度を高めた Gene Chip が開発されている。Gene Chip で解析された膨大なトランスクリプトームデータは研究目的の新規関連遺伝子の発見に大きなインパクトを与えている。最近、解析データを膨大な遺伝子間、タンパク質間の相互関係、高次生物学的プロセス関係の膨大なデータベースと網羅的に照合させる Ingenuity Pathway Analysis システムが開発されている、Gene Chip を用いて、ヒト骨芽細胞への低出力レーザー照射によって発現が変化する遺伝子のトランスクリプトーム解析を試みた。オントロジー解析した結果、細胞分裂、シグナル伝達関連遺伝子、成長因子、成長因子受容体、イオンチャネル関連因子、Ca 調節因子など多数の遺伝子の発現がレーザー照射によって変動していることを見出した。さらに、Ingenuity Pathway Analysis システムに解析データベースにデータマイニングした解析結果を送り、検索し、Wint/β-catenin シグナル系、PI3 kinase/JAKT シグナル系の関与が示唆されている。

【まとめ】

培養細胞系を応用してレーザー照射の生物学的効果が証明され、その機序としてレーザー照射は種々の遺伝子発現を変動することが明らかとなった。さらに、ゲノム/トランスクリプトーム データベースを応用した差分化遺伝子クローニングや cDNA マイクロアレイ、Affymetrix Gene Chip を応用してレーザー照射による発現促進する遺伝子の探索にも成功した。良くいわれてることではあるが、mRNA レベルは必ずしもタンパク質レベルとは一致しない。遺伝子発現をモニターする、これらの研究成果によってレーザー照射の生物学的効果の全貌が明らかになったわけではない。情報伝達系の構成分子のなかには、必ずしもレーザー照射の刺激によって遺伝子の転写を通じてタンパク質を発現させる必要はなく、例えばタンパク質としてはすでに存在しているが不活性状態であり、リン酸化されて始めて作用を発揮するものがある。あるいは半減期の長いタンパク質では高い遺伝子発現レベルを必要としない。したがってトランスクリプトーム解析によって全ての生命現象が明らかになるわけではない。二次元電気泳動ゲルをイメージアナライザーで分析して標的タンパク質スポットを切り出して、in gel digestion、ペプチド断片の精製を自動的に行うロボットと飛行時間型質量分析機器システムを用いてゲノムデータベース解析ツール MASCOT サーチを応用してレーザー照射によって増減するタンパク質、リン酸化タンパク質の網羅的解析を行っている。レーザー照射による生物学的効果の解明にプロテオミクス研究を応用している。近い、将来、(ゲノム)-(トランスクリプトーム)-(プロテオーム)データベース情報を駆使したバイオインフォーマティクス研究によってレーザー照射による生物学的作用の全貌が明らかになり、より効果的なレーザー医療の推進が期待できよう。今後、異なる波長の自由電子レーザー照射による生物学的効果の違いについてトランスクリプトーム-プロテオーム解析から得られるデータによって情報伝達系—遺伝子発現をコアーにした更なる解明が必要である。

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＜原　著＞

Effect of Low-Level Laser Irradiation on Macrophage Inhibitory Factor Gene Expression in Osteoblasts

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骨芽細胞の Macrophage Inhibitory Factor 遺伝子発現への低出力レーザー照射の影響

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The low-level laser irradiation (LLLI) therapy for bone healing has attracted strong interest in the field of clinical implantology. It has been reported that LLLI on bony implant sites might have positive effects on the integration of implants. The biostimulatory effect of bone formation by LLLI has been investigated, however, very little is known about the molecular basis of biostimulatory mechanisms. Since LLLI will be useful to support implant therapy, it is important to elucidate the mechanism of biostimulatory effect of LLLI on bone formation.

We previously constructed a cDNA library of mouse osteoblastic cells (MC3T3-E1), which enhanced gene expression by LLLI using a subtracted gene cloning technology. In the present study, we analyzed the DNA nucleotide sequence of gene clones MCL-176. The nucleotide sequence of MCL-176 DNA insert was determined and assessed in the standard nucleotide-nucleotide BLAST homology - search using NCBI DNA databases. DNA nucleotide sequences of clone MCL-176 cloned DNA exhibited high homology with macrophage (migration) inhibitory factor (MIF) gene. Reverse-transcription real-time PCR analysis showed that *MIF* mRNA level was enhanced by LLLI. MIF is known as a cytokine that mediates inflammatory processes in a variety of tissues. Since it has been reported that MIF is also presented in osteoblasts and MIF modulates the proliferation of osteoblasts, MIF is involved in the growth of osteoblasts and bone tissue remodeling. Our findings suggest that LLLI may enhance *MIF* mRNA transcription of osteoblasts and play a role in the osseous metabolism.

Key words : *osteoblast, low-level laser irradiation, macrophage migration inhibitory factor, gene expression*

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Introduction

Various biostimulatory effects of low-energy laser irradiation (LLLI) have been reported for wound healing[1], cell proliferation of fibroblasts[2] and chondrocytes[3], collagen synthesis[4] and nerve regeneration[5]. Of particular interest for implant research is the acceleration of bone regeneration by laser treatment[6~9]. Further, the recent advent of improved laser delivery systems has attracted interest in the application of lasers in implantology[10]. DÖRTBUDAK et al.[11] reported that LLLI stimulated osteocyte viability in bony implant sites after drilling and implant insertion in baboons, and suggested the positive effect of LLLI on the integration of implants. However, the lack of knowledge of the biological mechanism triggered by LLLI may delay the clinical use of laser therapy including clinical implantology. In order to develop LLLI therapy for dental implants, it is important and necessary to elucidate the mechanism of its biostimulatory effect on bone formation.

We previously reported that LLLI stimulated cellular proliferation, bone nodule formation, alkaline phosphatase activity, and osteocalcin gene expression in early-stage cultures of rat calvaria-derived osteoblasts[12]. Those findings suggested that laser irradiation may play two principal roles, stimulation of proliferation of bone nodule forming cells of osteoblast lineage and cellular differentiation. However, the molecular bases of these mechanisms leading to those findings have not been elucidated.

The MC3T3-E1 cell line, one of the most representative osteoblastic cell lines, satisfies the most important criterion for osteoblasts, because it forms mineralized nodules in vitro through a process analogous to bone formation in vivo[13]. We previously constructed a cDNA library of MC3T3-E1, which showed an enhanced gene expression by LLLI using a subtracted gene cloning procedure[14]. Among subtractive gene clones, we found a gene clone that exhibited a high homology nucleotide sequence with DNA replication licensing factors genes[15,16], ATP biosynthesis involving the mitochondrial enzyme FOA1-ATPase gene[17], and annexin gene Ⅲ [18].

Differentially expressed genes between the corresponding treatments with and without LLLI in osteoblasts can help us understand the molecular basis of the biostimulatory effect of LLLI. The identification and characterization of genes expressed exclusively or preferentially in osteoblasts will shed light on the mechanisms. In the present study, we further characterized a novel gene clone, MCL-176 from the subtracted gene library, by the homology search of nucleotide sequence technology using NCBI DNA databases.

Materials and Methods

1. Cell culture and laser irradiation

MC3T3-E1 cells established from newborn mouse calvaria by KODAMA et al.[19], were cultured in minimal essential medium (α-MEM ; Gibco BRL, USA) containing 10% fetal calf serum, 100 μg/ml of penicillin G (Sigma Chemical Co., USA), and 50 μg/ml of gentamicin sulfate (Sigma). The cultures were kept at 37℃ in a humidified incubator (Forma CO_2 incubator MIP-3326, Sanyo Electric Medica System Co., Tokyo, Japan) in the presence of 95% air and 5% CO_2.

LLLI was carried out according to a previous report[12]. Briefly, a Ga-Al-As diode laser device (Model ZH-M143DJP ; Matsushita Industrial Equipment Inc., Osaka, Japan) was used to irradiate cells plated in 100-mm tissue culture dishes at a density of 4×10^5 cells/dish at a wavelength of 805 nm. The laser beam was delivered by optical fibers and was irradiated uniformly in a circular area, 130 mm in diameter, 30 cm above the cell layer. We used 1 Hz pulsed irradiation with equal exposure and rest times. The experimental procedure allowed uniform irradiation of all the cells on the tissue culture dish. The actual total energy corresponding to an exposure of 5 minutes was 1.5 J/cm^2. Laser irradiation

was carried out in a CO_2 incubator.

2. Restriction endonuclease analysis

Plasmid DNA from subtracted gene clone was isolated and digested with *Mlu* I and *Not* I, and DNA fragments were run on 1% agarose gel-electrophoresis. The gel was stained with ethidium bromide under UV irradiation.

3. DNA nucleotide sequencing homology search

Dideoxy-chain termination sequencing[20] was performed with fluorescent dye-labeled T7 universal primers (Aloka Co. Ltd., Japan) and Sequi Therm™ Long-Read™ cycle sequencing kits for Li-Cor® Sequencing (Epicentre Technologies, USA). The reaction products were analyzed using a 4000LS Long ReadIR™ DNA sequencing system (LI-Cor, USA). Plasmid DNA of the MCL-176 clone was purified and the 5'-portion of the DNA insert was nucleotide sequenced. A homology-search of the DNA sequence was carried out by a standard nucleotide-nucleotide BLAST (blastn) homology-search using NCBI DNA databases.

4. Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from MC3T3-E1 cells with or without LLLI by acid guanidium thiocyanate-phenol-chloroform extraction[21]. cDNA synthesis and amplification by reverse transcription-polymerase chain reaction (RT-PCR) were carried out using a GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT, USA). The design of the oligonucleotide primers for the *MIF* gene was evaluated using Primer 3 software (http : //www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The PCR DNA primers were as follows : 5'-AAG CCC GCA CAG TAC ATC G-3'(the forward primer for mouse *MIF*) ; 5'-CAG GCC ACA CAG CAG CTT AC-3'(the reverse primer for mouse *MIF*), with an expected product size of 153 bp ; 5'-ATC ACC ATC TTC CAG GAG-3'(the forward primer for mouse GAPDH) ; 5'-ATG GAC TGT GGT CAT GAG-3'(the reverse primer for mouse GAPDH) ; with an expected product size of 318 bp. PCR products were electrophoresed on 1.5% agarose gel and subsequently stained with ethidium bromide under UV irradiation. The relative fluorescence intensity of the PCR amplified DNA band was semi-quantified using an image analyzer (ATTO Densitograph, ATTO Corp., Tokyo, Japan).

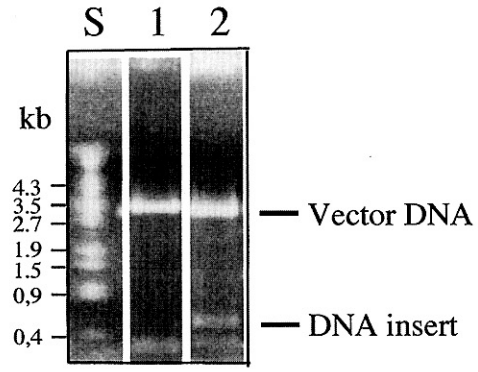
5. Real-time PCR analysis

Two-step quantitative detection of the *MIF* gene was performed as follows. Twenty micrograms of total RNAs was reverse-transcribed using Superscript II and Random Primer (Gibco BRL, Gaithersburg, MD, USA). PCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Chatsworth, CA, USA). The amplification reactions were performed in a final volume of 20 μl containing 10 μl of 2×QuantiTect SYBR Green PCR Master Mix, 0.25 μM of *MIF*-specific primers, and 5 μl of 500-fold diluted cDNA solution. To reduce variability between the replicates, PCR premixes, which contained all reagents except for cDNA, were prepared and aliquoted into 0.2 μl thin-wall strip tubes (MJ Research Inc., MA, USA). The thermal cycling conditions comprised an initial denaturation step at 95℃ for 15 minutes and 40 cycles at 94℃ for 15 seconds, 60℃ for 30 seconds, and 72℃ for 30 seconds. Experiments were performed in triplicate for each data point. All PCR reactions were performed using the OPTICON™ DNA Engine (MJ Research Inc., MA, USA).

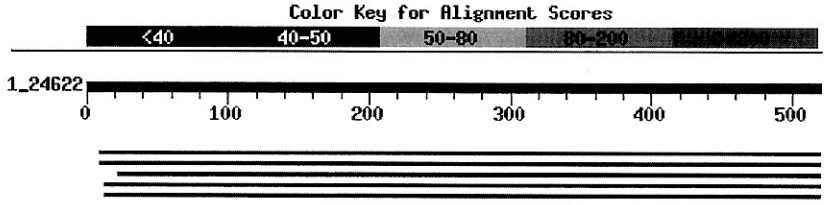
Results

Figure 1 summarizes the results of identification of the MCL-176 clone gene. The agarose electrophoresis pattern used for analyzing the DNA insert size is shown in Fig. 1A. Plasmid DNA from the MCL-176 clone was digested with restriction endonucleases *Mlu* I and *Not* I, and the DNA fragments were run on agarose gel-electrophoresis. The MCL-176 clone

A



B



C

Gene ID	Description
AK013328	mouse macrophage migration inhibitory factor
AK008939	mouse macrophage migration inhibitory factor
BC024895	mouse macrophage migration inhibitory factor
NM_010798	mouse macrophage migration inhibitory factor
L10613	mouse glycosylation-inhibiting factor

Fig. 1 Identification of MCL-176 gene. A, Endonuclease digestion pattern of plasmid DNA from MCL-176. S, Size marker of λ *EcoR* T14 I digestion ; 1, vector plasmid pAP3neo ; 2, plasmid DNA from MCL-176. B, Homology-search of nucleotide sequences of DNA insert in MCL-176, Distribution of blastin homology-search hits on the query sequence. C, Genes identified by blastin homology-search. D, Comparison of DNA sequences between MCL-176 cloned gene (Sbjct) and AK013328 mouse *MIF* gene (Query). E, Amino acid sequence of MIF from 100% match nucleotide sequence from MCL-176. * , stop codon.

has an approximate insert size of 0.5 kb, based on calculation of DNA size standards. Next, the nucleotide sequence of DNA insert in MCL-176 was analyzed. Then, a blastin search for the 511 bp nucleotide sequences from MCL-176 was carried out using the NCBI database. Many blast hits on the query sequence with a high homology were found, and the top 5 genes are shown in Fig. 1B. The top 4 genes had a greater than 99% homology with the nucleotide sequence of the MCL-176 DNA insert, and they were identical with mouse *MIF* genes (Fig. 1C). Fig. 1D showed a 100% match of nucleotide sequences between the MCL-176 insert and AK013328 mouse *MIF* gene, and the amino acid sequence of mouse MIF is shown in Fig. 1E.

To confirm the enhanced mRNA level of the *MIF* gene in irradiated cells, RT-PCR analysis was performed. Figure 2 shows that higher levels of *MIF* RT-PCR products were detected 6 and 24 hours after LLLI, while mRNA levels of GAPDH, the housekeeping control, showed almost no change regardless of irradiation. Further, the PCR endproducts of *MIF* and *GAPDH* showed the expected sizes of 153 bp and 318 bp, respectively, suggesting that both PCR endproducts were correctly amplified by the designed DNA primers. In addition, a semi-quantitative assay using fluorescence intensity scanning demonstrated that *MIF* mRNA levels in irradiated cells were 2.0-

D

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Score = 1013 bits (511), Expect = 0.0 : Identities = 511/511 (100%)

Query: 10  agctcaggtccctggcttgggtcacaccgcgctttgtaccgtcctccggtccacgctcgc 69
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2   agctcaggtccctggcttgggtcacaccgcgctttgtaccgtcctccggtccacgctcgc 61

Query: 70  agtctctccgccaccatgcctatgttcatcgtgaacaccaatgttccccgcgcctccgtg 129
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 62  agtctctccgccaccatgcctatgttcatcgtgaacaccaatgttccccgcgcctccgtg 121

Query: 130 ccagaggggtttctgtcggagctcacccagcagctggcgcaggccaccggcaagcccgca 189
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 122 ccagaggggtttctgtcggagctcacccagcagctggcgcaggccaccggcaagcccgca 181

Query: 190 cagtacatcgcagtgcacgtggtcccggaccagctcatgacttttagcggcacgaacgat 249
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 182 cagtacatcgcagtgcacgtggtcccggaccagctcatgacttttagcggcacgaacgat 241

Query: 250 ccctgcgccctctgcagcctgcacagcatcggcaagatcggtggtgcccagaaccgcaac 309
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 242 ccctgcgccctctgcagcctgcacagcatcggcaagatcggtggtgcccagaaccgcaac 301

Query: 310 tacagtaagctgctgtgtggcctgctgtccgatcgcctgcacatcagcccggaccgggtc 369
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query: 370 tacatcaactattacgacatgaacgctgccaacgtgggctggaacggttccaccttcgct 429
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 362 tacatcaactattacgacatgaacgctgccaacgtgggctggaacggttccaccttcgct 421

Query: 430 tgagtcctggccccacttacctgcaccgctgttctttgagcctcgctccacgtagtgttc 489
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 422 tgagtcctggccccacttacctgcaccgctgttctttgagcctcgctccacgtagtgttc 481

Query: 490 tgtgtttatccaccggtagcgatgcccacct 520
           ||||||||||||||||||||||||||||||
Sbjct: 482 tgtgtttatccaccggtagcgatgcccacct 512
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E

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MCL-176
AK013328      1 GGGTCACGTAGCTCAGGTCCCTGGCTTGGGTCACACCGCGCTTTGTACCGTCCTCCGGTC   60
Amino acid    1                                                              1
MCL-176                 ---------AGTCTCTCCGCCACCATGCCTATGTTCATCGTGAACACCAATGTTCCCCGC
AK013328     61 CACGCTCGCAGTCTCTCCGCCACCATGCCTATGTTCATCGTGAACACCAATGTTCCCCGC   120
Amino acid    1                              M  P  M  F  I  V  N  T  N  V  P  R   12
MCL-176                 GCCTCCGTGCCAGAGGGGTTTCTGTCGGAGCTCACCCAGCAGCTGGCGCAGGCCACCGGC
AK013328    121 GCCTCCGTGCCAGAGGGGTTTCTGTCGGAGCTCACCCAGCAGCTGGCGCAGGCCACCGGC   180
Amino acid   12  A  S  V  P  E  G  F  L  S  E  L  T  Q  Q  L  A  Q  A  T  G    32
MCL-176                 AAGCCCGCACAGTACATCGCAGTGCACGTGGTCCCGGACCAGCTCATGACTTTTAGCGGC
AK013328    181 AAGCCCGCACAGTACATCGCAGTGCACGTGGTCCCGGACCAGCTCATGACTTTTAGCGGC   240
Amino acid   32  K  P  A  Q  Y  I  A  V  H  V  V  P  D  Q  L  M  T  F  S  G    52
MCL-176                 ACGAACGATCCCTGCGCCCTCTGCAGCCTGCACAGCATCGGCAAGATCGGTGGTGCCCAG
AK013328    241 ACGAACGATCCCTGCGCCCTCTGCAGCCTGCACAGCATCGGCAAGATCGGTGGTGCCCAG   300
Amino acid   52  T  N  D  P  C  A  L  C  S  L  H  S  I  G  K  I  G  G  A  Q    72
MCL-176                 AACCGCAACTACAGTAAGCTGCTGTGTGGCCTGCTGTCCGATCGCCTGCACATCAGCCCG
AK013328    301 AACCGCAACTACAGTAAGCTGCTGTGTGGCCTGCTGTCCGATCGCCTGCACATCAGCCCG   360
Amino acid   72  N  R  N  Y  S  K  L  L  C  G  L  L  S  D  R  L  H  I  S  P    92
MCL-176                 GACCGGGTCTACATCAACTATTACGACATGAACGCTGCCAACGTGGGCTGGAACGGTTCC
AK013328    361 GACCGGGTCTACATCAACTATTACGACATGAACGCTGCCAACGTGGGCTGGAACGGTTCC   420
Amino acid   92  D  R  V  Y  I  N  Y  Y  D  M  N  A  A  N  V  G  W  N  G  S   112
MCL-176                 ACCTTCGCTTGAGTCCTGGCCCCACTTACCTGCACCGCTGTTCTTTGAGCCTCGCTCCAC
AK013328    421 ACCTTCGCTTGAGTCCTGGCCCCACTTACCTGCACCGCTGTTCTTTGAGCCTCGCTCCAC   480
Amino acid  112  T  F  A  *                                                  116
MCL-176                 GTAGTGTTCTGTGTTTATCCACCGGTAGCGATGCCCACCT
AK013328    481 GTAGTGTTCTGTGTTTATCCACCGGTAGCGATGCCCACCT                     520
Amino acid  116                                                              116
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Fig. 1 Continued.

and 2.5-fold higher after 6 and 24 hours, respectively, than those without LLLI.

Endpoint RT-PCR analysis can only provide semi-quantitative results, while with real-time PCR analysis accumulation of the PCR product is measured and reported during each amplification cycle, making the analysis quantitative and more accurate. Next, we re-confirmed the mRNA levels for *MIF* and *GAPDH* us-

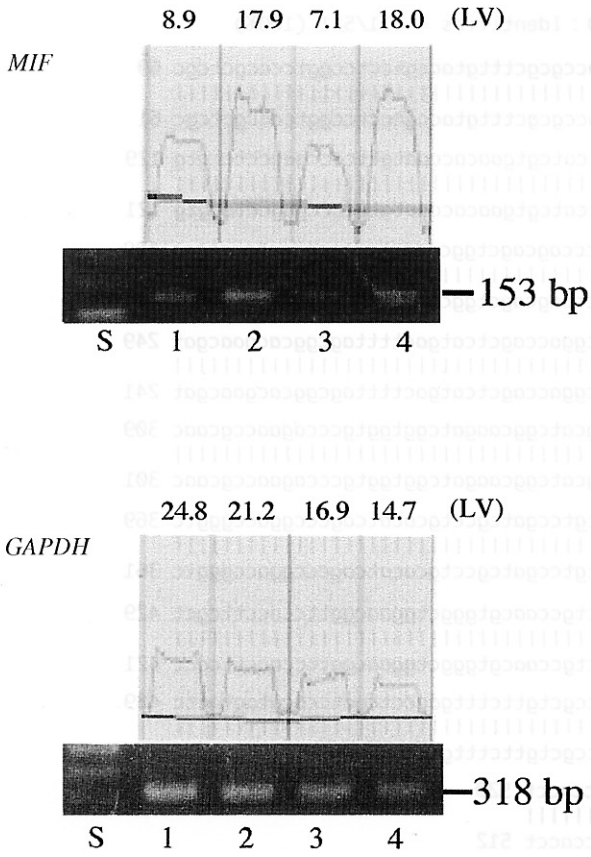

Fig. 2 RT-PCR analysis of *MIF* mRNA level. Ethidium bromide staining pattern of amplified PCR endproducts on agarose gel electrophoresis and fluorescence intensity scanning. S, size standard ; LV, luminescence value of the DNA band in gel. 1, LLLI (−) after 6 hours ; 2, LLLI (+) after 6 hours ; 3, LLLI (−) after 24 hours ; 4, LLLI (+) after 24 hours.

ing real-time PCR analysis with the same DNA primers used in the endpoint RT-PCR experiments. Figure 3 shows the results of real-time PCR, including the exponential curves at the initial reaction (Fig. 3A), the standard curve with high linearity (Fig. 3B), and the melting curve profiles of the PCR endproducts (Fig. 3C). The melting curve profiles of the PCR endproducts of the *MIF* samples all showed the same characteristic curves with fluorescence acquisition in each cycle at 86℃ during the amplification program, which eliminated the nonspecific fluorescence signal and ensured accurate quantification of the desired gene product. These results sug-

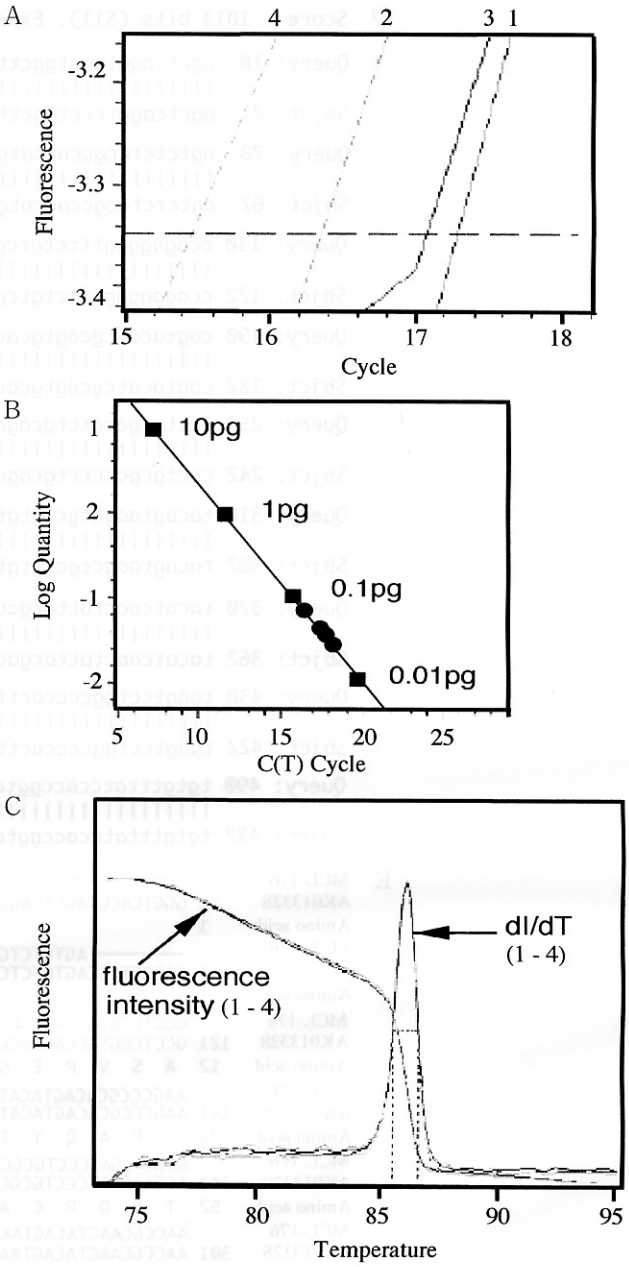

Fig. 3 Real-time RT-PCR analysis of *MIF* mRNA level. A, Exponential curves of the initial PCR reaction. 1, LLLI (−) after 6 hours ; 2, LLLI (+) after 6 hours ; 3, LLLI (−) after 24 hours ; 4, LLLI (+) after 24 hours. B, Standard curve of fluorescence intensity detected during the PCR cycle. Four standard dilutions of the copy number standard (0.01, 0.1, 1, 10 pg) were compared with the template samples of amplified *MIF* DNA. C, Melting curves of the PCR-amplified MIFDNA show the extinction of fluorescence intensity. Tm values were determined by calculating the negative first derivative of the fluorescence intensity divided by temperature (dI/dT).

gested that the real-time PCR experiments provided reliable quantification results.

MIF mRNA transcripts introduced at the start of the reaction were calculated by plotting the cycle threshold on the standard curve. The final result was then normalized, and expressed as the amount of amplified DNA (Fig. 4). Twenty-four hours later after MC3T3 E1 cells were treated with LLLI, the *MIF* mRNA level was higher (83.2 ± 10 fg/ μl, n=4) than in the case without LLLI as a control level (47.3 ± 9.3 fg/μl, n=4). LLLI had a significant effect on the expression of *MIF* mRNA of MC3T3-E1 cells ($p<0.05$), as the expression of *MIF* mRNA in the cells 24 hours following irradiation was 1.8-fold higher than in the control.

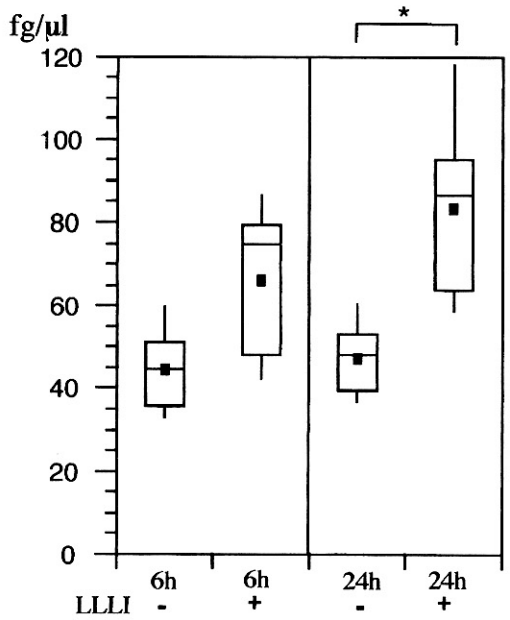

Fig. 4 Comparison of *MIF* mRNA levels from real-time PCR analysis by Box and Whiker plots. Results represent the means (solid square) ± S.E. (outer box) of the relative expression of the *MIF* DNA obtained in 4 experiments. LLLI significantly enhanced *MIF* mRNA level at 24 hours (*$p<0.05$).

Discussion

Osteogenesis and the process of bone healing following dental implant therapy are very important for a good prognosis. Thus, the most important biostimulatory effect of LLLI for implant therapy is likely to be the acceleration of bone formation. In an in vivo study, SILVA et al.[22] reported that Ga-Al-As laser irradiation stimulated femur bone repair in a rat experimental model, and their computerized morphometric results showed a significant increase in areas of newly mineralized bone in the LLLI groups. More recently, GUZZARDELLA et al.[23] reported that Ga-Al-As LLLI enhanced the healing rate of defect areas in the epiphysis femora explanted from rats in an organ culture experimental system, and they also showed that alkaline phosphatase and Ca content were significantly increased in LLLI groups.

In vivo animal experimental studies are considered reliable to show the stimulation of bone repair due to LLLI. However, in vivo results sometimes cannot easily prove actual evidence at the molecular level, because of the great variety of cell populations and biochemical factors involved. Although results from in vitro studies cannot entirely be extrapolated to in vivo conditions, in vitro cell experiments have a much greater level of control in the absence of nerve and vascular systems and the environmental extracellular matrix, and can highlight the sequence of events and parameters involved with tissue healing.

In the present study, we found that the coding DNA nucleotide sequence in a gene clone, MCL-176 from a subtractive gene library in which gene expression was enhanced by LLLI, had a high homology to mouse *MIF*. RT-PCR analysis demonstrated elevated mRMA levels in irradiated MC3T3-E1 cells. These findings suggest that LLLI enhanced the transcription of *MIF* in the osteoblastic cell line MC3T3-E1. There are some problems underlying the endpoint PCR method for measurement of mRNA levels, as such analysis can only provide semi-quantitative results, and differences between varying concentrations of PCR reaction components are difficult to discern due to reagents that may be limiting as the reaction progresses. During real-time PCR analysis,

the accumulation of PCR products is measured and reported during each amplification cycle, making quantitative analysis of data possible. We examined *MIF* mRNA levels in cells with and without LLLI using real-time quantitative RT－PCR analysis and confirmed the enhancement of *MIF* mRNA levels by LLLI.

MIF is a lymphokine that was first reported to prevent random migration of macrophages out of blood vessels[24]. It was long believed to be exclusively secreted by T-lymphocytes, however, MIF was recently shown to exist in a variety of inflammatory tissues and cells including macrophages, and is now considered to be a major mediator of inflammation and immunological reactions[25]. In addition, high MIF expression has been identified in non-inflammatory, highly proliferative tissues, and its involvement in physiological cellular proliferation is also strongly suggested[26,27]. ONODERA et al.[28] first reported the presence of MIF protein and mRNA in MC3T3-E1 cells and mouse calvarial osteoblasts.

Osteoblasts proliferation is promoted by various growth factors such as transforming growth factor-β, basic fibroblast growth factor, insulin-like growth factor-Ⅱ, and fetal calf serum, which also were shown to remarkably up-regulate *MIF* gene expressions in MC3T3-E1[29]. Furthermore, after the cells in that study were synchronized in the G0 phase by serum-starvation, *MIF* mRNA expression gradually increased from G0 to reach its maximum at the S phase when genes are duplicated by DNA synthesis. In addition, recombinant MIF upregulated the expression of urokinase plasminogen activator inhibitor-1 (PAI-1) mRNA in human osteoblastic cells. Since plasminogen activator (PA) is known to play an important role in bone metabolism, such as activation of pro-collagenase or growth factors, and in the mitogenic activity for osteoblastic cells, these findings suggest that MIF is associated with the proliferation of osteoblasts and bone tissue remodeling through the PA and plasmin systems.

Recently, the effects of LLLI on the mitochondria, nucleus, and cytoskeleton of CHO K-1 cells were investigated by the use of specific fluorescent probes, and it was found that LLLI was capable of providing positive biomodulation, and prevented apoptosis[30]. Furthermore, LLLI increased the mitochondrial activity and promoted a bio-stimulation effect on the early stages of liver regeneration without any detectable damage of the cells[31].

The present study is the first to demonstrate that LLLI enhanced the expression of *MIF* mRNA in a mouse osteoblastic cell line, MC3T3-E1. Since MIF modulates osteoblasts proliferation and bone remodeling, LLLI may have positive effects on the integration of implants and may be useful to reduce healing times and speed up osseointegration of dental implants through enhancement of *MIF* gene expression.

Conclusion

The DNA nucleotide sequence of MCL-176, a subtracted gene clone, the gene expression of which in mouse osteoblastic cell MC3T3-E1 was enhanced by LLLI, was analyzed and its DNA sequence assessed by homology-search using NCBI DNA databases. DNA nucleotide sequences of MCL-176 cloned DNA exhibited high homology with mouse *MIF* gene. The enhanced *MIF* mRNA levels in MC3T3-E1 cells by LLLI were also confirmed by endpoint RT-PCR and real-time PCR. Since MIF modulates the proliferation of osteoblasts and bone tissue remodeling, LLLI may enhance *MIF* mRNA transcription of osteoblasts and play a role in the osseous metabolism.

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13307059) from Japan Society for the Promotion of Science.

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ORGINAL ARTICLE

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Effect of low-level laser irradiation on osteoglycin gene expression in osteoblasts

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Abstract Many studies have attempted to elucidate the mechanism of the biostimulatory effects of low-level laser irradiation (LLLI), but the molecular basis of these effects remains obscure. We investigated the stimulatory effect of LLLI on bone formation during the early proliferation stage of cultured osteoblastic cells. A mouse calvaria-derived osteoblastic cell line, MC3T3-E1, was utilised to perform a cDNA microarray hybridisation to identify genes that induced expression by LLLI at the early stage. Among those genes that showed at least a twofold increased expression, the osteoglycin/mimecan gene was upregulated 2.3-fold at 2 h after LLLI. Osteoglycin is a small leucine-rich proteoglycan (SLRP) of the extracellular matrix which was previously called the osteoinductive factor. SLRP are abundantly contained in the bone matrix, cartilage cells and connective tissues, and are thought to regulate cell proliferation, differentiation and adhesion in close association with collagen and many other growth factors. We investigated the time-related expression of this gene by LLLI using a reverse transcription polymerase chain reaction (RT-PCR) method, and more precisely with a real-time PCR method, and found increases of 1.5–2-fold at 2–4 h after LLLI compared with the non-irradiated controls. These results suggest that the increased expression of the osteoglycin gene by LLLI in the early proliferation stage of cultured osteoblastic cells may play an important role in the stimulation of bone formation in concert with matrix proteins and growth factors.

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Keywords Gene expression · Laser · Osteoblast · Osteoglycin/mimecan · Real-time PCR

Introduction

Several biostimulatory effects of LLLI have been described, but the precise mechanism and molecular basis of such effects remain unclear. In this study we attempted to elucidate the effect of LLLI on bone formation. Previously, the effects of LLLI at various growth stages of cultured osteoblastic cells were investigated and cellular proliferation, bone nodule formation, alkaline phosphatase activity and osteocalcin gene expression were found to be enhanced only at the early proliferation stage [1]. A cloned mouse calvaria-derived osteoblastic cell line, MC3T3-E1, was used to construct a subtracted cDNA library by LLLI. The gene coding for minichromosome maintenance (MCM)-3, which is thought to function as a licensing factor for initiation of replication, exhibited an increased expression level [2]. We also found an enhanced expression of the annexin III gene in an early growth stage of MC3T3-E1 cells [3]. Following that study, we utilised a newly introduced cDNA microarray hybridisation technology to investigate the expression rates of other genes influenced by LLLI at the early growth stage of MC3T3-E1 cells [4]. More than 3800 genes were screened, and among those that showed an increased expression of at least twofold at 2 h after LLLI, the osteoglycin/mimecan gene was found to be upregulated 2.3 times more than in the non-irradiated controls. Osteoglycin is a member of the class III type of SLRP found in the extracellular matrix, which was previously called the osteoinductive factor [5]. SLRP have been shown to interact with collagen and regulate collagen fibrillogenesis, an essential process for growth, development, tissue repair and metastasis [6]. SLRP have also been reported to interact with many growth factors by proteoglycan chains and modulate those biological activities [7]. We attempted to determine time-related changes for the enhanced expression of

the osteoglycin/mimecan gene caused by LLLI using RT-PCR and, more precisely, with a real-time PCR method.

Materials and methods

Cell culture and laser irradiation

MC3T3-E1 cells, established from newborn mouse calvaria [8], were cultured in minimum essential medium (α-MEM; Invitrogen Corporation, USA) containing 10% fetal calf serum and antibiotics comprising 100 μg/ml penicillin G (Sigma Chemical Co., USA) and 50 μg/ml gentamicin sulfate (Sigma) in multiwell plates. The cultures were kept at 37 °C in a humidified incubator (Forma CO_2 incubator MIP-3326, Sanyo Electric Medica System Co., Japan) in the presence of 95% air and 5% CO_2.

LLLI was carried out according to a previous report [1]. A gallium–aluminium–arsenide (Ga-Al-As) diode laser device (Model Panalas 1000; Matsushita Industrial Equipment Inc., Japan) was used as a low-power laser source. This laser therapy system utilises a wavelength of 830 nm and output power of 100–700 mW, which is variable in a continuous wave (c/w). In the present study, an output power in a c/w of 500 mW was selected. The probe was fixed at 550 mm from the cells to be irradiated, giving a spot size of 78.5 cm^2 and an incident power density of approximately 6.4 mW/cm^2. The irradiation time was 20 min, which equalled an incident energy density of 7.64 J/cm^2, and was performed on a clean bench. After irradiation, the cells were maintained in a CO_2 incubator. The control cells were also placed on a clean bench for the same period without any irradiation.

RNA preparation and RT-PCR

Total cellular RNA was isolated from the MC3T3-E1 cells with or without LLLI by an acid guanidinium thiocyanate–phenol–chloroform extraction method [9]. cDNA synthesis was carried out using a Superscript II RNaseH(–) reverse transcriptase system (Invitrogen) with oligo $d(T)_{12\text{-}18}$ primer at 42 °C for 1 h.

PCR was carried out using a Gene Amp PCR reagent kit (Applied Biosystems, USA) as instructed by the manufacturer under the following conditions: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, for 30 cycles. The primers for PCR amplification were designed by referring to the cDNA sequences reported for mouse osteoglycin [10] to produce a 347 bp fragment (forward primer 5′-TGA TGC TGT ACC ACC ATT GC -3′; reverse primer: 5′-ATT CCA GGT CGT TAT GGT CC -3′), and for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard housekeeping gene to produce a 318 bp fragment (forward primer: 5′-ATC ACC ATC TTC CAG GAG -3′; reverse primer: 5′-ATG GAC TGT GGT CAT GAG -3′), respectively. PCR-amplified DNA fragments were checked by electrophoresis on a 1.5% agarose gel and subsequently stained with ethidium bromide.

Real-time PCR

Real-time PCR reactions were carried out using a DNA Engine Opticon system (MJ Research Inc., USA) with a QuantiTect SYBR Green PCR kit (Qiagen GmbH, Germany). PCR was started with an initial incubation at 95 °C for 15 min to activate the Taq DNA polymerase, then set at 94 °C for 15 s, 56 °C for 30 s and 72 °C for 30 s, for 40 cycles. The fluorescent signals were measured at the end of each elongation step and the start points of their exponential curves were determined for conversion of the cycle number into the amount of PCR products using standard purified cDNA to make the standard curves. The PCR efficiency of the primer sets was checked to confirm that the dilution rate of the samples was not affected. First, the annealing temperature of the eight wells of the PCR reaction plate on the apparatus was changed gradiently from 55 °C to 65 °C to determine the optimal annealing temperature between the two sets of PCR primers, after which 56 °C was decided on. After the final PCR step, the temperature was gradiently elevated to 95 °C while monitoring the fluorescent signals to form the melting curves in order to check the specificity of the PCR amplification. Values were calculated as means ± standard deviation (SD). Comparisons between groups were made using Student's *t*-test. Differences were accepted as significant when $p < 0.01$.

Results

To elucidate the effect of LLLI on the expression profile of the gene during the early proliferation stage of developing osteoblastic MC3T3-E1 cells, total RNA were prepared after 0, 2, 4 and 8 hours with or without LLLI. cDNA were also prepared and the expression profile was searched. In the cDNA microarray experiment the osteoglycin/mimecan gene was found to be have enhanced expression, which increased 2.3-fold at 2 h after LLLI.

To determine the enhanced mRNA level of the osteoglycin gene by LLLI, RT-PCR was carried out. Figure 1 shows that higher levels of osteoglycin mRNA were detected after 2 h and were maintained for 4–8 h after LLLI in MC3T3-E1 cells compared to the non-LLLI cells, whereas mRNA levels of GAPDH, the housekeeping control, showed almost no change regardless of LLLI or incubation time.

Further experiments to determine the exact rates of enhancement of the osteoglycin gene by LLLI were performed using real-time PCR. The PCR exponential curves of the osteoglycin gene are shown in Fig. 2a, and around the detection points are expanded and shown in Fig. 2b. The same reaction was performed for the GAPDH gene, after which the data were converted to relative mRNA amounts and the ratio of osteoglycin/GAPDH was calculated for standardisation (see Materials and methods) and is described in Table 1. A significant increase of osteoglycin gene expression occurred at 2 and 4 h after LLLI ($p < 0.01$). The elevated expression of the osteoglycin gene returned to a normal range in the non-irradiated control at 8 h after LLLI.

The specificity and accuracy of these PCR amplifications were also tested to draw their melting curves, one of which about the osteoglycin gene is shown in Fig. 2c.

Discussion

Osteoglycin/mimecan is a member of the SLRP family and found in many connective tissues. It was originally isolated as an osteoinductive factor from the organic matrix of bovine bone [6], and was later shown to induce ectopic bone formation [11]. A cDNA analysis showed that bovine osteoglycin is synthesised as a larger 299 aa precursor, which exhibits a 94% identity to that of

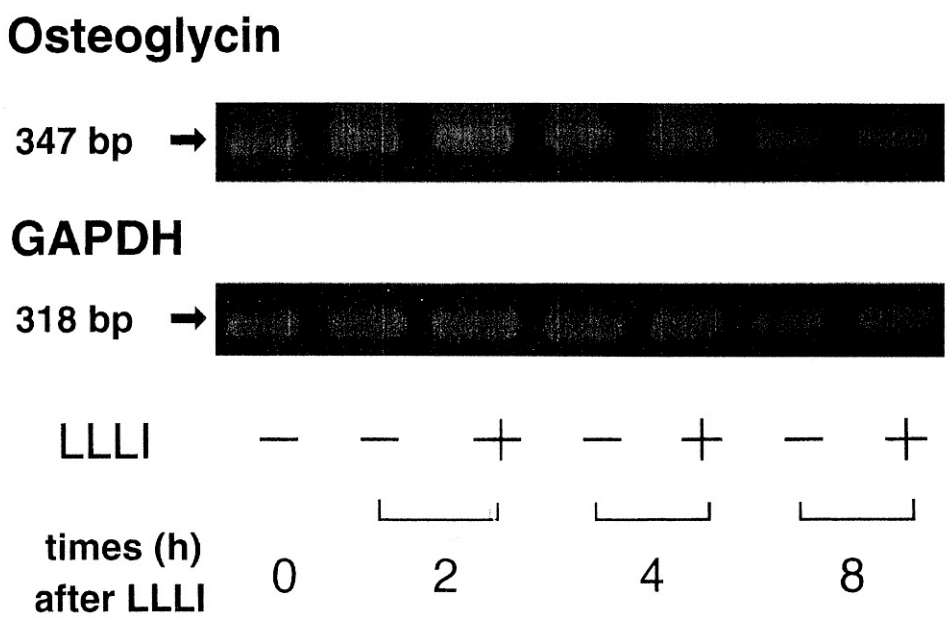

Fig. 1 RT-PCR analysis of osteoglycin mRNA levels. Osteoglycin mRNA was highly expressed at each time point following LLLI compared to the non-irradiated groups. The ethidium bromide staining pattern of amplified PCR products on agarose gel electrophoresis is shown. The RT-PCR method and DNA primers used are described in Materials and methods. The GAPDH gene was used as a control

humans and an 86% identity to its mouse counterparts [10]. Bovine osteoglycin is a single copy gene which can be transcribed into eight different mRNA by differential splicing and alternative transcription initiation [12]; however, they all encode the same protein [13].

The bovine cornea contains three unique keratan sulphate proteoglycans (KSPG), one of which, the 25-kDa protein (KSPG 25), has been determined to be the same gene product of osteoglycin [14]. KSPG 25 protein comprises 223 aa with keratan sulphate chains, whereas osteoglycin only makes up the c-terminal 105 aa that exists in KSPG 25 protein. Because of the difference between osteoglycin and KSPG 25 with regard to tissue expression, the molecular mass and the existence of keratan sulphate, KSPG 25 was named as a mimecan for its gene and its products [14]. Osteoglycin/mimecan-related proteins not modified with keratan sulphate have also been detected in several connective tissues [14].

The SLRP gene family is comprised of three classes by their structural resemblance to the number of leucine-rich repeats (LRR) and genomic organisation. They are synthesised as either glycoproteins or proteoglycans containing chondroitin/dermatan sulphate or keratan sulphate chains. LRR are thought to be structural modules used in molecular recognition processes for cell adhesion, signal transduction, DNA repair and RNA processing [7], and the SLRP family shares common physiological roles.

Osteoglycin/mimecan is a member of the class III type of SLRP with epiphycan/PG-Lb, a 42.5% identity existing in the aa sequences between mouse PG-Lb and mouse osteoglycin/mimecan [15]. Epiphycan/PG-Lb is largely expressed in chick embryo cartilage and mammalian epiphyseal cartilage, and is thought to regulate chondrocyte differentiation and osteogenesis [15,16]. The class II group includes fibromodulin, lumican, keratocan, PRELP and osteoadherin, and especially lumican and keratocan form the major parts of KSPG with mimecan in the bovine cornea, which are considered to function for the maintenance of corneal transparency [14]. Lumican is also a major proteoglycan component of bone matrix [17], though it has been reported to be expressed mainly during the mineralisation and differentiation stages of MC3T3-E1 development, and not during the proliferation stage [18]. The class I group includes decorin and biglycan, and decorin is reported to interact with collagen and regulate collagen fibre formation [19]. In a targeted disruption of the biglycan gene the experimental animals showed reduced bone mass, hence biglycan is thought to act as a positive regulator of the cellular processes of bone formation [20].

SLRP are abundant in the bone matrix, cartilage cells and connective tissues, and thought to play an important role in the regulation of cell proliferation, differentiation, adhesion and, especially, mineralisation of bone in a close association with collagen and many other growth factors [15]. SLRP have also been shown to interact with transforming growth factor (TGF)-β to modulate its activity [6]. The synthesis of bone matrix components by osteoblasts is an essential process for the formation of mineralised bone, and stimulation of this synthesis of decorin and biglycan by TGF-β treatment by MC3T3-E1 cells has been reported [21].

In recent studies, analysis of the promoter region of the human osteoglycin/mimecan gene revealed the existence of many potential transcription factor-binding sites, besides enhancer and silencer elements, such as p53, a metal response element, and NF-κB recognition sites [22, 23]. These regulatory elements may be responsible for cell type-specific regulation of osteoglycin/mimecan gene transcription [23]. In the present

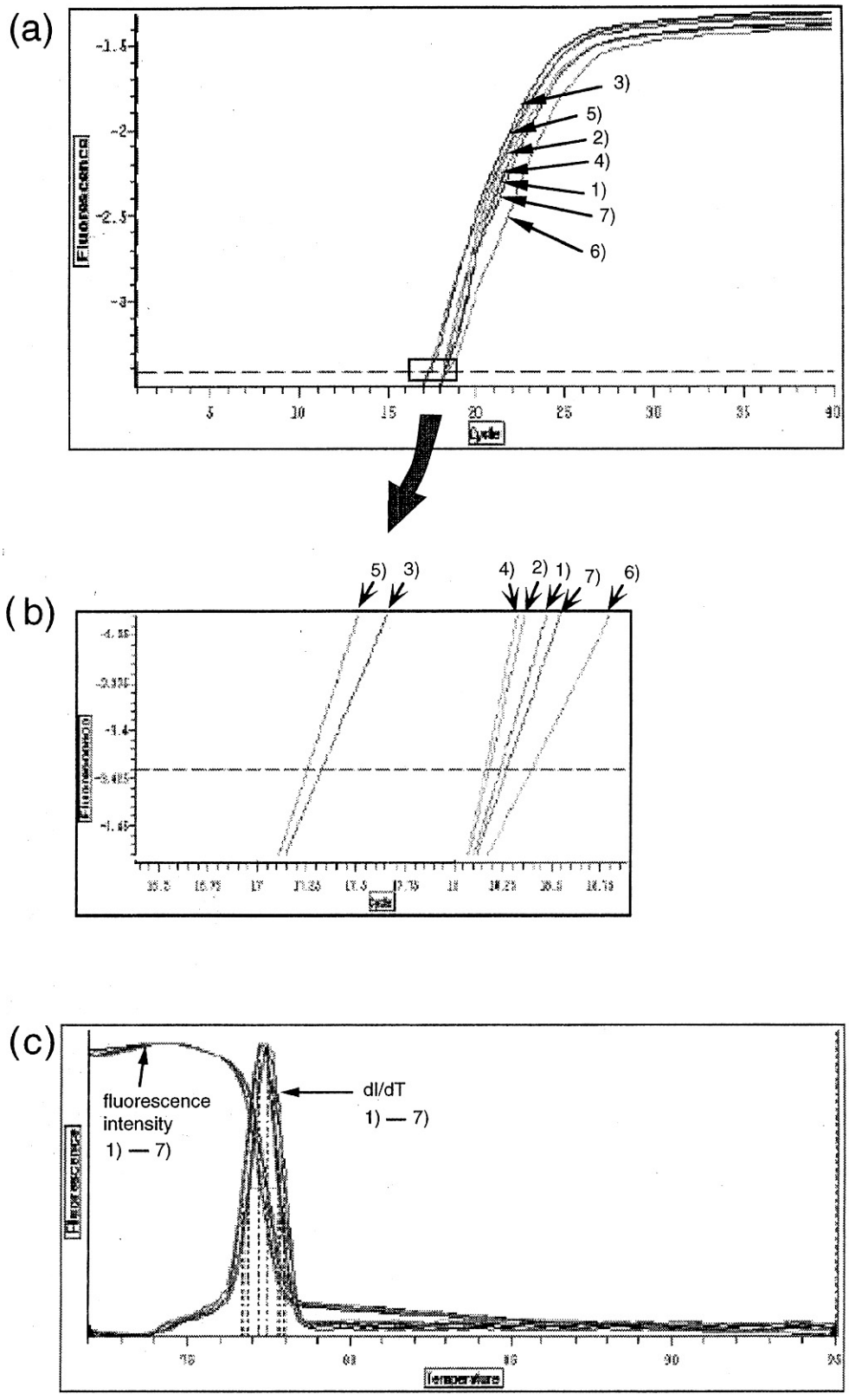

Fig. 2 (a) Real-time PCR detection of osteoglycin gene amplification. The PCR method is described in Materials and methods. One of three experiments regarding real-time PCR amplification of osteoglycin genes expressed after 0, 2, 4 and 8 h of incubation with (+) or without (–) LLLI is shown. Times (h) after (+) or (–) LLLI are shown as follows: 1); 0 h–, 2); 2 h–, 3); 2 h+, 4); 4 h–, 5); 4 h+, 6); 8 h–, and 7); 8 h+. Exponential curves of fluorescence intensity detected at the end of each elongation step during the cycle are shown by a logarithmic scale on a vertical axis. The horizontal broken line shows the cycle threshold line for converting the crossing points to the PCR exponential curves to the starting cycle numbers of the curves described in (b). Detection points at the beginning of the exponential curves are shown magnified. PCR samples containing a higher level of osteoglycin cDNA entered the logarithmic growth phase first. (c) Melting curves of the PCR-amplified osteoglycin gene are shown as the extinction of fluorescence intensity. Tm values were determined by calculating the negative first derivative of the fluorescence intensity divided by temperature (dI/dT). Nearly identical curves were obtained from the PCR products of this gene, which demonstrated the specificity and accuracy of the PCR amplification. Tm values were in a range of 77.2–77.6 °C

study, a significant increase of osteoglycin/mimecan gene expression was seen after 2 and 4 h of LLLI (Figs. 1 and 2, Table 1). The increased expression of the osteoglycin/mimecan gene by LLLI may occur transiently only in the early proliferation stage of MC3T3-E1 cells, or also biphasically in other stages. It would be

Table 1 Expression of the osteoglycin gene as determined by real-time PCR data, which are shown as normalised values divided by results of GAPDH (osteoglycin/GAPDH). Significant increases (* $p < 0.01$, $n = 3$) of osteoglycin gene expression were seen at 2 and 4 h after LLLI

Times after LLLI (±)	Osteoglycin/GAPDH (±S.D.)	Rate of increase
0 h	1.889 (±0.5390)	
2 h−	1.6742 (±0.1638)	
2 h+	3.2196 (±0.3515)	×1.923*
4 h−	2.3296 (±0.1311)	
4 h+	3.6912 (±0.2318)	×1.584*
8 h−	1.4622 (±0.2038)	
8 h+	1.4949 (±0.1539)	×1.022

interesting to know whether this increased expression was a direct or secondary effect of LLLI.

In conclusion, the increased expression of osteoglycin may be associated with other effectors or extracellular matrix molecules that have an important effect for the proliferation and differentiation of MC3T3-E1 cells. Further studies are necessary to clarify the effect of LLLI on the expression of SLRP during the early stage of bone formation.

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培養ヒト顎関節滑膜細胞に低出力レーザー照射が及ぼす影響について

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抄録 低出力レーザー照射（Low-Level Laser Irradiation，以下LLLIと略す）は顎関節症の理学療法で用いられており，筋障害や疼痛の緩和に有効であるとされているが，その作用機序については不明な点が多い。本研究では，LLLIが顎関節症症状改善に有効であるかどうかを検討することを目的とし，顎関節滑膜細胞に顎関節症患者の滑液中で高値に検出されているIL-1βを作用させた後にLLLIを行い，好中球やマクロファージの遊走・活性化に関与する代表的なケモカインであるIL-8およびMCP-1の産生に及ぼす影響を検討した。

インフォームド・コンセントを行った顎関節突起骨折患者由来の顎関節滑膜細胞を継代培養し，コンフルエントになった細胞にIL-1βを作用させた後，LLLIを行ったグループおよびLLLIを行わなかったグループに分け，3，6および9時間後の培養上清中のIL-8およびMCP-1産生量をELISA法にて測定した。

顎関節滑膜細胞が産生するIL-8およびMCP-1はIL-1β刺激で増加するが，LLLIにより産生が有意に減少した。以上の結果から，LLLIは顎関節滑膜細胞のIL-8およびMCP-1産生抑制効果があることが認められた。。

キーワード 顎関節滑膜細胞，低出力レーザー，ケモカイン

緒　言

低出力レーザー照射（Low-Level Laser Irradiation，以下LLLIと略す）は，創傷治癒促進[1]，疼痛緩和[2]，局所血流量の改善[3]などの効果が知られており，慢性関節リウマチを中心とした関節炎の治療などに用いられている[4]。口腔領域においても，象牙質知覚過敏症[5]，口腔内手術後の治癒促進[6]などのほか，顎関節症の理学療法として疼痛緩和[7,8]に用いられているが，その作用機序については不明な点が多い。

Interleukin（以下ILと略す）-1βは変形性顎関節症患者の滑液中で上昇していることが報告され[9]，疼痛との関連も示唆されていることから，顎関節症の発症および進展において重要な役割を担っているものと考えられている。当研究グループでは，ヒト顎関節滑膜細胞の培養系を確立し，培養ヒト顎関節滑膜細胞に及ぼすIL-1βの影響を検討してきた[10,11]。

IL-8，monocyte chemoattractant protein（以下MCPと略す）-1は代表的なケモカインで，主にIL-8は好中球，MCP-1は単球の遊走，活性化に関与している。近年，顎関節症患者の滑液でもIL-8量が上昇していることが報告されている[12]。これらのケモカインは生体の恒常性維持に必須であるものの，産生が過剰になると炎症の増大，組織破壊を引き起こすものと考えられる。

本研究では，近年注目されているLLLIが顎関節症症状改善に有効であるかどうかを検討することを目的とし，顎関節滑膜細胞にIL-1βを作用させた後にLLLIを行い，IL-8およびMCP-1の産生を測定した。

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材料および方法

1. 顎関節滑膜細胞の採取および培養

インフォームド・コンセントを行った左側顎関節突起骨折患者の下顎頭摘出術および顎関節形成術施行時に顎関節滑膜を採取し，顎関節滑膜細胞の初代培養を行った。すなわち，メスにて細分した滑膜を35 mmディッシュ内にカバーグラスにて固定し，20% fetal calf serum（以下FCSと略す：INTERGEN），penicillin G 100 μg/ml（萬有製薬），kanamycin 100 μg/ml（明治製薬），fungizone 250 ng/ml（CHROMOGENIX）を含む，Ham F 12培地（Gibco）にて，37°C，5% O_2，5% CO_2，90% N_2（サンヨーマルチガスインキュベーターMCO-175M）条件下で培養を行った。35 mmディッシュ内にoutgrowthしてきた顎関節滑膜細胞がコンフルエントになった時点を継代数1代とした。得られた顎関節滑膜細胞は，10% FCSおよび抗生物質を含むHam F 12培地を用い37°C，5% O_2，5% CO_2，90% N_2条件下にて継代培養を行った。培地は3日ごとに交換し，実験には，継代数8～10代の細胞を用いた。

2. ELISA法によるIL-8およびMCP-1の測定

顎関節滑膜細胞を24 wellマルチプルプレートに3×10^4 cells/wellにて播種し，コンフルエントになったことを確認後，2% FCSおよび抗生物質を含むHam F 12培地に交換し24時間培養した。次いで，1.0 U/ml IL-1β（Cistron Biotechnology）を作用させ，コントロールを作用させない場合とし，3，6，9および24時間後の培養上清を採取した。また，LLLIの影響をみるため，IL-1βを作用させた後にクリーンベンチ内でLLLIを10分間行い，IL-1β作用後より3，6および9時間後の培養上清を採取し，それぞれ使用まで−80°C下にて保存した。この培養上清中のIL-8およびMCP-1をELISAキット（Endogen）にて測定した。IL-8およびMCP-1の産生量については，顎関節滑膜細胞の数，10^5個あたりの培養上清中の量に換算して表示した。

3. レーザー照射装置および照射条件

LLLIには波長830 nm，出力500 mWのGa-Al-As半導体レーザー照射装置（パナラス1000，松下産業機器）を用いた。Uedaら[13]は，本研究にて使用したレーザー照射装置と同機種を用いて，培養骨芽細胞に総照射エネルギー密度3.84 J/cm²にて低出力レーザーを照射した場合に，最も細胞の生化学的な反応が促進されたと報告している。この条件に準じて，レーザーは培養プレートの細胞表面に対して垂直方向から出力100%で，総照射エネルギー密度3.84 J/cm²となるよう10分間連続照射した（Fig. 1）。

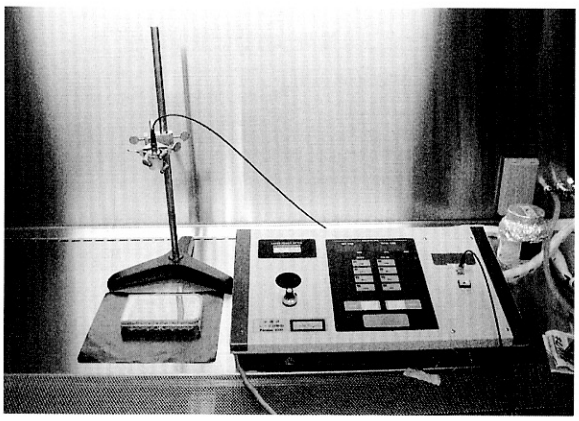

Fig. 1 The low-level Ga-Al-As diode laser apparatus used in this study

結　果

1. IL-1β作用後の培養上清中のIL-8およびMCP-1量

顎関節滑膜細胞に1.0 U/ml IL-1βを3，6，9および24時間作用させたときの培養上清中のIL-8およびMCP-1量をTable 1およびTable 2に示す。

IL-8およびMCP-1は，経時的に上昇していく傾向が認められたが，いずれの時間においてもIL-1βを作用させた系においては，作用させないコントロールと比較して有意に上昇した。

2. LLLIによるIL-8およびMCP-1産生への影響

顎関節滑膜細胞にIL-1βを作用させた後にLLLIを10分間行い，3，6および9時間後に培養上清中のIL-8

Table 1 Time course of IL-8 levels in the conditioned medium from TMJ synovial cell treated with IL-1β

(ng/10^5 cells)

	Time (hours)			
	3	6	9	24
Control	3.72±0.77	5.46±1.11	5.78±0.36	7.42±1.92
IL-1β	24.75±1.84*	26.23±2.00*	35.01±2.78*	54.33±3.64*

Mean±S.D. (n=4), *：$p<0.001$

Table 2 Time course of MCP-1 levels in the conditioned medium from TMJ synovial cell treated with IL-1 β

(ng/10^5 cells)

	Time (hours)			
	3	6	9	24
Control	10.82±1.56	11.75±1.52	12.56±1.68	19.26±2.78
IL-1 β	35.42±3.15*	42.18±5.78*	52.45±3.19*	73.89±5.26*

Mean±S.D. (n=4), *：$p<0.001$

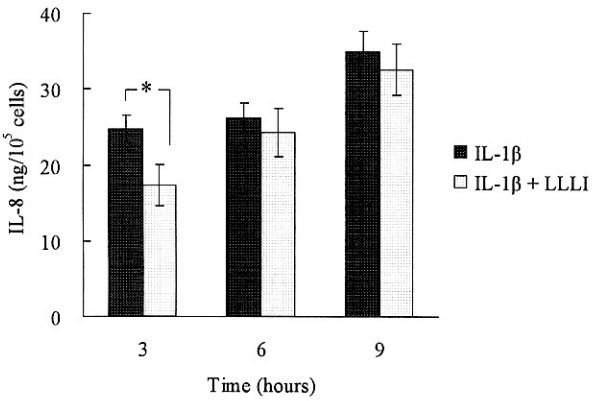

Fig. 2 Effects of LLLI on IL-8 production in TMJ synovial cells
Results are expressed as mean±S.D. (n=3).
*$p<0.01$, and was compared to the medium treated with IL-1 β without irradiation.

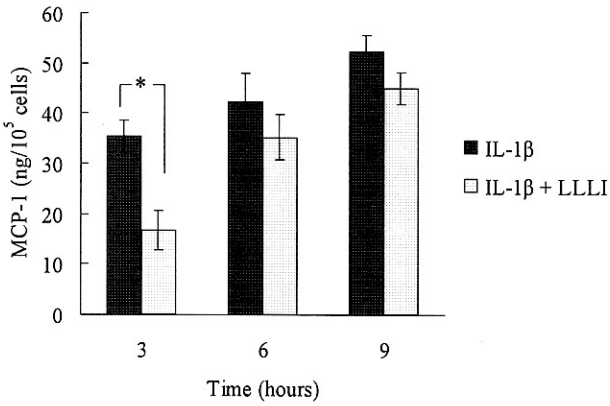

Fig. 3 Effects of LLLI on MCP-1 production in TMJ synovial cells
Results are expressed as mean±S.D. (n=3).
*$p<0.01$, and was compared to the medium treated with IL-1 β without irradiation.

およびMCP-1量を測定した結果をFig. 2およびFig. 3に示す。

LLLIを行うことにより，IL-1 βを作用させただけの場合と比較して，IL-8およびMCP-1量は低下する傾向が認められた。特にLLLIを行った後の早期である3時間後では，IL-8，MCP-1量のいずれにおいても有意差を認めた。

考　　察

LLLIを用いた治療法は，生体組織に対する光刺激作用による鎮痛や創傷治癒促進などの効果を期待して行われている。特に顎関節症に対する治療においては，咀嚼筋障害を主徴候とした場合に有効[14]とされているが，その作用機序には末梢循環の促進が関与すると考えられている[15]。

顎関節症は咬合性の慢性外傷性因子，外来性外傷，咀嚼筋の緊張や異常習癖および精神的ストレスなどが複合して発症すると考えられているが，その発症のメカニズムは不明な点が多い。近年，顎関節滑液解析が行われるようになり，顎関節内障や変形性顎関節症の滑液中ではIL-1 βやIL-6[16]，tumor necrosis factor（以下TNFと略す）-α[17]などの炎症性サイトカイン濃度が，正常な人の滑液と比較して高値を示すことが報告され，これらの因子が軟骨や骨破壊の一端を担っていると考えられている。よって，滑膜組織の培養細胞を用いた細胞学的研究は有用なものであると思われる。

一般にレーザー光は，組織表面における反射，組織内における吸収あるいは拡散により，組織を透過した際のエネルギーは減衰される[18]ことが知られている。しかしながら，本実験で使用したGa-Al-As半導体レーザーの波長は830 nmであり，この波長は体内でヘモグロビンや水分に吸収されにくく，生体への透過性が非常によいとされており，深達度は約3 cm[19]といわれている。よって，本研究における照射条件では，十分に顎関節滑膜細胞に低出力レーザーが到達することは可能であると考えられる。

顎関節滑膜細胞にIL-1 βを作用させると，IL-8およびMCP-1産生が上昇したが，IL-1 β作用後にLLLIを行うと，IL-8およびMCP-1のいずれにおいても照射しないときに比べて，照射後3時間で有意に産生量が少なかった。IL-1 βはマクロファージや線維芽細胞などの種々の細胞から産生され，細胞外基質分解酵素[20]やプロ

スタグランディンE_2[21]（以下，PGE_2と略す）などの炎症性因子の産生を上昇させる。この炎症性サイトカインであるIL-1βは変形性関節症患者の滑液中で高値に検出[22]されることから，関節疾患への関与が示唆されている。また，破骨細胞の骨吸収機能を誘導する[23]ことから，細胞外マトリックスや骨代謝において重要な因子として作用していると考えられている。

顎関節滑膜細胞にIL-1βを作用させると，IL-8およびMCP-1産生が上昇することは，顎関節滑膜細胞が顎関節症における炎症の進行や関節破壊に深く関与していることを示唆している。本研究において測定したIL-8およびMCP-1は，好中球やマクロファージを遊走・活性化させるケモカインである[24,25]。これら免疫担当細胞は，恒常性維持に重要であるものの，活性酸素や浸潤した炎症性細胞が産生する種々の酵素などにより，組織破壊を引き起こす[25]。また，多量のIL-1βやTNF-αを産生するため，顎関節滑膜細胞が刺激されケモカイン産生が上昇するという連鎖が起こるものと示唆される。よって，IL-8およびMCP-1産生量を減少させ，このような連鎖を抑制させることにより，消炎，基質破壊抑制効果があると示唆される。メカニカルストレスを加えた歯根膜細胞に一定条件で低出力レーザーを照射した検討では，レーザー照射量に依存してPGE_2やIL-1βの産生が抑制されること[26]や，プラスミノーゲンアクチベーター活性が顕著に抑制されること[27]が報告されており，使用された細胞は異なるものの，LLLIが抗炎症作用を有することを示唆しているという点で同様の結果が得られた。

LLLIによってIL-8およびMCP-1の産生量に有意な差が認められたのは照射後3時間の場合だけであったが，これはLLLIの効果が短時間しか持続しないことを示すとも考えられる。しかしながら，今回の実験では炎症刺激として作用させた高濃度のIL-1βが，実験開始から終了まで培養上清中に存在しており，このことが6，9時間後に有意差が認められなかったことに関係すると思われる。すなわち，IL-1βは滑膜細胞を刺激してIL-8およびMCP-1以外に種々のサイトカインの産生も誘導することから，滑膜組織においては複雑なサイトカインネットワークが形成されている[28]。また，滑膜組織にはIL-1βレセプターアンタゴニストも存在しており，IL-1βの作用を阻害して病態を修飾していることも知られている[28]。よって，LLLIによって産生が抑制されたIL-8およびMCP-1も，高濃度で残留していたIL-1β，あるいは他のサイトカインによって再び産生されるようになったのではないかと考えられる。LLLIの作用機序をさらに詳細に知るためには，サイトカインネットワークを考慮した多角的な検討が必要である。本研究で得られた結果は*in vitro*における実験によるものであり，ケモカイン産生抑制によって好中球やマクロファージの遊走・活性化に，実際にどのような影響が及ぼされるかについては，動物などを用いた*in vivo*での実験で検討する必要があろう。また，今回はLLLIが滑膜細胞に及ぼす影響を知るため，1回だけの照射によって検討を行ったが，臨床での効果を考えた場合，短期間あるいは長期間にわたって継続した照射も行い検討しなければならないと考えている。

結　語

LLLIが顎関節症症状改善に有効であるかどうかを検討することを目的とし，顎関節滑膜細胞に顎関節症患者の滑液中で高値に検出されているIL-1βを作用させた後にLLLIを行い，ケモカイン産生に及ぼす影響を検討した。その結果，顎関節滑膜細胞にIL-1β刺激を与えたことによって増加したIL-8およびMCP-1の産生は，LLLIを行うことによって抑制された。よって，LLLIが炎症反応の抑制に有用である可能性が示唆された。

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原　著

培養ヒト顎関節滑膜細胞に近赤外線照射が及ぼす影響について

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Effects of Near-Infrared Irradiation in Cultured Synovial Cells from Human TMJ

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Abstract : Biostimulatory effects of near-infrared irradiation, such as anti-inflammation and relieving pain have been reported. However, the molecular based mechanisms are not elucidated yet.

Human synovial tissues were obtained from the patient with a condylar process fracture of the TMJ undergoing extraction of the mandibular head and arthroplasty. The cells were isolated from TMJ synovial tissues and primary cultured using outgrowth method. The confluent-stage cells were treated with IL-1β, as the same time, near-infrared irradiation was treated to the cells. Three levels of band pass filtering were set at the 700-800 nm, 800-900 nm, and 900-1000 nm wavelengths and irradiation was conducted. The amounts of chemokines in conditioned medium were measured by ELISA kit.

After synovial cells were exposed to IL-1β, the production of IL-8 and MCP-1 was elevated, though the amounts of increase were reduced at early time after irradiation as compared to without irradiation. The

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production of IL-8 were significantly reduced at the 900-1000 nm wavelength, and the production of MCP-1 were significantly reduced at the 700-800 nm, 800-900 nm, and 900-1000 nm wavelengths.

These findings suggest that near-infrared irradiation may have anti-inflammatory effect on TMJ disorder through the reduction of chemokines production. And the results at each filter waveband were different, which suggested that a difference in potency may be likely.

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Key words=Synovial cells, Near-infrared, Chemokine

キーワード=滑膜細胞，近赤外線，ケモカイン

緒　言

近年，簡便で副作用の少ない低出力レーザーや近赤外線を用いた光線療法が，種々の疾患に対する治療に応用されるようになってきている[1-3]。近赤外線照射は，光作用と輻射熱とによる組織血流の改善[4]，神経興奮性の抑制[5]，筋の弛緩および生体活性物質産生促進[6]などにより，創傷治癒の促進や疼痛緩和の効果があるとされており，疼痛をはじめとする顎機能障害の諸症状に対しても有効であることが臨床研究により報告されている[7-12]。しかしながら，顎関節症に対する近赤外線照射作用機序の詳細については，いまだ不明な点が多い。

顎関節症は咬合性の慢性外傷性因子，外来性外傷，咀嚼筋の緊張や異常習癖および精神的ストレスなどが複合して発症すると考えられている。近年，顎関節の滑液解析が行われるようになり，変形性顎関節症患者の滑液中ではInterleukin（IL)-1βなどの炎症性サイトカイン濃度が正常者よりも高値を示すことが報告され[13]，疼痛との関連が示唆されている。このことから，IL-1βは顎関節症の発症および進展において重要な役割を担っているものと考えられている。すでに我々は，培養ヒト顎関節滑膜細胞にIL-1βを作用させると，IL-8[14]およびMonocyte Chemoattractant Protein（MCP)-1産生量が増加し，さらに3.8 J/cm²のエネルギー密度の低出力半導体レーザーを照射することによりIL-8およびMCP-1産生量が減少するという結果を得ている。

IL-8およびMCP-1は代表的なケモカインで，IL-8はおもに好中球，MCP-1は単球の遊走，活性化に関与しており，顎関節症患者の滑液ではIL-8量が上昇していることが報告されている[15]。これらのケモカインは生体の恒常性維持に必須であるものの，産生が過剰になると炎症の増大，組織破壊を引き起こすものと考えられる。

本研究では，近赤外線照射が顎関節滑膜細胞にどのような影響を及ぼすのかを検討することを目的とし，培養ヒト顎関節滑膜細胞にIL-1βを作用させた後に，低出力半導体レーザー照射にてIL-8およびMCP-1産生が抑制されたエネルギー密度（3.8J/cm²）になるよう設定した3種類の波長帯の近赤外線照射を行い，IL-8およびMCP-1の産生を測定し検討を行った。

実験方法

1.　培養ヒト顎関節滑膜細胞

インフォームド・コンセントを行った左側顎関節突起骨折患者の下顎頭摘出術および顎関節形成術施行時に顎関節滑膜を採取し，顎関節滑膜細胞の初代培養を行った。すなわち，メスにて細分した滑膜を35mmディッシュ内にカバーグラスにて固定し，20% 牛胎児血清（Fetal calf serum ; FCS)，penicillin G 100μg/ml（萬有製薬)，kanamycin 100μg/ml（明治製薬)，fungizone 250ng/ml（CHROMOGENIX）を含む，Ham F12培地（Gibco Laboratories）にて，37℃，5％O_2，5％CO_2，90% N_2（サンヨーマルチガスインキュベーター MCO-175M）条件下で培養を行った。35mm ディッシュ内にoutgrowthしてきた顎関節滑膜細胞がコンフルエントになった時点を継代数1代とした。得られた顎関節滑膜細胞は，10% FCSおよび抗生物質を含むHam F12培地を用い37℃，5％O_2，5％ CO_2，90％N_2条件下にて継代培養を行った。培地は3日ごとに交換し，実験には継代数8～10代の細胞を用いた。

2.　顎関節滑膜細胞の免疫染色法

顎関節滑膜細胞を2穴ラブテックチャンバースライド（Nalge Nunc International）に5×10^4cells/wellにて播種し，10% FCSおよび抗生物質含有Ham F12培地で24時間培養後，2％FCSおよび抗生物質含有Ham F12培地に交換し，さらに24時間培養した。その後，培地を取り除きphosphate buffered saline（PBS）で2回洗浄後，4％パラホルムアルデヒド溶液にて10分間固定した。さらにPBSで2回洗浄し乾燥後，免疫染色に使用するまで−80℃にて保存した。細胞の免疫染色は，DAKO LSABキット（DAKO）を用いて行った。一次抗体として細胞

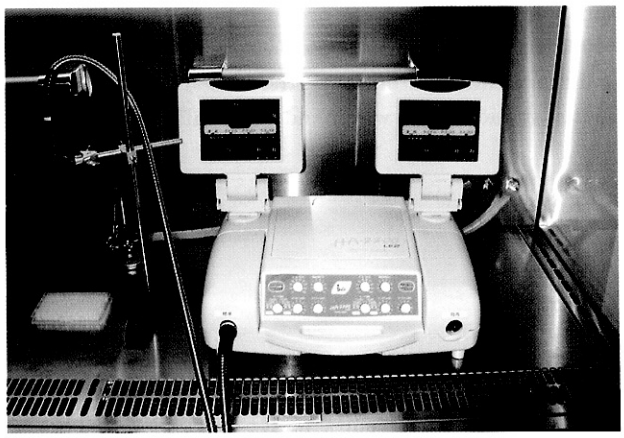

図1　SUPER LIZER HA-2200 LE2改良型

特性マーカーである抗ヒト線維芽細胞マーカー抗体（Diagnostic Biosystems），抗ヒトビメンチン抗体（COSMO BIO Co., LTD），抗ヒトHLA class Ⅱ抗体（YLEM S.R.L.），抗ヒトマクロファージマーカー抗体（YLEM S.R.L.）および抗ヒト樹状細胞マーカー抗体（COSMO BIO Co.,LTD）を用いた。最後にマイヤーのヘマトキシリンにて核染後，脱水，透徹，封入した。

3.　IL-1β刺激および近赤外線照射装置

顎関節滑膜細胞を，24穴マルチプルプレートに3×10^4 cells/wellにて播種し，コンフルエント確認後，2％FCSおよび抗生物質を含むHam F12培地に交換し24時間培養した。次いで，1.0U/ml IL-1βを作用させ，インキュベーター内で近赤外線照射を行った。近赤外線照射装置には，波長600～1600nm，最大出力2,200mWのSUPER LIZER HA-2200 LE2改良型（東京医研）（図1）を用いた。波長は700-800nm，800-900nmおよび900-1000nmとなるよう3種類のバンドパスフィルターを設置し，それぞれの波長における近赤外線照射を培養プレートの細胞表面に対して高さ18cmより垂直方向に光パワー0.5Wにて1秒間照射，1秒間停止のサイクル照射（平均パワー密度：0.056W/cm²）で11分25秒間行った。この照射条件で3種類の波長帯のエネルギー密度は，いずれも3.8J/cm²である。培養液の存在下および非存在下でレーザー照射を行って調べたところ，両条件でのエネルギー量に差は認められなかった。このことから培養液中の赤色物質による吸収は軽微と考えられ，照射時に培養液を取り除かず実験を行った。

4.　ELISA法によるIL-8およびMCP-1の測定

近赤外線照射を行ってから2，4，6時間後に培養上清を採取し，使用まで－80℃下にて保存した。IL-8およびMCP-1の測定は，ELISAキット（Biosource International）を用いた。IL-8およびMCP-1の産生量については，滑膜細胞数10^5個あたりの培養上清中の量に換算して表示した。

5.　統計学的検索

得られたデータは，IL-1β刺激のみで近赤外線未照射の群とIL-1β刺激後に700-800nm，800-900nm，900-1000nmの3種類のバンドパスフィルターを設置した近赤外線を照射した合計4群について多重比較（Scheffe's test）を行い，危険率5％未満を有意な差とした。

結　果

1.　顎関節滑膜細胞の細胞マーカー

顎関節滑膜細胞に対して各種細胞マーカーによる免疫染色を行ったところ，線維芽細胞のマーカーである線維芽細胞マーカーとビメンチンは染色されたが，マクロファージマーカー，HLA class Ⅱおよび樹状細胞マーカーは染色されなかった（図2）。

ネガティブコントロールは，一次抗体を作用させず，二次抗体のみ反応させた。結果は示さないが，細胞マーカーによる免疫染色では，IL-1βを作用させたときと作用させないときとでは差は認められなかった。

2.　近赤外線照射によるIL-8およびMCP-1産生への影響

顎関節滑膜細胞にIL-1βを作用させた後に3種類の波長帯の近赤外線照射をそれぞれ行い，2，4，6時間後の培養上清中のIL-8およびMCP-1量を測定した。

IL-1βを作用させることにより，コントロールと比較してIL-8およびMCP-1産生量は増加するが，さらに近赤外線を照射することにより，IL-8産生量は2時間後で900-1000nmの波長帯において他の3群と比較して有意な低下が認められたが，4，6時間後では大きな差は認められなかった（表1）。また，MCP-1産生量は，2時間後においてIL-1βのみ作用させた場合と比較して3種類すべての波長帯で有意に低下しており，4，6時間後においてもIL-1βのみ作用させた場合と比較して3種類すべての波長帯で低下する傾向であったが有意差は認められなかった（表2）。

考　察

今回我々は，顎関節滑膜細胞にIL-1βを作用させた後に近赤外線照射を行い，ケモカイン産生に対してどのような影響を及ぼすかについて検討した。

滑膜細胞は，電子顕微鏡的にマクロファージ様細胞のA型滑膜細胞と，線維芽細胞様細胞のB型滑膜細胞の2種類が存在するといわれている[16]。そこで，得られた滑膜細

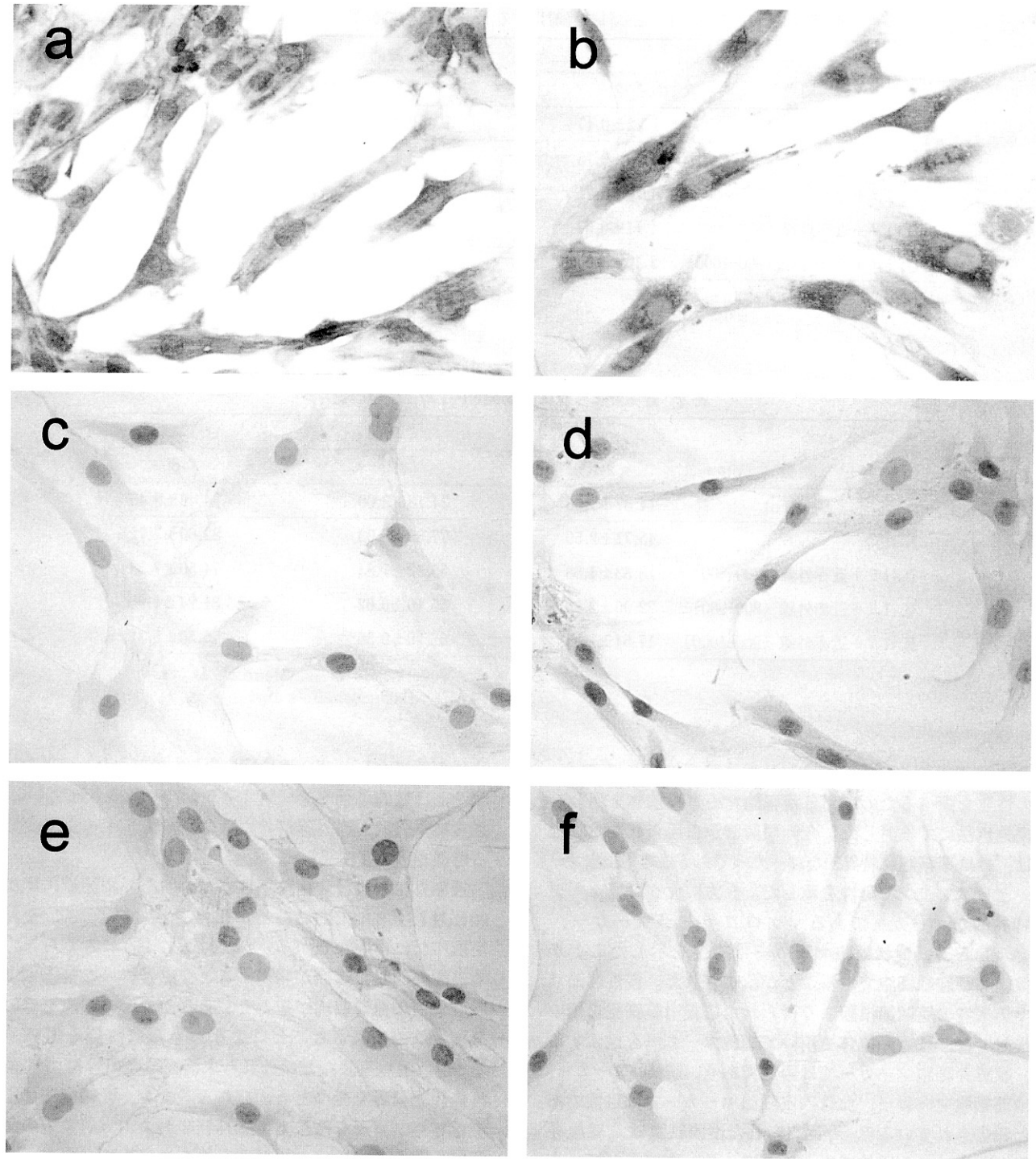

図 2　顎関節滑膜細胞の免疫染色
a. 線維芽細胞マーカー，b. ビメンチン，c. マクロファージマーカー，d. HLA class Ⅱ，e. 樹状細胞マーカー，f. ネガティブコントロール（倍率；×400）
線維芽細胞マーカーおよびビメンチンのみ陽性を示した。

表 1　近赤外線照射による IL-8 産生への影響

刺激	時間 2	時間 4	時間 6
Control	1.22±0.17	1.60±0.39	1.69±0.07
IL-1β	4.56±0.75	8.57±1.17	20.08±2.20
IL-1β＋近赤外線（700-800）	4.19±0.71	8.87±0.93	22.28±2.55
IL-1β＋近赤外線（800-900）	4.11±0.67	7.95±0.35	21.65±1.39
IL-1β＋近赤外線（900-1000）	2.49±0.05	8.02±0.57	21.18±1.54

単位：ng/10^5 cells，Mean±S.D.（n=3）
*$p<0.05$：Scheffe's test

表 2　近赤外線照射による MCP-1 産生への影響

刺激	時間 2	時間 4	時間 6
Control	14.37±1.66	34.18±3.00	34.46±9.45
IL-1β	45.71±2.50	77.39±8.03	82.60±7.72
IL-1β＋近赤外線（700-800）	18.33±4.58	55.52±1.84	74.50±7.38
IL-1β＋近赤外線（800-900）	22.96±2.60	55.56±6.62	81.24±4.90
IL-1β＋近赤外線（900-1000）	17.54±1.52	62.70±9.38	76.50±9.32

単位：ng/10^5 cells，Mean±S.D.（n=3）
*$p<0.05$：Scheffe's test

胞がA型滑膜細胞であるのか，B型滑膜細胞であるのか，その性質を調べるために，各種細胞マーカー抗体を用い，免疫染色法にて検討した。今回顎関節から得られた滑膜細胞は，線維芽細胞に特異的なマーカーである線維芽細胞マーカーとビメンチンで陽性を示した。一方，マクロファージに特異的なマーカーであるマクロファージマーカー，HLA class Ⅱ，樹状細胞マーカーは陰性を示したことから線維芽細胞様細胞であることが示唆された。慢性関節リウマチ患者の膝関節滑膜より得られた培養滑膜細胞を用いた検討では，種々の抗体を用いて細胞マーカーを検索すると，線維芽細胞マーカーでは陽性であり，筋細胞マーカー，平滑筋細胞マーカー，血管内皮細胞マーカーでは陰性であると報告されている[17]。今回得られた滑膜細胞は，線維芽細胞マーカーのみ陽性であることで一致した。よって，本実験で初代および継代培養した細胞を，ヒト顎関節滑膜細胞として実験に供することとした。

近赤外線照射による疼痛治療における作用機序については明確にはされていないが，近赤外線は遠赤外線を含む通常赤外線の中でも水にも血液にも吸収されにくいため生体深達度が高く，その波長域は半導体やHe-Neなどの単一波長の低出力レーザー波長域を含むものであり，低出力レーザーとほぼ同じ効果を発現する[18]ものと考えられている。さらに，光源が赤外線灯のために高エネルギーを供給できる特徴を有している。

近赤外線治療は，歯科領域においては顎関節症の疼痛緩和に対して有効であることが報告されている[7-12]。その作用機序には不明な点が多いが，以下のように考えられている。すなわち，疼痛が発現すると交感神経系の緊張により四肢末梢の血管収縮が起こり，それが組織の虚血を導いて発痛物質を放出することにより，さらに疼痛を増強させる[18]が，温熱療法などはこれら末梢組織の血管拡張により疼痛の悪循環を絶つと考えられる。また，光線療法による交感神経ブロック療法も交感神経のおよぶ末梢領域の血管収縮を改善し，疼痛の悪循環を絶ち疼痛緩和を導き，これが全身性の交感神経過緊張の抑制に働き全身性の効果を認めるものと考えられている[18]が詳細は不明である。

顎関節症に対する近赤外線治療については，臨床的な研究に比較して生化学的および細胞学的レベルでの研究は，ほとんどないのが現状である。近年，顎関節症患者の滑液を用いた生化学的な研究から，IL-1β，Tissue necrosis

factor-α（TNF-α）[19]，IL-6[20]などの炎症性サイトカイン濃度が正常者と比較して高い値を示すことが報告されており，関節疾患への関与が示唆されている。これらは破骨細胞の骨吸収機能を誘導することから，細胞外マトリックスや骨代謝において重要な因子として作用していると考えられている。IL-1βを作用させることにより，コントロールと比較してIL-8およびMCP-1産生量は増加したが，近赤外線照射を行うと，IL-8産生量は2時間後で900-1000nmの波長帯において有意に低下し，MCP-1産生量は2時間後において3種類すべての波長帯で有意に低下した。IL-8およびMCP-1は，好中球やマクロファージが遊走・活性化させるケモカインである。これら免疫担当細胞は，恒常性維持に重要であるものの，活性酸素や種々の酵素を産生することにより，組織破壊を引き起こす。また，多量のIL-1βやTNF-αを産生するため，滑膜細胞が刺激されケモカイン産生が上昇するという連鎖が起こるものと考えられる。よって，IL-8およびMCP-1産生量を減少させ，このような連鎖を抑制させることにより，消炎，基質破壊抑制効果があると示唆される。

本実験で使用した近赤外線照射装置は，痛みの治療に用いられる光線の波長である600-1600nmの範囲の近赤外線を，さらに3つの波長帯に分けて，それぞれ先端ユニットを変えることによって照射できるものである。波長600nm以下のものは血中ヘモグロビンに吸収されやすく，波長1600nm以上のものは水分の吸収率が高くなることから，波長の違いによる生体への影響は，生体深達度と波長の持つ固有な光子エネルギーに反映されると考えられる。よって，波長帯の違いによりケモカイン産生量に違いが認められたことは，影響を受けやすい特異的な波長があるものと考えられが，近赤外線による熱作用も考慮する必要があり，今後さらに詳細に波長帯を分けるような条件を設定するとともに，培養上清中の温度変化についても検討する必要があると考えられる。

結　論

培養ヒト顎関節滑膜細胞において，IL-1β刺激で増加したIL-8およびMCP-1の産生は，近赤外線照射を行うことによって早期に抑制され，近赤外線照射が炎症反応の抑制に有用であることが示唆された。また，その効果は近赤外線の波長の違いによって異なる可能性が示唆された。

謝　辞

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<原　著>

Enhanced Gene Expression by Low-Level Laser Irradiation in Osteoblast

——Identification of Annexin III Gene by Subtractive Gene Cloning——

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低出力レーザー照射による骨芽細胞の遺伝子発現の変動

——その1　遺伝子差分化法による Annexin III 遺伝子の同定——

山本　雅久[1]　河原　三明[1]　安孫子宜光[1,2]

The recent advent of improved low-level laser irradiation (LLLI) therapy has promoted interest in clinical implantology. It has been reported that LLLI on bony implant sites might have positive effects on the integration of implants. The biostimulatory effect of cell proliferation and bone formation by LLLI has been investigated, but little is known about the molecular basis of biostimulatory mechanisms. Since LLLI will be useful to support implant therapy, it is important to elucidate the mechanism of the biostimulatory effect of LLLI on bone formation.

We previously constructed the cDNA library of mouse osteoblastic cells (MC 3 T 3-E 1), which enhanced gene expression by LLLI using a subtracted gene cloning technology. In the present study, we further analyzed the DNA nucleotide sequence of gene clones, and focused on a gene clone designated MCL-174. The nucleotide sequence of MCL-174 insert was determined and assessed in the standard nucleotide-nucleotide BLAST (blastn) homology-search using NCBI DNA databases. DNA nucleotide sequences of clone MCL-174 inserted DNA exhibited 99% homology with *Mus musculus* annexin III gene. Reverse-transcription PCR analysis showed that the mRNA level was enhanced by LLLI. These findings suggest that LLLI may enhance mRNA transcription and play a role in stimulating proliferation of osteoblasts through the enhancement of annexin III gene expression. Annexin III was detected in secretory ameloblasts and odontoblasts, and it was thought to be involved in the regulation of cell calcium. These findings suggest that the biostimulatory effect of LLLI on bone formation may relate through gene expression of annexin III.

Key words : *osteoblast, low-level laser, nucleotide sequence, annexin III, gene expression*

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Introduction

Various biostimulatory effects of low-energy laser irradiation (LLLI) have been reported that included wound healing[1], cell proliferation of fibroblasts[2] and chondrocytes[3], collagen synthesis[4], and nerve regeneration[5]. Of particular interest for implant research, the acceleration of bone regeneration by laser treatment has been investigated[6~9]. The recent advent of improved laser delivery systems promoted interest in the application of lasers in implantology[10]. DÖRTBUDAK et al.[11] reported that LLLI on bony implant sites, after drilling and implant insertion in baboons, stimulated osteocyte viability, and suggested a positive effect on the integration of implants. Since LLLI will be useful to support implant therapy, it is important to elucidate the mechanism of the biostimulatory effect of LLLI on bone formation.

We studied the effects of LLLI at various cell culture stages of osteoblast cells, and found that cellular proliferation, bone nodule formation, alkaline phosphatase activity, and osteocalcin gene expression were only enhanced at the early stage[12]. These findings suggest that laser irradiation may principally play two roles in cell proliferation of bone nodule-forming cells of osteoblast lineage, and stimulation of cellular differentiation. However, the molecular bases of mechanisms leading to these findings are not elucidated.

To accomplish this, we previously constructed the cDNA library of MC 3 T 3-E 1, a clonal osteoblastic cell line, which enhanced gene expression by LLLI using a subtracted gene cloning procedure[13]. Among subtractive genes, we found a gene clone, exhibited a high homology nucleotide sequence with DNA replication licensing factors MCM 3[14] and MCM 5[15] genes, and ATP biosynthesis involving mitochondrial enzyme FOA 1-ATPase gene[16].

In the present study, we further characterized the gene library by the DNA nucleotide sequence of gene clones, and focused on a gene clone encoding a high homology nucleotide sequence with *Mus musculus* annexin III gene.

Materials and Methods

1. Cell culture and laser irradiation

MC 3 T 3-E 1 cells, established from newborn mouse calvaria by KODAMA et al.[17], were cultured in minimal essential medium (α-MEM ; Gibco BRL, USA) containing 10% fetal calf serum, 100 μg/m*l* of penicillin G (Sigma Chemical Co., USA), and 50 μg/m*l* of gentamicin sulfate (Sigma). The cultures were kept at 37°C in a humidified incubator (Forma CO_2 incubator MIP-3326, Sanyo Electric Medica System Co., Tokyo, Japan) in the presence of 95% air and 5% CO_2.

The LLLI was carried out according to a previous report[12]. Briefly, a Ga-Al-As diode laser device (Model Panalas® 1000 ; Matsushima Industrial Equipment Inc., Osaka, Japan) was used. Laser irradiation was performed so that the axis of the three beams met at right angles to the cell monolayer. The specification of this laser device was wavelength 830 nm and output power 100~700 mW variable. Laser irradiation was performed at a distance of 550 mm (area of spot size : 78.5 cm^2) from the probe to the cell layer. Continuous wave, at 500 mW output power for 20 min (power density : 7.64 J/cm^2), was used.

2. Restriction endonuclease analysis

Plasmid DNA from subtracted gene clones was isolated and digested with *Mlu* I and *Not* I. DNA fragments were run on 1% agarose gel-electrophoresis. The gel was stained with ethidium bromide under UV irradiation.

3. DNA nucleotide sequencing homology search

Dideoxy-chain termination sequencing[18] was performed with fluorescent dye-labeled T 7 universal primers (Aloka, Japan) and Sequi Therm™ Long-Read™ cycle sequencing kits for Li-Cor® Sequencing (Epicentre Technologies, USA). The reaction products were analyzed by a 4000 LS Long

ReadIR™ DNA sequencing system (LI-Cor, USA). Representative clones were randomly selected from a subtracted cDNA library, and a 5′-portion of each insert was nucleotide-sequenced.

The homology-search of the DNA sequence of cloned DNA was carried out by the standard nucleotide-nucleotide BLAST (blastn) homology-search, using NCBI DNA databases.

4. Reverse transcription polymerase chain reaction

Total cellular RNA was extracted from MC 3 T 3-E 1 cells with or without LLLI by acid guanidium thiocyanate-phenol-choloroform extraction[19]. cDNA synthesis and amplification by reverse transcription-polymerase chain reaction (RT-PCR) were carried out, using a GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT, USA). The PCR DNA primers were as follows : 5′-TCT ATC TGG GTT GGA CCT CGA GGA-3′ (the forward primer for mouse annexin III) ; 5′-CTT CTA CCA TCT GCC AGA GTC AGC-3′ (the reverse primer for mouse annexin III) ; 5′-ATC ACC ATC TTC CAG GAG-3′ (the forward primer for mouse GAPDH) ; 5′-ATG GAC TGT GGT CAT GAG-3′ (the reverse primer for mouse GAPDH) PCR products were electrophoresed on 1.5% agarose gel and subsequently stained with ethidium bromide.

Results

Figure 1 shows the agarose electrophoresis pattern for the screening of DNA insert size from subtracted gene clones. Plasmid DNAs with different sizes of inserts were selected and nucleotide sequenced.

The endonuclease digestion pattern of plasmid DNA from MCL-174 is shown in Fig. 2. The result suggests that MCL-174 clone has a 1.5 kb-size insert.

Then, nucleotide sequence homology of MCL-174 insert was examined. A blastin search showed many blast hits on the query sequence, with a variety of homology, as shown in Fig. 3.

Among hit genes, MCL-174 DNA exhibited with annexin III genes of *Mus musculus* (99%), *Rattus norvegicus* (89%), and *Homo sapiens* (85%), as shown in Table 1. Figure 4 showed the identification of nucleotide sequences between MCL-174 and *Mus musculus* annexin III gene.

To confirm the enhanced mRNA level of annexin III gene in LLLI cells, RT-PCR analysis was carried out. Figure 5 showed higher levels of annexin III mRNA level in LLLI MC 3 T 3-E 1 cells compared with non-LLLI, while mRNA levels of GAPDH, as a house keeping control, were the same between with or without LLLI cells.

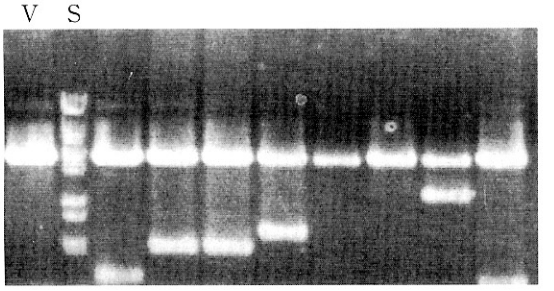

Fig. 1 Agarose gel electrophoresis pattern for the screening of DNA insert size from a subtracted gene library
V, Vector plasmid pAP 3 neo DNA ; S, Size marker of λ DNA *Hind* III digestion ; other lanes, subtracted gene clone plasmid DNA

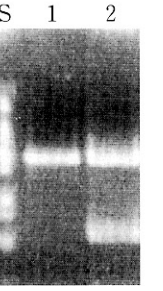

Fig. 2 The endonuclease digestion pattern of plasmid DNA from MCL-174
S, Size marker λ *Hind* III digestion ; 1, Vector plasmid pAP 3 neo ; 2, plasmid DNA from MCL-174

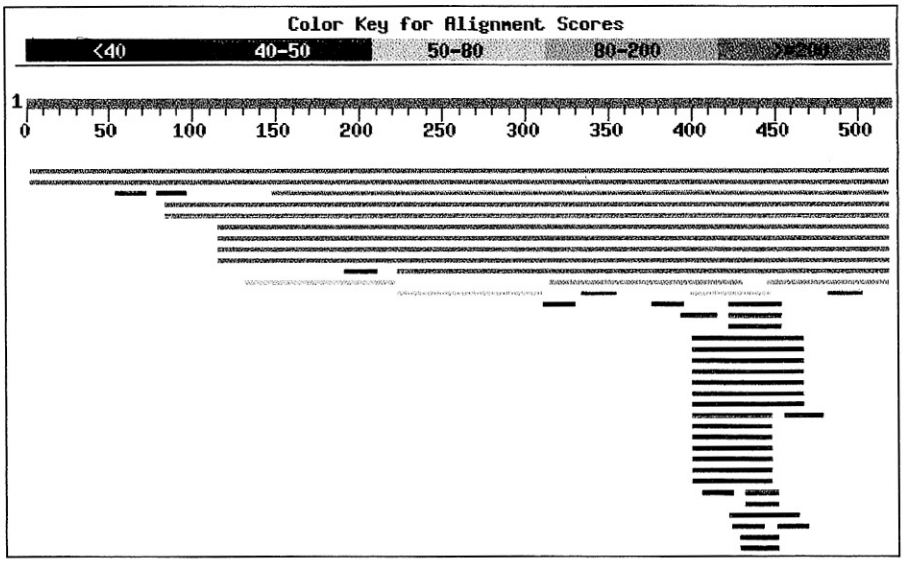

Fig. 3 Distribution of Blastin search hits on the Query sequence
Number showed nucleotide bp. Lines showed the identical DNA sequences region.

Discussion

In this study, we found the annexin III gene in a subtractive gene library, which enhanced its gene expression by LLLI. The nucleotide sequence of DNA insert of MCL-174 had high homology with mouse annexin III. Further, RT-PCR analysis results confirmed an elevated mRMA level in LLLI cells. These findings suggested that LLLI enhanced the transcription of annexin III in the osteoblastic cell line MC 3 T 3-E 1.

Mouse annexin III cDNA was first characterized as expressed sequence tag clones by molecular sequencing, chromosomal mapping, and systematic analysis. cDNA sequences extended the location of intron 7, with respect to the human gene, and mapped to the middle of mouse chromosome 5[20]. Our gene clone MCL-174 has a high nucleotide sequence homology with mouse annexin III reported in this literature.

Annexins are soluble proteins capable of binding to phospholipid membranes in a calcium-dependent manner in neutrophils[21]. By immunofluorescence microscopy observation, annexin III was more likely to be associated with specific granules in neutrophils, suggesting annexins could be implicated in processes of granule fusion.

Study of the annexin protein family on hard tissues, immunoblot analyses, and ultrastructural immunogold experiments have been conducted on secretory ameloblasts and odontoblasts of rat incisor. GOLDBERG et al.[22] found that annexins I and II were seen in soluble and particulate fractions of enamel-related portion, but not in dentin-related portion, and annexins III, IV and V were detected in both soluble and particulate fractions of enamel- and dentin-related portions. They speculated that annexins are implied in the regulation of cell calcium. These findings suggest that the mechanism of the biostimulatory effect by LLLI on bone formation may relate through annexin III gene expression in osteoblasts.

While laser treatment has been utilized in oral surgery for many years, the recent advent of improved laser delivery systems has promoted interest in the application of lasers in implantology. WALSH[10] reviewed the use of lasers in laboratory and clinical techniques used in implantology, and provided useful information with particular refer-

ence to carbon dioxide, Nd : YAG, argon, and erbium : YAG lasers. It is of interest to determine whether screws were able to osseointegrate in a laser-prepared bone defect, and to compare the pattern of bone healing around these screws. Osseointegration of titanium screws can be achieved using an erbium : YAG laser to prepare the implant

Table 1 Result of Blastin search

Homologous gene	Species	Gene ID	Similarity (%)	Identity (bp)
Annexin III	mouse	NM 013470	99	514/518
Annexin III	mouse	AJ 001633	99	514/518
Annexin III	rat	NM 012823	89	394/440
Annexin III	human	BC 000871	85	345/405

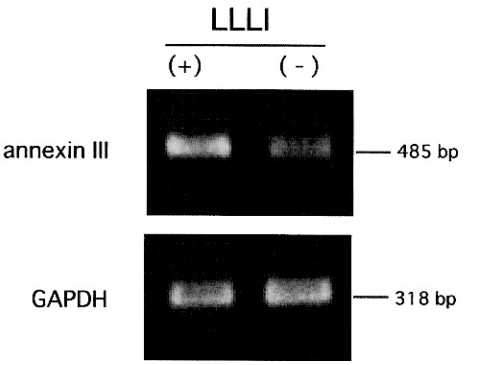

Fig. 5 RT-PCR analysis of the annexin III mRNA level Ethidium bromide staining pattern of amplified PCR products on agarose gel electrophoresis. RT-PCR method and DNA primers used were described in Materials and Methods.

```
Score =  979 bits (494), Expect = 0.0
Identities = 514/518 (99%), Gaps = 2/518 (0%)
Strand = Plus / Plus

Query: 3   gggagctacggccggccgaggctgatctttcaccttcgctgagcttctgcctgggcttcg 62
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 87  gggagctacggccggccgaggctgatctttcaccttcgctgagcttctgcctgggcttcg 146

Query: 63  cctccgcggccccaaggattgctgtgatctcggcttgagagaaaggtggactgtcatggc 122
           | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 147 c-tccgcggccccaaggattgctgtgatctcggcttgagagaaaggtggactgtcatggc 205

Query: 123 ctctatctgggttggacctcgaggaaccataaaagattatccaggctttagccccgtcgg 182
           |||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct: 206 ctctatctgggttggacctcgaggaaccataaaagattatccaggctttagccc-gtcgg 264

Query: 183 tggatgccgaagctatccggaaagcgatcagaggacttgggactgacgagaaaaccctca 242
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 265 tggatgccgaagctatccggaaagcgatcagaggacttgggactgacgagaaaaccctca 324

Query: 243 tcaacattctgacggagcggtcgaacgcgcagcgacagctgattgtcaagcagtaccaag 302
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 325 tcaacattctgacggagcggtcgaacgcgcagcgacagctgattgtcaagcagtaccaag 384

Query: 303 cagcgtatgaacaggagctgaaagatgacttgaagggtgatctctctggccacttcgagc 362
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 385 cagcgtatgaacaggagctgaaagatgacttgaagggtgatctctctggccacttcgagc 444

Query: 363 acgtcatggtggctcttgttactgcaccagccctgtttgatgcgaagcaactgaagaaat 422
           |||||||||||||||||||||||||||||||||||||||||||||  ||||||||||||
Sbjct: 445 acgtcatggtggctcttgttactgcaccagccctgtttgatgcgaacgaactgaagaaat 504

Query: 423 ccatgaagggaactggcacagatgaagatgccctgattgaaatcctaacaaccaggtcaa 482
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 505 ccatgaagggaactggcacagatgaagatgccctgattgaaatcctaacaaccaggtcaa 564

Query: 483 gcaggcagatgaaggaaatctcgcaggcctattataca 520
           ||||||||||||||||||||||||||||||||||||
Sbjct: 565 gcaggcagatgaaggaaatctcgcaggcctattataca 602
```

Fig. 4 Identification of nucleotide sequences between MCL-174 and mouse annexin III genes
Query, MCL-174 ; Sbjct, mouse annexin III

bed[23].

Among the many physiological effects of low-energy laser irradiation, anti-inflammatory functions have also been reported. HONMURA et al.[24] reported the therapeutic effect of Ga-Al-As laser irradiation on experimental carrageenin-induced inflammation. We previously reported that Ga-Al-As diode LLLI caused inhibition of prostaglandin E2 (PGE2) and IL-1β[25], as well as plasminogen activator production[26] in mechanically stressed human periodontal ligament cells.

Osteogenesis and the bone healing process following implant therapy are very important to lead successful result. Thus, the most important biostimulatory effect of LLLI for implant therapy should be the acceleration of bone formation. DÖRTBUDAK et al.[11] examined the effects of LLLI on osteocytes and bone resorption at bony implant sites in iliac crest of baboon. Holes for accommodating implants were drilled and irradiated with LLLI, then immediately inserted implants. Histochemical studies showed that osteocyte viability was significantly higher in LLLI subjects. In contrast, the bone resorption rate was not affected by LLLI. These findings suggest that more vital bone tissue is present in the LLLI area compared with control sites. Tissue engineering can be defined as one of the most challenging fields in medicine. Bone tissue engineering, using bone marrow derived mesenchymal cells, has been heralded as an alternative strategy to regenerate bone. It has been reported that LLLI stimulated bone matrix formation in osteoblasts derived from bone marrow cells[27].

Taken together of these investigations, the biological effects of bone formation and anti-inflammation, the utilizing of LLLI can be expected to reduce healing times and speed up osseointegration of implants.

The lack of knowledge of the biological mechanism about LLLI may bring a delay in clinical use of laser therapy, including clinical implantology. The findings presented here clearly provide evidence that LLLI alters the transcription level in osteoblasts. Further studies for another candidate genes, which involve bone formation, are progress in our laboratory.

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原　著

差分化遺伝子クローニングによる骨芽細胞への低出力レーザー照射の生物学的効果の機序解明

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Elucidation of Mechanisms for the Biostimulatory Effect of Low Level Laser Irradiation on Osteoblasts by Subtractive Gene Cloning

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Abstract : Various biostimulatory effects of low-energy laser irradiation (LLLI) have been reported that involve the acceleration of bone regeneration. We reported that LLLI stimulated cellular proliferation, bone nodule formation, alkaline phosphatase activity, and osteocalcin expression. However, the molecular basis of the mechanisms leading to these findings has not been elucidated. To accomplish this, we constructed the cDNA library of MC3T3-E1, a clonal osteoblastic cell line, which enhanced gene expressions by LLLI using a subtracted gene cloning procedure. In the present study, we further characterized gene library by DNA nucleotide sequence and homology-search with a DNA database. Among 88 subtractive genes, several clones exhibited high homology with mitochondrial protein genes, signal transduction-related genes, and EST genes. These findings may help to elucidate the molecular-based mechanisms for the biostimulatory effect of LLLI on osteoblasts.

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Key words=Low-energy laser irradiation, Osteoblast, Gene expression

キーワード=低出力レーザー，骨芽細胞，遺伝子発現

緒　言

低出力レーザー照射が創傷や難治性潰瘍に治癒効果があると言及されて以来，炎症抑制，疼痛減少，骨折治癒促進など広範な臨床効果が報告されている[1,2]。しかしながら，低出力レーザー照射による生物学的効果の作用機序は不明な点が多い。とくに分子レベルでの実証科学的な解明は遅れている。

歯科医学領域にも積極的な応用が期待されているなかで，レーザー医療をさらに推進，発展させるためには，有用性

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の高いレーザー照射の機種，照射法を開発するとともに，生物学的効果を実証科学的に解明していく必要があると思われる。

従来の分子レベルでの研究アプローチは，生理活性物質の発見→精製→遺伝子クローニング→機能解析→タンパク質一次構造の解析が行われてきた。しかし，これらの研究方法では，すでによく知られている生理活性物質あるいは同定，精製に成功した分子しか捉えることはできない。当然ながら，目的とする生命現象に関わる未知の遺伝子については研究することは不可能である。

近年，ゲノムサイエンス研究の飛躍的な進展に伴い，ヒトを含む種々の生物のゲノム計画が進められている[3]。ゲノムデータベースの充実に伴って，細胞内で変動する遺伝子のトランスクリプトーム解析は，生命現象を理解することに多くの情報を与え得ると期待されている。

遺伝子発現の変動を捉える方法として，遺伝子バンクを作成し，対照のmRNAで差し引きするサブトラクション遺伝子クローニング法が開発されている[4]。レーザー照射した細胞からmRNAを分離してcDNA遺伝子バンクを調製し，非レーザー照射細胞のmRNAを用いて同一の遺伝子を差し引いた（差分化）残りの遺伝子群は，レーザー照射によって遺伝子発現が増大した遺伝子ということになる。この差分化遺伝子ライブラリーを作成し，得られた遺伝子クローンの塩基配列を解読してゲノムデータベースの遺伝子配列とホモロジー検索することによって関与する遺伝子を同定し，その遺伝子の遺伝子産物の機能を明らかにすることが可能となる。

本研究では，骨芽細胞に低出力レーザー照射して差分化遺伝子ライブラリーを構築して新たに得た88遺伝子クローンについて塩基配列を解読し，低出力レーザー照射の生物学的効果の作用機序の解明を試みた。

実験方法

1.　細胞培養

骨芽細胞として，マウス新生仔の頭蓋骨から樹立され，骨結節形成能を有するMC3T3-E1[5]を用いた。細胞は，10% 牛胎児血清，100μg/mlペニシリンG，50μg/mlゲンタマイシン硫酸を添加したα-MEM培地で，95% air-5 % CO_2，37℃の条件化，CO_2インキュベーターで培養した。培養ディシュ（75cm²）に培養液10mlの培養液を加えて培養を行った。

2.　レーザー装置

低出力レーザー照射装置は，Ga-Al-As半導体レーザー装置（モデルPanalas-1000，波長 830nm，最大出力500 mW；松下電器）を用いた。細胞表面から5 cmの距離に照射部を設定し，500mWで培養ディシュ75cm²に対して20分間照射した。この照射条件で，エネルギー密度は7.6 J/cm²になる。培養液の存在下および非存在化でレーザー照射を行って調べたところ，両条件でのエネルギー量に差は認められなかった。このことから培養液中の赤色物質による吸収は軽微と考えられ，照射時に培養液を取り除かず実験を行った。また，この照射条件で培養液の温度の変化に有意差は認められない。

3.　cDNA ライブラリーの作成

本実験では，細胞増殖への影響も調べるために，MC3T3-E1細胞をセミコンフルエントまで培養し，レーザー照射後，1，6，12，24時間後に細胞から総RNAを回収した。総RNAを混合して，mRNAを精製し，cDNAライブラリーを作成した。5μgのポリA-RNAをテンプレートにして，1.6μg *Not* Ⅰサイトをもつoligo dTをプライマーに，メチル化デオキシヌクレオチドを用いて逆転写酵素（Superscript Ⅱ）反応によってcDNAを合成した。合成試料からRNA をRNase Hを用いて取り除いた後，*Bgl*Ⅱ-*Sma*Ⅰアダプターを付加してベクタープラスミドに一方向で挿入できるようにした。300bp以下の短いDNAフラグメントをスピンカラムで除き，ベクタープラスミドpAPneoに挿入し，T4リガーゼでキメラプラスミドを作成した[6]（図1）。

4.　差分化遺伝子クローニング

キメラプラスミドを宿主細胞 *Escherichia coli* DH5αF 7IQにエレクトロポーレーション法で形質転換し，R408ファージを感染させて一本鎖化して回収した。次いで，レーザー非照射MC3T3-E1から回収，精製したmRNAをビオチン標識し，一本鎖化キメラプラスミド ライブラリーとハイブリダイズさせ，さらにストレプトアビジンを結合させた。共通するmRNA結合キメラプラスミドをフェノール法で除去し，残ったキメラプラスミド，すなわちレーザー照射でmRNA レベルが上昇した遺伝子を含むキメラプラスミドを回収した。この操作を二度繰り返した。差分化キメラプラスミドにBcaBEST DNA ポリメラーゼを用いて二本鎖DNAプラスミドにした後，*E. coli* MC1061Aに形質転換して差分化遺伝子クローン ライブラリーを作成した[6]（図2）。

5.　塩基配列の解読

単一コロニーの*E. coli*遺伝子クローンをランダムに回収後，自動プラスミド精製装置（Kurabo，PI-100）でプラスミドを回収，精製した。各キメラプラスミドの挿入DNA断片の塩基配列の解読は，dideoxy chain termination法[7]で行った。塩基配列の解読は蛍光自動DNAシーケンサー（LiCor，Epicenter Technolgy社），蛍光標識

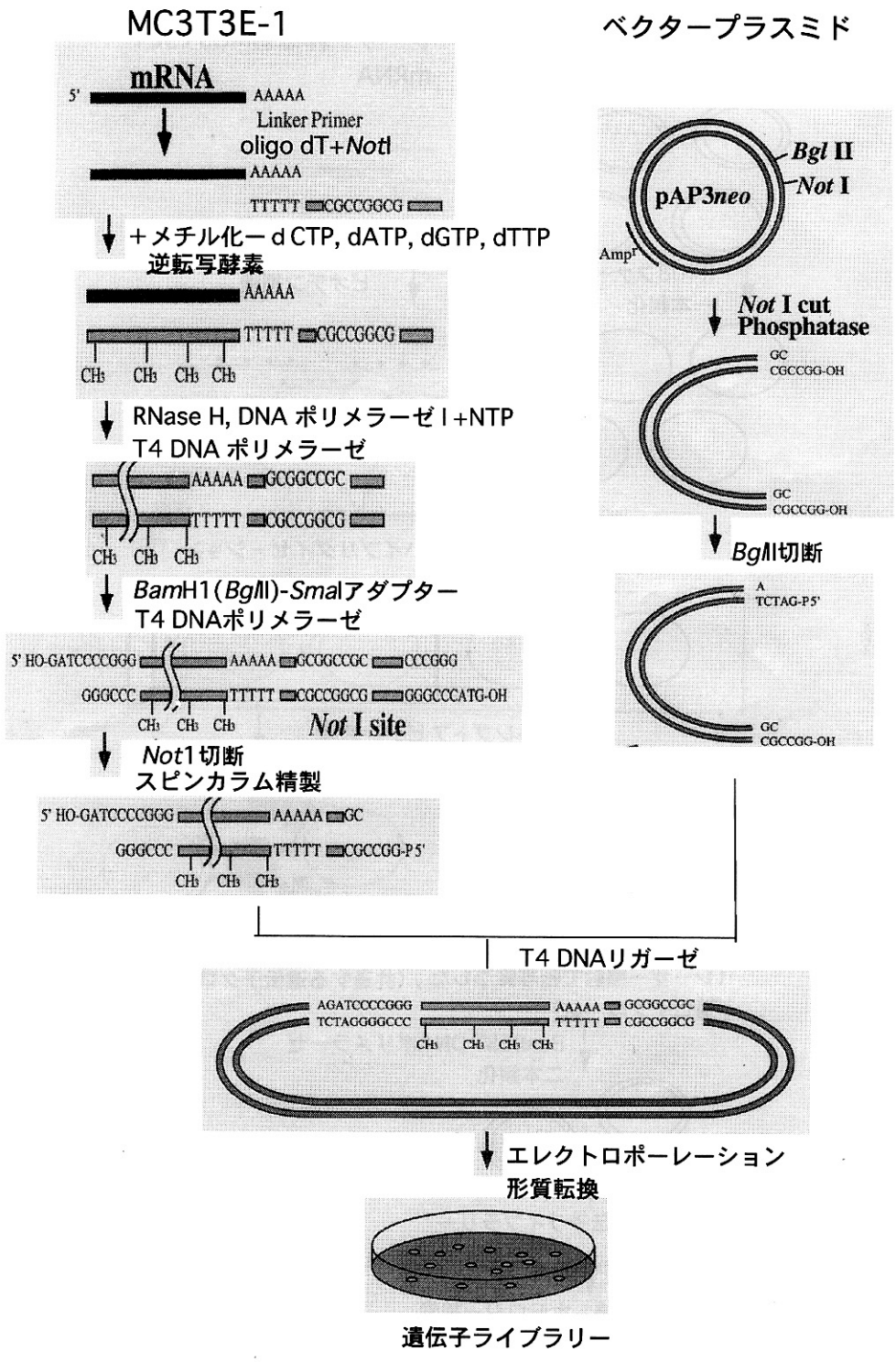


図 1　非レーザー照射細胞遺伝子バンク作成の概略
制限酵素リンカー付加の工夫によって cDNA 断片の挿入方向は一定となり，差分化する際の非レーザー照射細胞 mRNA に対するアンチセンス鎖がプラスミド DNA の一本鎖として残る。

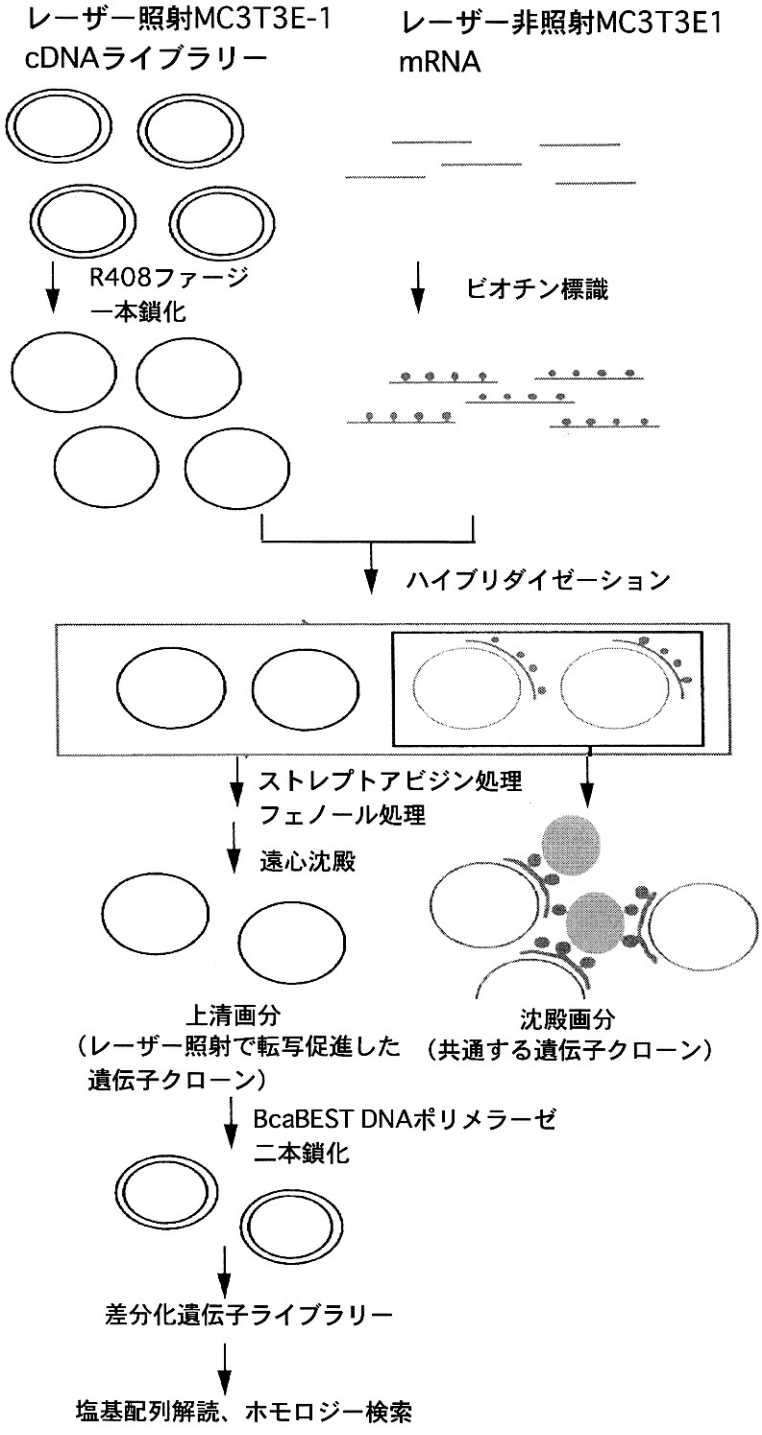


図 2　差分化遺伝子ライブラリー作成の概略
プラスミド遺伝子バンクの一本鎖化されたDNAにビオチン標識した非レーザー照射細胞mRNAが結合する。ビオチンにストレプトアビジンが結合したプラスミドはフェノール処理で取り除かれる。上清画分に残ったプラスミドDNAを二本鎖に戻して大腸菌宿主に形質転換して差分化遺伝子ライブラリーを得る。

DNA プライマー，Sequi therm Long-Read cycle sequencing kit（LiCor, Epicenter Technolgy 社）を用いて行った。

6. データベース検索

National Center for Biotechnology Information（NCBI）の BLAST（Basic Local Alignment Search Tool）を利用して，塩基配列のホモロジー検索（Nucleotide BLAST；blastn）を行った。約 520bp の塩基配列のホモロジー検索の結果で既知遺伝子と一部の相同性が見られたものを Known gene クローン，アノテーションがない EST（Expression Sequence Tag）gene とのホモロジーが見られたものを EST クローン，30bp 以上相同性が見られないのを Unknown gene クローンとした。

結　果

差分化遺伝子ライブラリーからプラスミド DNA を抽出，精製し制限酵素で切断してアガロースゲル電気泳動を行い，挿入断片を調べた。図 3 にその一部を示す。

300bp 以上の挿入断片をもち，かつ制限酵素消化パターンが異なる 88 遺伝子クローンを選び，プラスミド DNA を精製し，それぞれの挿入 DNA 断片の塩基配列について 520bp 解読した。得られた塩基配列情報を NCBI の BLAST プログラムで塩基配列のホモロジー検索を行った。図 4 は，No. 1-178 クローンを一例として示したもので，図 4A が塩基配列，図 4B が DNA データベースとのホモロジー検索で相同性のあった遺伝子とのアライメントスコアー，図 4C がホモロジー検索で相同性の極めて高いトップ 4 遺伝子のアノーテーション情報である。そして，図 4D は，No. 1-178 の塩基配列（Query）と NM02553.1 遺伝子（Sbjct）の DNA 塩期配列ホモロジー検索結果である。相同性の高かった 4 遺伝子のアノーテーションは，いずれもマウスのミトコンドリアのリボゾームタンパク質 L11 であった。

88 遺伝子クローンについてのホモロジー検索の結果を表1にまとめた。22 クローンが Known 遺伝子，38 クローンが EST クローン，30 クローンが Unknown 遺伝子であった。また，表 2 に 22 クローンの既知遺伝子についてのアノテーション情報を示した。

考　察

低出力レーザー照射の生物学的効果として，炎症抑制，疼痛減少，創傷治癒促進，骨折治癒促進などが報告されている。しかしながら，一方ではレーザー照射の効果について心理的なプラセボー効果が大きいとする懐疑的な見方や単なる温度上昇効果によるものであるとする意見もある。

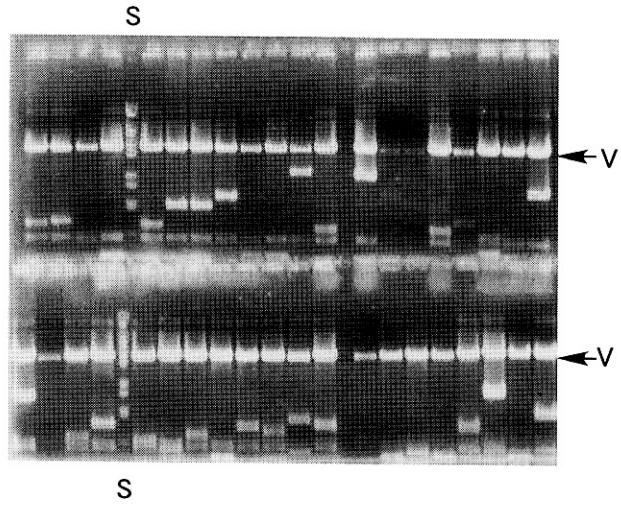


図 3　差分化遺伝子クローンのスクリーニング
差分化遺伝子ライブラリーの大腸菌クローンを培養し，集菌後，プラスミド DNA を分離する。制限酵素 *Not* I, *Bgl* II で切断し，アガロース電気泳動を行う。300bp 以上の挿入 DNA をもつクローンを選択する。S, サイズスタンダード DNA；V, ベクタープラスミド DNA 断片

このような背景のなかで，歯科領域への低出力レーザーの積極的な応用をさらに推進，発展させるためには実証科学的な方法で作用メカニズムを解明する基礎研究が必要であると考えられる。

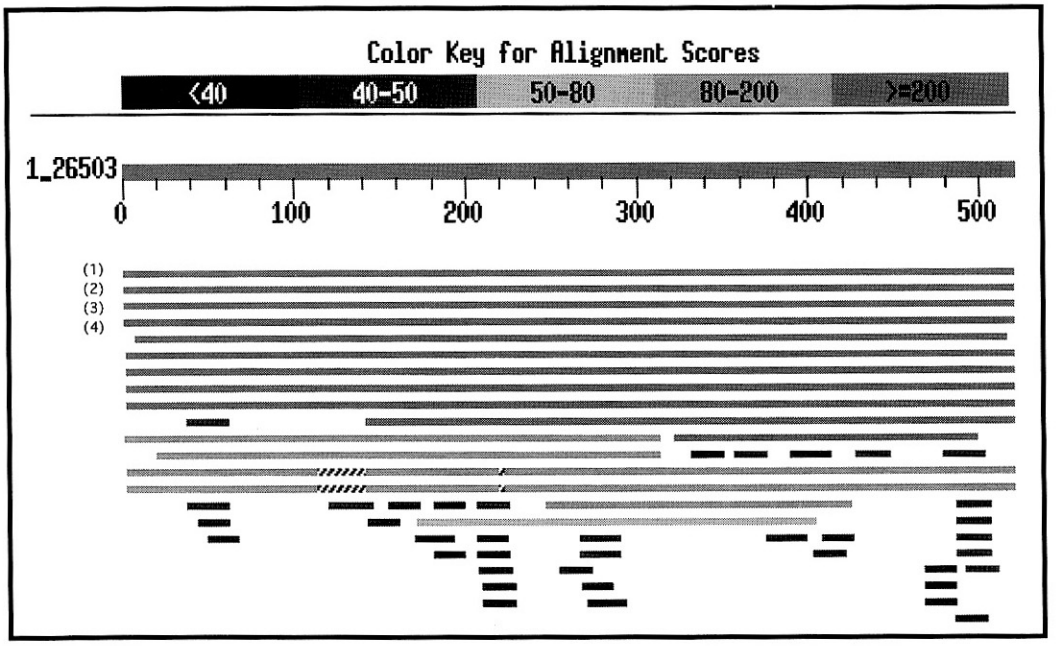
われわれは，レーザー照射の骨形成促進メカニズムを解明するために in vitro 実験系を設定し検討を行っている。ラット胎仔の頭蓋骨から採取した骨芽細胞に低出力レーザー照射を行い，骨結節数が照射量に比例して増大し，アルカリホスファターゼ活性，コラーゲン産生が促進されることを見いだしている[8]。さらに，培養早期のレーザー照射がより強い骨形成促進効果を有することを報告している[9]。この結果は，レーザー照射が未分化間葉系細胞の増殖と骨芽細胞への分化を促進することを示唆する。

近年，ゲノム科学を基盤とする研究技術が進展し，ゲノム情報をすべて解読するゲノムプロジェクトの進展とその応用によって先進的な研究が行われている。細胞・組織から mRNA を抽出して，ランダムプライマーを用いて mRNA の遺伝子増幅を行って遺伝子発現レベルの差を同定するデファレンシャル ディスプレイ法や遺伝子バンクを作成して対照の mRNA で差し引くサブトラクション遺伝子クローニング法などが開発されている。そして，得られた遺伝子の塩基配列情報をゲノムデータベースと照合することで，その生命現象に関与する遺伝子発現を未知遺伝子も含めて同定することが可能である。

A 塩基配列

TACATCATGTCCAAGCTAAGCCGGGCCACTCGGACCCTCAAGAAGCCCGAGGCCGGCGGCGTGATCCGGTCCATCG
TGCGAGCAGGCCAAGCTATTCCTGGGCCTCCACTAGGTCCCATCTTGGGTCAGCGAGGTGTCTCTATCAACCAGTT
CTGCAAAGAGTTCAACGAGAAGACAAAGGACATCAAAGAAGGCATTCCCCTGCCTACAAAAATTTTTATAAAGCCC
GACAGGACATTTGAGCTCAAGATTGGGCAGCCCACTGTTTCTTACTTTTTGAAGGCAGCTGCTGGGATCGAGAAGG
GGGCCCGGCATACAGGGAAAGAGGTGGCAGGCCTGGTGAGTTTGAAGCACGTATATGAGATTGCCTGTGTCAAAGC
TAAGGATGATGCTTTTACCATGCAAGATGTGCCCCTGTCTTCTGTGGTCCGTTCCATCATTGGCTCTGCCCGTTCC
CTGGGCATTCGAGTGGTGAAGGACCTCAGTGCAGAAGAACTGGAGGCTTTCCAGAAGGAACGAG

B ホモロジー検索でヒットした遺伝子



C ホモロジーの高い４遺伝子のアノテーション

(1) gi|13384979|ref|NM_025553.1|
Mus musculus mitochondrial ribosomal protein L11　Identities = 519/520 (99%)

(2) gi|12845690|dbj|AK010329.1|AK010329
Mus musculus ES cells, mitochondrial ribosomal protein L11　Identities = 519/520 (99%)

(3) gi|12833044|dbj|AK002797.1|AK002797
Mus musculus kidney, mitochondrial ribosomalprotein L11　Identities = 519/520 (99%)

(4) gi|13559368|dbj|AB049639.1|AB049639
Mus musculus MRPL11, mitochondrial ribosomal protein L11　Identities = 519/520 (99%)

D 塩基配列のホモロジー

```
(1) gi|13384979|ref|NM_025553.1
Query:   1 tacatcatgtccaagctaagccgggccactcggaccctcaagaagcccgaggccggcggc 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  74 tacatcatgtccaagctaagccgggccactcggaccctcaagaagcccgaggccggcggc 133

Query:  61 gtgatccggtccatcgtgcgagcaggccaagctattcctgggcctccactaggtcccatc 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 134 gtgatccggtccatcgtgcgagcaggccaagctattcctgggcctccactaggtcccatc 193

Query: 121 ttgggtcagcgaggtgtctctatcaaccagttctgcaaagagttcaacgagaagacaaag 180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 194 ttgggtcagcgaggtgtctctatcaaccagttctgcaaagagttcaacgagaagacaaag 253

Query: 181 gacatcaaagaaggcattcccctgcctacaaaaatttttataaagcccgacaggacattt 240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 254 gacatcaaagaaggcattcccctgcctacaaaaatttttataaagcccgacaggacattt 313

Query: 241 gagctcaagattgggcagcccactgtttcttacttttgaaggcagctgctgggatcgag 300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 314 gagctcaagattgggcagcccactgtttcttacttttgaaggcagctgctgggatcgag 373

Query: 301 aaggggcccggcatacagggaaagaggtggcaggcctggtgagtttgaagcacgtatat 360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 374 aaggggcccggcatacagggaaagaggtggcaggcctggtgagtttgaagcacgtatat 433

Query: 361 gagattgcctgtgtcaaagctaaggatgatgcttttaccatgcaagatgtgcccctgtct 420
           |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct: 434 gagattgcctgtgtcaaagctaaggatgatgcttttgccatgcaagatgtgcccctgtct 493

Query: 421 tctgtggtccgttccatcattggctctgcccgttccctgggcattcgagtggtgaaggac 480
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 494 tctgtggtccgttccatcattggctctgcccgttccctgggcattcgagtggtgaaggac 553

Query: 481 ctcagtgcagaagaactggaggctttccagaaggaacgag 520
           |||||||||||||||||||||||||||||||||||||||
Sbjct: 554 ctcagtgcagaagaactggaggctttccagaaggaacgag 593
```

図 4　差分化遺伝子クローンのホモロジー検索の一例
1-178 と名付けた差分化遺伝子クローンの塩基配列を NCBI の BLAST サーチを行った結果，マウスのミトコンドリアのリボゾームタンパク質 L11 遺伝子と高い相同性が得られた。A，No. 1-178 クローンの塩基配列解読結果：B，BLAST 解析した結果のホモロジーをもつ遺伝子のアライメント結果：C，相同性の極めて高いトップ 4 遺伝子のアノーテーション情報 D，塩基性配列のホモロジー情報

表 1　88 遺伝子クローンのホモロジー検索結果

Unknown	EST		Known
1-87	1-79	332/332 (100%)	1-81 /32kDa
1-93	1-83	129/130 (99%)	1-84 /Thirod reductase
1-97	1-102	35/37 (94%)	1-85 /Myosin
1-100	1-112	291/306 (95%)	1-95 /TF
1-109	1-114	30/31 (96%)	1-104 /ESNRA
1-113	1-116	71/76 (93%)	1-122 /Msx Zn finger
1-121	1-125	239/254 (94%)	1-126 /sn RNA
1-143	1-127	54/54 (100%)	1-133 /galactin
1-149	1-128	297/300 (99%)	1-155 /c-jun
1-152	1-131	509/512 (99%)	1-164 /hydrolase
1-160	1-132	431/442 (97%)	1-167 /Centromere
1-163	1-136	199/218 (91%)	1-174 /Annexin Ⅲ
1-165	1-147	233/251 (92%)	1-178 /Mt rRNA protein
1-168	1-150	237/240 (98%)	1-191 /hydrolase
1-169	1-151	494/516 (95%)	1-198 /EST／MHC
1-171	1-157	181/192 (94%)	1-199 /G protein
1-172	1-161	507/521 (97%)	2p-12 /phosphatase
1-173	1-162	156/171 (91%)	2p-16 /GDP／disof.
1-180	1-176	518/518 (100%)	2b-59 /Tyr-phosphatase
1-182	1-177	487/494 (98%)	2b-46 /silence Med-EST
1-183	1-194	519/520 (99%)	
1-187	1-195	473/473 (100%)	
1-192	1-197	413/420 (98%)	
2b-45	2b-51	82/82 (100%)	
2b-47	2b-52	418/466 (89%)	
2b-50	2b-54	520/520 (100%)	
2b-55	2b-56	83/85 (97%)	
2b-57	2b-58	410/453 (90%)	
2b-58	2p-1	516/518 (99%)	
2p-20	2p-2	517/517 (100%)	
	2p-6	517/518 (99%)	
	2p-7	182/191 (95%)	
	2p-8	516/517 (99%)	
	2p-10	127/134 (94%)	
	2p-11	509/516 (98%)	
	2p-13	418/425 (98%)	
	2p-18	301/304 (99%)	
	2p-19	515/515 (100%)	
30 clones	38 clones		20 clones

表 2　22-Known クローンの既知遺伝子のアノテーション情報

Gene ID No.	Description	Identities
AB049639	Mus musculus MRPL 11 mRNA for mitochondrial ribosomal protein L11	515/516 (99%)
AA285552	Mus musculus GDP-dissociation inhibitor	513/518 (99%)
AJ001633	Mus musculus mRNA for annexin Ⅲ	513/517 (99%)
AI006497	Human protein phosphatase PP2A, 65kD regulatory subunit	511/518 (98%)
AF039567	Mus musculus Msx-interacting-zinc finger protein 1 (Miz 1)	492/494 (99%)
AF112439	Mus musculus Fanconi anemia group G protein	465/472 (98%)
AA856395	Mus musculus Hypotical 32.8kD pritein in PDE1-CSE1 interigenic region	363/375 (96%)
BF714528	Mus musculus SnRNA activating protein 50kD subunit	352/408 (86%)
AF116187	Mus musculus inner centromere protein	278/296 (93%)
AF218069	Mus musculus galectin-8	267/270 (98%)
BE334277	Mus musculus c-jun protein oncogene	259/263 (98%)
AF230356	Mus musculus U2 small nuclear ribonucleoprotein A	240/258 (93%)
U28807	Mus musculus lymphoid-specific transcription factor NFATc3	239/244 (97%)
AF110520	Mus musculus major histocompatibility complex NG27, 28, RPS28	190/194 (97%)
BF730197	Human Ubiquitin carboxyl-terminal hydrolase 16	158/170 (92%)
AF013490	Mus musculus protein-tyrosine phosphatase	128/133 (96%)
BC006075	Mus musculus Myosin, heavy polypeptide 9	121/134 (90%)
AA166294	Mus musculus Silencing mediator of retinoid & thyroid action	71/ 72 (98%)
AA611370	Mus musculus Bleomycin hydrolase	60/ 61 (98%)
AW743450	Mus musculus Thioredoxin-dependent peroxide reductase	46/ 49 (93%)

われわれはすでに，低出力レーザー照射したマウス骨芽細胞様細胞から遺伝子バンクを作成し，レーザー非照射細胞から回収したmRNAを結合させて取り除き，レーザー照射によって発現促進した約50遺伝子クローンを単離している。DNA ホモロジー検索の結果から，細胞周期のライセンス因子[10]やATP合成酵素のF0F1-ATPase[11]などの遺伝子を同定することに成功している。

本研究では，新たにレーザー照射MC3T3E-1の差分化88遺伝子クローンについて塩基配列を解読した。その結果，特にホモロジーが高かった既知遺伝子として，mitochondria ribosomal protein L11, GDP-dissociation inhibitor, annexin Ⅲ, protein phosphatase, MSX-interacting-zinc finger protein, Fraconi anemia group G proteinなどが同定された。また，ホモロジーが極めて高いESTクローンが数個同定された。

ribosomal protein L11は，EF-Gの機能に関与することが報告されており，タンパク質合成過程で重要な働きをすると考えられている[12]。GDP-dissociation inhibitor, protein phosphatase, G proteinはいずれも情報伝達系において重要な役割を果たしている。Annxin Ⅲは，エナメル芽細胞や象牙芽細胞に見いだされており[13]，細胞カルシウムの調節に関与するといわれている[14]。MSX-2は，顎顔面の発育に関与することが知られているが[15]，MSX-interacting-zinc finger proteinは，MSX-2の応答性エレメントDNAへの結合に関与すると示唆されている[16]。今後，RT-PCR法やノーザンブロット法によってmRNAレベルが上昇しているかを確認し，これら遺伝子の転写の促進が，どのように骨芽細胞の増殖，分化に関与するかの研究を進める必要がある。

また，ESTクローンのなかには，ホモロジーが極めて高いEST遺伝子が多く同定されている。マウスのゲノムプロジェクトが進展しており，新規に同定され機能が判明する遺伝子の情報がデータベースに蓄積されていくことから，ホモロジー検索を続けることによって新規遺伝子の発見が期待できる。

結　論

骨芽細胞MC3T3E-1にGa-Al-As半導体レーザー（モデルPanalas-1000，波長 830nm）照射し，非照射細胞のmRNA を用いて差分化遺伝子ライブラリーを作成した。新たに88遺伝子クローンの塩基配列を解読し，DNAデータベースとホモロジー検索を行った。その結果，情報伝達系，タンパク合成系などに関与する遺伝子が同定された。これらの結果から，低出力レーザー照射は，これら遺伝子の転写促進により，骨芽細胞の増殖，分化に関与している可能性が示唆された。

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Stimulation of MCM3 Gene Expression in Osteoblast by Low Level Laser Irradiation

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Abstract. Biostimulatory effect of cell proliferation and bone formation by laser irradiation has been reported, however, very little is known about the molecular basis of mechanisms. We previously constructed the cDNA library of mouse osteoblastic cells (MC3T3-E1) which enhanced gene expression by laser irradiation using a subtracted gene cloning procedure. In the present study, we focused on a gene clone, designated as MCL-140, which exhibited the high homology of DNA sequence with mouse minichromosome maintenance (MCM) 3 gene. MCM3 is involved in the initiation of DNA replication as licensing factor in eukaryotic cells. Nucleotide sequence of MCL-140 insert was determined and assessed in the nucleic acid databases. The transcription level of MCL-140 was examined by Northern blot analysis. The DNA sequences of clone MCL-140 insert exhibited 96.2% homology with MCM 3 gene coding P1 protein. Higher MCM3 mRNA levels were observed in laser-irradiated cells compared to the levels in non-irradiated cells; furthermore, radiolabelled thymidine incorporation was increased by laser irradiation. These findings suggest that low-level laser irradiation may enhance DNA replication and play a role in stimulating proliferation of osteoblast through the enhancement of the MCM3 gene expression.

Keywords: DNA replication; Ga-Al-As diode laser; MCM3; Osteoblast; Proliferation

INTRODUCTION

Various biostimulatory effects of low-level laser irradiation have been reported that involve wound healing [1,2], chondral proliferation [3], collagen synthesis [4], anti-inflammation [4,5] and nerve regeneration [6]. In particular, the acceleration of bone regeneration by laser treatment has been investigated by in vivo study [7–11]. In vitro study, cell growth, DNA synthesis [12] and bone nodule formation [13] were significantly stimulated by low-level laser irradiation. However, the molecular basis mechanisms of laser effects on osteoblast cells are not fully elucidated and clinical laser therapy may not be efficiently applied. Therefore, it is important to clarify the mechanism of the biostimulatory effects of laser irradiation.

We investigated the effects of low-level laser irradiation at various cell culture stages of osteoblast cells and found cellular proliferation, bone nodule formation, alkaline phosphatase activity and osteocalcin gene expression were only enhanced at an early stage [14]. These findings suggest that laser irradiation may principally play two roles in cell proliferation of nodule-forming cells of osteoblast lineage and stimulation of cellular differentiation, especially to committed precursors, resulting in an increase in bone formation. However, the molecular bases of mechanisms leading to these findings have not yet been elucidated. To accomplish this, we previously constructed the cDNA library of MC3T3-E1, a clonal osteoblast cell line, which enhanced gene expression by low-level laser irradiation using a subtracted gene cloning procedure [15]. Among subtractive genes, we found a gene clone, designated as MCL-140, which exhibited the high homology DNA sequence with minichromase maintenance (MCM)3 gene. The replication of the whole genome could occur only once in each eukaryotic cell cycle. No genome DNA is re-replicated until passage through G1 phase into the next S-phase. MCM3 has been identified as a licensing factor involved in the initiation of replication [16].

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In the present study, to understand the mechanism of biostimulatory effect of cell proliferation by laser, we further characterised the MCL-140 clone by partial nucleotide sequencing. Furthermore, Northern blot analysis of MCM3 mRNA level and [^{3}H]thymidine incorporation in laser irradiated MC3T3-E1 cells were examined.

MATERIALS AND METHODS

Cell Culture

MC3T3-E1 cells, established from newborn mouse calvaria by Kodama et al. [17], were cultured in minimal essential medium (α-MEM; Gibco BRL, USA) containing 10% fetal calf serum and antibiotics comprising 100 μg/ml penicillin G (Sigma Chemical Co. USA) and 50 μg/ml gentamicin sulphate (Sigma) in multiwell plates. The cultures were kept at 37°C in a humidified incubator (Forma CO_2 incubator MIP-3326, Sanyo Electric Medica System Co., Tokyo, Japan) in the presence of 95% air and 5% CO_2. When the cells that grew out from the explants reached confluence, they were detached with 0.05% trypsin (Gibco) in phosphate-buffered saline (PBS) and subcultured in flasks.

Laser Irradiation

As the low-power laser source, a Ga-Al-As diode laser device (Model Panalas® 1000; Matsushima Industrial Equipment Inc., Osaka, Japan) was used. This laser apparatus has three beam elements gathered at the tip of the probe and expanded to the outside. Laser irradiation was performed so that the axis of the three beams met at right angles to the cell layer and the centre of the well. The technical specification of this laser device was as follows: wave length, 830 nm; output power, 100–700 mW, variable. Laser irradiation was performed at a distance of 550 mm (area of spot size: 78.5 cm^2) from the probe to the cell layer. Continuous wave at 500 mW output power for 20 min (power density: 7.64 J/cm^2) was used.

DNA Sequencing

Dideoxy-chain termination sequencing [18] was performed with fluorescent dye-labelled T7 universal primers (Aloka, Japan) and Sequi Therm™ Long-Read™ cycle sequencing kits for Li-Cor® Sequencing (Epicentre Technologies, USA). The reaction products were analysed by a 4000LS Long ReadIR™ DNA sequencing system (LI-Cor, USA).

Northern Blot Analysis

Total cellular RNA was extracted from MC3T3-E1 cells using acid guanidium thiocyanate–phenol–chloroform [19]. Guanidinum thiocyanate (4 M) containing 0.1 M 2-mercaptoethanol was added to the cells exposed or not to laser irradiation. The final RNA precipitate was stored in ethanol at −135°C. Total RNA samples obtained were denatured with 1 M glyoxal in 50% dimethyl sulphoxide (Sigma) in 10 mM phosphate buffer (pH 7.0) at 50°C for 60 min. The total RNA was then electrophoresed on 0.8% agarose gel containing 10 mM phosphate buffer (pH 7.0) and then transferred to Hybond-N+ nylon membranes (Amersham). The membranes were prehybridised for 2 h at 45°C in hybridisation buffer containing 50% formamide, 5 × saline–sodium phosphate–EDTA buffer (SSPE) (20 × SSPE=0.2 M sodium phosphate, 3.6 M NaCl, 0.02 M EDTA-Na_2 pH 7.7), 100 mg/ml sonicated salmon sperm DNA (Takara Shuzou Co. Ltd, Japan), 0.1% sodium dodecyl sulphate (SDS), and 5 × Denhardt's solution. Hybridisation proceeded overnight with 5′-[α-^{32}P]-dCTP (Amersham) for MCL-140 inserted DNA and glyceraldehydephosphate dehydrogenase (GAPDH). The membranes were then washed at 65°C in 2 × SSPE and 0.1% SDS (15 min × 2), at 65°C in 1 × SSPE and 0.1% SDS (30 min × 2), and at room temperature in 0.1 × SSPE and 0.1% SDS (15 min × 2), and they were then exposed to X-ray film with an intensifying screen at −20°C.

Assay of DNA Synthesis

DNA synthesis was determined by incorporation of [^{3}H]thymidine (47 Ci/mmol, Amersham). Following laser irradiation, the cells were further cultured for 6 h with [^{3}H]thymidine (0.5 μCi/ml). 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) tricitrate solution and subsequently with ethanol–ether (3:1, v/v). The cells were

```
                    10         20         30         40         50
MCL-140       1  AGGGATGG-A GCTGATGGTG AGACGATCAG GACTTGGTGG AAGCTTTGTG  50
P1 (mouse)    1  AGGGATGGAA GCTGATGGTG AGATGATCAG GACTTGGTG- AAGCTTTGTG  50

                    60         70         80         90        100
MCL-140      51  TATGACTTCT GCCTTGGGGC AAGAGCTGGA GGGAGGGCCA GGACTCAGGC 100
P1 (mouse)   51  TATGACTTCT GCCTTGGGGC AAGAGCTGGA GGGAGGGCCA GGACTCAGGC 100

                   110        120        130        140        150
MCL-140     101  AGAAGGCCTG CCTCACAAGC CCCAGTCCTA AACATGGGGA GTCTTCATGA 150
P1 (mouse)  101  AGAATGCCTG CCTCACAAG- CCCAATCCTA AACATGGGGA GTCTTCATGA 150

                   160        170        180        190        200
MCL-140     151  TTTGAGCTGT GTTTCTGGGC ACAGCTGTGT CCTGCGTTTG TTACTTGTGT 200
P1 (mouse)  151  TATGAGCTGT GTTTCTGGGC ACAGCTGTGT CCTGCG-TTG TTACTTGTGT 200

                   210        220        230        240        250
MCL-140     201  TTTTACCCCC ACCCCCAGGC ACTTTAGTCC AGGAAGCTTT ..........  250
P1 (mouse)  201  TTTTACCCCC A-CCCCAGGC ACTTTAGTCC AGGAAGCTTT ..........  250
```

Fig. 1. Homology search of the nucleotide sequences of the cDNA inserted in MCL-140. Sequence similarity was examined by using the GenBank and EMBL nucleic acid databases and compared with mouse MCM3. Square boxes indicate perfect match sequences.

dissolved in 0.5 M NaOH–10% SDS solution. The radioactivity was measured using a scintillation counter (Aloka, Japan).

RESULTS

Sequencing of cDNA Insert

Figure 1 shows DNA sequence data of inserted DNA from MCL-140. Square boxes indicate identical nucleotide. There is high homology (96.2%) with MCM3 gene encoding mouse P1 protein.

Northern Blot Hybridisation

To confirm whether the transcription level of the MCM3 gene was enhanced by low-level laser irradiation, the total RNA was isolated from both laser-irradiated and non-irradiated MC3T3-E1 cells, and examined mRNA level by Northern blot hybridisation using radiolabelled DNA insert in MCL-140.

As shown in Fig. 2, control housekeeping gene β-actin mRNA level in MC3T3-E1 cells showed a change with or without laser irradiation. It is apparent that the MCM3 gene expression was higher in laser-irradiated cells than in non-irradiated cells.

DNA Synthesis

The effect of laser irradiation on DNA replication in MC3T3-E1 cells was examined by

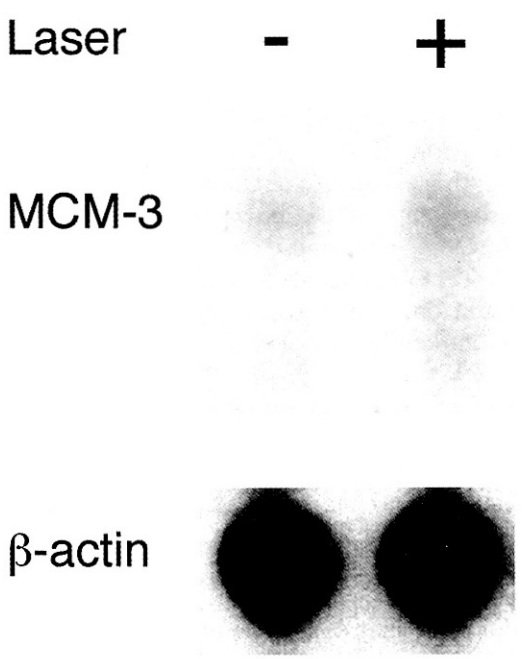

Fig. 2. Northern blot analysis of MCM3 gene expression. Each lane contained 4 μg of total RNA from MC3T3-E1 cells with or without laser-irradiation. The probes used were MCL-140 insert DNA, and β-actin gene was used as control.

incorporation rate of radiolabelled thymidine. Table 1 shows that [^{3}H]thymidine incorporation into MC3T3-E1 cells was markedly stimulated by laser irradiation.

DISCUSSION

The biostimulatory mechanisms of laser irradiation are not fully understood. As a result, this lack of knowledge may bring about a delay in the application of laser therapy in

Table 1. Effect of laser irradiation on DNA synthesis

	[^{3}H]Thymidine incorporation (dpm/10^5 cells)
Control	420.65 ± 123.95
Laser	2208.87 ± 269.95*

Values are mean ± SD for three cultures.
*Significant difference from non-irradiation control $p<0.001$.

clinical application. Therefore, it is important to clarify the mechanism of the biostimulatory effects of laser irradiation by molecular and cell biologically based studies in order to provide new approaches using laser therapy. Low-level laser irradiation has several biostimulatory effects, the principal of which is promotion of cell proliferation [20,21]. Several studies on osteoblasts showed that DNA synthesis was stimulated by low-power laser irradiation [12,22].

In this study, subtractive gene cloning strategy, one of the powerful genome science technologies, was used for the identification of genes that are expressed preferentially by laser irradiation. Here we report the results of this molecular approach, focusing on the characterisation of one of the novel genes identified by the subtraction. The nucleotide sequence of 240 bp DNA insert of MCL-140 had highly homology with mouse MCM3 gene (Fig. 1). Northern blot hybridisation analysis using radiolabelled DNA insert of MCL-140 confirmed that MCM3 gene was highly expressed in laser irradiated cells when compared with that of non-irradiated cells (Fig. 2). Furthermore, stimulation of DNA synthesis by laser irradiation was also confirmed by the thymidine incorporation experiment (Table 1). These findings strongly indicate that mouse MCM3 gene expression was enhanced by low-level laser irradiation.

The MCM gene family is conserved from yeast to mammals and consists of at least six members [23–27]. Six MCM proteins (MCM2–7) play a distinct role in DNA replication. This gene family is known as 'licensing factor' [28], and plays important roles in the once-per-cell-cycle DNA replication. Replication licensing factor ensures that eukaryotic chromosomal DNA is replicated exactly once in each cell cycle. On exit from metaphase, replication-licensing factor is activated and binds to or modifies chromatin. This modification is required for subsequent DNA replication; the license is also inactivated in the process of replication. Active replication licensing factor is not imported into the nucleus, so further DNA replication cannot occur until the DNA is relicensed by passage throughout mitosis. A number of proteins implicated in the licensing reaction have been identified including the MCM2–7 protein complex, one of which is a Xenopus homologue of the yeast MCM3 protein. Xenopus MCM3 associates with chromatin in G1 and is removed during replication, consistent with its being a component of the replication licensing factor system [29,30]. A soluble heterohexameric MCM complex appears to be made by association of two distinct subcomplexes: one containing MCM3 and MCM5 proteins that are in tight association with each other and another containing MCM4, -6 and -7 with which MCM2 is weakly associated [31].

These findings suggested that MCM proteins have an important role in the regulation of DNA replication during the S phase. Our previous study demonstrated that mRNA level of a MCM family member, MCM5 was enhanced by laser irradiation [32]. Furthermore, Karu et al. [33] 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. Thus, laser irradiation may play an important role in stimulating cell proliferation of osteoblasts through enhancement of MCM family gene expression. Since several MCM proteins make a large complex and play a role in the initiation of DNA replication, it would be of interest to study the effect of laser irradiation on the gene expression of other MCM family genes.

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Inhibition of Interleukin-1β Production and Gene Expression in Human Gingival Fibroblasts by Low-energy Laser Irradiation

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Abstract. Human gingival fibroblast (hGF) cells reside in gingival tissues which are challenged frequently by oral bacteria. Lipopolysaccharide (LPS) from periodontal pathogens can penetrate gingival tissues and stimulate the production of interleukin-1β (IL-1β), which has been implicated in inflammation and bone resorption. The anti-inflammatory effects of low-energy laser irradiation have been reported, but the mechanisms of this biostimulatory effect have not been fully elucidated. Primary cultured hGF cells were challenged with LPS isolated from *Campylobacter rectus*, a known periodontal disease-associated pathogen, and irradiated by a Ga-Al-As diode low-energy laser (830 nm, 3.95–7.90 J/cm^2). The hGF cells cultured medium showed a marked elevation of IL-1β production by LPS, which was significantly inhibited by laser irradiation in a dose-dependent manner. By reverse transcription-polymerase chain reaction (RT-PCR) analysis, this inhibitory effect was involved in the reduction of IL-1β mRNA levels but not that of the IL-1β converting enzyme.

Keywords: Ga-Al-As diode laser; Gene expression; Gingival fibroblast; Interleukin-1β; Lipopolysaccharide; Periodontal disease

INTRODUCTION

The main causative factor of tooth loss is periodontitis associated with bacterial infection, and one of the characteristics of this disease is alveolar bone loss. Interleukin (IL)-1 is a key mediator involved in a variety of activities in immune and acute-phase inflammatory responses [1], one of which is known to stimulate bone resorption [2]. Recently, high levels of IL-1β were identified in the gingiva [3] and crevicular fluid [4] of periodontitis patients, implicating this potent cytokine in the disease process. Using a cell culture system, Sismey-Durrant and Hopps [5] demonstrated that lipopolysaccharide (LPS) from periodontal disease-associated pathogens stimulated IL-1β production in human gingival fibroblast (hGF) cells.

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Among the many physiological effects of low-energy laser irradiation, anti-inflammatory functions have been reported. Honmura et al. [6] reported the therapeutic effect of Ga-Al-As laser irradiation on experimental carrageenin-induced inflammation. Histological evaluations of the effect of laser irradiation on rheumatoid arthritis have also been described [7]. Furthermore, Kana et al. [8] reported the enhancement of wound healing by laser irradiation using an in vivo experimental system. In an in vitro experimental system, we previously reported that laser irradiation caused the inhibition of prostaglandin E_2 (PGE_2) and IL-1β [9], as well as plasminogen activator production [10] in mechanically stressed human periodontal ligament cells. Recently, we found that Ga-Al-As diode laser irradiation reduced *Campylobacter rectus* LPS stimulated PGE_2 [11] and plasminogen activator [12] production in hGF cells.

Little is known regarding the biological mechanism of the anti-inflammatory effect of laser irradiation in oral diseases, which may cause a delay in the active application of laser therapy. It is known that IL-1β stimulates

PGE_2 [13] and plasminogen activator [14] production in hGF cells. In the present study, we attempted to clarify the effects of laser irradiation on IL-1β production and gene expression levels stimulated by *C. rectus* LPS in hGF cells. Since the IL-1β converting enzyme (ICE) is known to cleave the IL-1β precursor to mature IL-1β and because IL-1β is capable of extracellular secretion [15], the effect of laser irradiation on ICE gene expression was also examined.

MATERIALS AND METHODS

Bacterial Culture and LPS Preparation

C. rectus was grown in Todd–Hewitt broth supplemented with 0.5% yeast extract (BBL; Becton Dickinson Microbiology Systems, Cockeysville, MD, USA), 0.2% ammonium formate, and 0.3% sodium fumarate at pH 7. The bacterium was grown at 37°C in an anaerobic chamber containing 80% N_2, 10% H_2 and 10% CO_2. LPS was extracted and partially purified from *C. rectus* cells as previously described by Koga et al. [16].

hGF Cell Preparation and Culture

hGF cells were established from the cellular outgrowth of healthy papillary gingival tissue explants removed from patients undergoing routine dental surgery (hGF1, female, age 11; hGF2, male, age 10; hGF3, female, age 14; hGF4, male, age 12; hGF5, male, age 13) according to the method of Somerman et al. [17]. Informed consent was obtained from all patients before beginning the study. Gingival epithelium was removed, and the tissue was washed twice in phosphate-buffered saline (PBS). The tissue was dissected into approximately 1 mm cubes and transferred to 35 mm tissue-culture dishes containing α-MEM (Gibco, Grand Island, NY, USA) supplemented with 100 μg/ml penicillin G (Sigma Chemical Co, St Louis, MO, USA), 50 μg/ml gentamicin sulphate (Sigma), 0.3 μg/ml amphotericin B (Flow Laboratories, Mclean, VA, USA), and 10% fetal calf serum (FCS, Cell Culture Laboratories, Cleveland, OH, USA). The cultures were kept at 37°C in a humidified incubator (Forma CO_2 incubator MIP-3326, Sanyo Electric Medica System Co, Tokyo, Japan) in the presence of 95% air and 5% CO_2. When the cells that grew out from the explants reached confluence, they were detached with 0.05% trypsin (580 BAEE U mg^{-1}, Gibco) in PBS for 10 min and subcultured in flasks.

hGF cells were seeded at 5×10^3 per well in 24-well culture plates (well size: 1 cm diameter, 3.14 cm^2, Corning Glass Works, Corning, NY, USA) and cultured for 3 days until reaching confluence. The confluent-stage cells were incubated for 24 h in medium containing 2% FCS, after which they were treated with the LPS and incubated at 37°C, under an atmosphere of 5% CO_2 and 95% air.

Cell Counting

hGF cells were detached with 0.025% (w/v) trypsin (Gibco) containing 0.02% (w/v) EDTA, and the number of cells per well was counted with a Coulter Counter (Model 2M, Electronics Ltd, Luton, Beds, UK).

Laser Irradiation

A Ga-Al-As diode laser (Panalas-1000, Matsushita, Inc., Tokyo, Japan), which has a continuous wavelength of 830 nm and a maximum power output of 700 mW, was used in this study. The laser beam was delivered by an optical fibre 0.6 mm in diameter that had been expanded at the tip of the fibre to irradiate a circular area 130 mm in diameter at the cell-layer level. The power density in the central area (65 mm in diameter) of the irradiation circle was uniformly measured with a laser power meter. In this manner, four wells of each six-well plate were simultaneously and uniformly irradiated within the central area. The exposure time was 10 min, and the total energy expended corresponded to 7.90 J/cm^2 (19.4 J/well). After irradiation, the cells were washed twice with PBS and detached with trypsin (Gibco). The cell numbers per well were then counted with a Coulter Counter (Model ZM, Electronics).

Assay for IL-1β Production

The concentration of IL-1β was determined in duplicate with a ^{125}I-labelled IL-1β assay system, which used the anti-human IL-1β specific antibody (Amersham). This assay is based on the competition between unlabelled IL-1β and

Table 1. DNA primers used in PCR analysis

Target gene	Direction	Sequence	Amplified fragment size
IL-1β	Forward	5′-GAT CAC TGA ACT GCA CGC-3′	369 bp
	Reverse	5′-CAT CAG CAC CTC CAA GC-3′	
ICE	Forward	5′-ACA ACC CAG CTA TGC CCA CA-3′	815 bp
	Reverse	5′-TTC AGT GGT GGG CAT CTG CG-3′	
GAPDH	Forward	5′-ATC ACC ATC TTC CAG GAG-3′	318 bp
	Reverse	5′-ATG GAC TGT GGT CAT GAG-3′	

a fixed quantity of ^{125}I-labelled IL-1β for a limited number of binding sites on IL-1β-specific antibodies. The test sample was mixed in a tube with antiserum and incubated for 4 h at room temperature. ^{125}I-labelled IL-1β was then added into the tube and the mixture was incubated for 24 h at 4°C. After a 10 min incubation with the secondary antibody reagent, the antibody-bound fraction was separated by magnetic separation. The radioactivity in each tube was then determined with a gamma scintillation counter (Aloka).

RNA Preparation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated by an acid guanidinium thiocyanate–phenol–chloroform extraction method [18]. The final RNA precipitate was stored in ethanol at −135°C. cDNA synthesis and amplification by RT-PCR was carried out using a GeneAmp RNA kit (Perkin-Elmer, New Jersey, USA). Briefly, cDNA synthesis was carried out at 42°C for 15 min in a final volume of 20 µl containing 4 µl of $MgCl_2$ solution (25 mM), 2 µl of 10 × PCR buffer II (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 2 µl of dNTP (10 mM each), 1 µl of RNase inhibitor (20 U/ml), 1 µl of MuLV reverse transcriptase (25 U/ml), 1 µl of random hexamer (25 mM), 1 µl of oligo d(T)16 (25 mM), and 2 µl of total RNA (1 mg/ml). The PCR mixture contained 20 µl of the cDNA solution, 4 µl of 25 mM $MgCl_2$, 8 µl of 10 × buffer II, 1 µl of forward and reverse primers, 65.5 µl of distilled H_2O, and 0.5 µl of AmpliTaq DNA polymerase. The mixture was subjected to amplification using a GeneAmp PCR system 9600 (Perkin-Elmer) set at 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min, for 27–33 cycles.

The primers for PCR amplification were designed by referring to the sequences of cDNA reported for IL-1β [19], ICE [15], and the internal standard housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [20], as shown in Table 1. PCR-amplified DNA fragments were analysed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

Statistical Methods

Values were calculated as the mean ± standard deviation (SD) from six cultures. Where comparisons were made between two groups, Student's t-test was used. The data were subjected to an one-way analysis of variance (ANOVA). Differences were considered as significant when $p<0.05$.

RESULTS

The effects of LPS on IL-1β production and different irradiation doses on IL-1β production in hGF cells were examined using hGF cells from hGF1. As shown in Fig. 1, hGF cells without LPS challenge constitutively synthesised some IL-1β, whereas hGF1 with the LPS treatment showed a significant increase in IL-1β production ($p<0.001$). LPS-challenged hGF cells were laser-irradiated for 3, 6, 10 or 20 min. Irradiation for 3 min inhibited PA activity ($p<0.01$) and irradiation for more than 6 min significantly inhibited IL-1β production ($p<0.001$) when compared to the non-irradiation group. Irradiation for 20 min reduced IL-1β production to the control level. The inhibitory effect was dependent on laser dose as determined by one-way ANOVA ($p<0.01$).

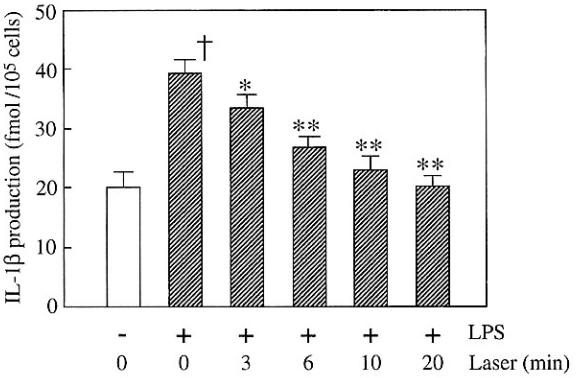

Fig. 1. Effects of laser irradiation time on the inhibition of IL-1β production. Values are mean±SD for six cell cultures. Significantly different from corresponding without LPS treatment (†$p<0.001$), non-laser irradiation as control (*$p<0.01$, **$p<0.001$). Laser irradiaion inhibited LPS-stimulated IL-1β production in a laser irradiated time dependent manner (one way ANOVA, $p<0.01$).

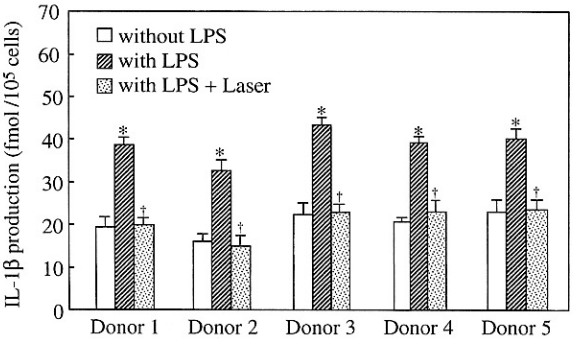

Fig. 2. Reproducibility of IL-1β production and inhibition by laser irradiation. Five different hGF cells from different individual donors (hGF 1–5) were described in Materials and Methods. Values are mean±SD for six cell cultures. Significantly different from corresponding without LPS treatment (*$p<0.01$), non-laser irradiation as control (†$p<0.01$).

We examined the inhibitory effect of laser irradiation on LPS-stimulated IL-1β production using only one hGF sample (hGF1), as shown in Fig. 1. Next, we attempted to confirm the reproducibility in other hGF cells from different donors. Figure 2 shows that a 10-min laser irradiation inhibited LPS-stimulated IL-1β production in the five hGF samples (hGF1–hGF5), which were obtained from five different donors.

Since ICE is required for IL-1β processing, to elucidate the mechanism by which IL-1β production is altered by laser irradiation, we examined the effect of laser irradiation on IL-1β and ICE mRNA levels in LPS-challenged hGF cells by RT-PCR analysis. Figure 3 represents the results from at least two similar experiments using two different hGF cells. Neither LPS challenge nor laser irradiation to

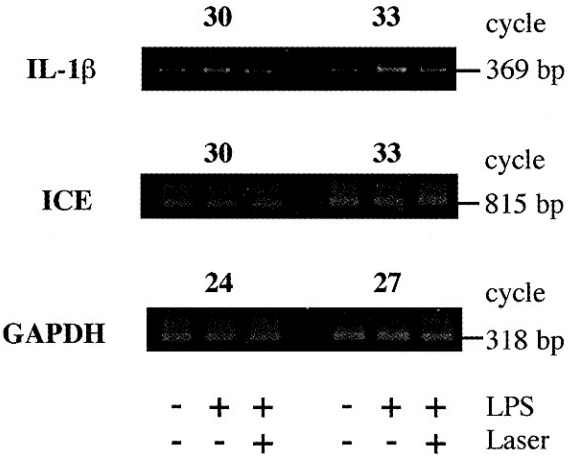

Fig. 3. Ethidium bromide staining pattern of simultaneously amplified PCR products on agarose-gel electrophoresis. Total RNA was extracted from each cell culture, and 2 μg of total RNA was reverse transcripted and the cDNA obtained was amplified.

the cells affected the bands for ICE or the internal standard GAPDH. The DNA bands corresponding to IL-1β mRNA were visible, and the DNA bands of the LPS-challenged cells were more intense than those for the corresponding controls. Further, the IL-1β DNA band was significantly reduced in the laser-irradiated hGF cells.

DISCUSSION

The biostimulatory mechanisms of laser irradiation are not fully understood. As a result, this lack of knowledge may bring about a delay in the application of laser therapy in clinical application. Therefore, it is important to clarify the mechanism of the biostimulatory effects of laser irradiation by molecular and cell biologically based studies in order to provide new approaches using laser therapy.

In the present study using a cell culture system, low-level laser irradiation significantly inhibited LPS stimulated IL-1β production in hGF cells. This inhibitory effect was dependent on irradiation time, with an almost complete inhibition seen for 20-min. RT-PCR results demonstrated that IL-1β mRNA was constitutively expressed in hGF cells and further enhanced the gene expression level in response to LPS, whereas ICE mRNA was unchanged in hGF cells with or without LPS. Although ICE is known to cleave the IL-1β precursor to mature IL-1β, the reduction of in IL-1β production by laser irradiation was not responsible

for the augmentation of ICE mRNA, though it was for that of IL-1β mRNA.

The anti-inflammatory effects of low-level laser irradiation in in vivo experimental models have been previously reported. Honmura et al. [6] demonstrated that Ga-Al-As diode laser irradiation, as used in the present study, inhibited carrageenin-induced inflammation in rats. Furthermore, low-level laser therapy is widely used clinically for rheumatoid arthritis with satisfactory results [6,7]. These findings suggest that the inhibitory effect of low-energy laser irradiation on IL-1β production presented in the present study can be considered as an anti-inflammatory action reflecting an in vivo event.

We recently reported that laser irradiation reduced LPS-stimulated PGE_2 [11] production through an inducible reduction of cyclooxygenase-2, which is an inducible and key enzyme for PGE_2 synthesis [21]. It has also been shown that the plasminogen activator has important roles in metalloprotease activation [22] and kinin production [23]. Laser irradiation inhibited the production of plasminogen activator in hGF cells through a reduction of the transcription level event. IL-1β induces signalling pathways, which lead to a transient expression of the transcription factors of inflammatory mediators. Matrix metalloproteinases have been detected in crevicular fluids and inflamed gingival tissues along with IL-1β, and it is believed that IL-1β regulates the transcription of metalloproteinases [24]. These findings suggest that the ability of laser irradiation to reduce the production of mediators in inflammatory and tissue destruction events contributes to the inhibition of IL-1β expression.

In conclusion, low-energy laser irradiation significantly inhibited IL-1β production that was stimulated by LPS in hGF cells through a reduction of IL-1β gene expression. Our results suggest that low-level laser irradiation may be of therapeutic benefit against the aggravation of gingivitis and periodontitis that accompanies bacterial infection.

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Inhibitory effect of low-level laser irradiation on LPS-stimulated prostaglandin E_2 production and cyclooxygenase-2 in human gingival fibroblasts

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It has been reported that lipopolysaccharide (LPS) from periodontal pathogens can penetrate gingival tissues and stimulate the production of prostaglandin E_2 (PGE_2), which is known as a potent stimulator of inflammation and bone resorption. Although biostimulatory effects of low-level laser irradiation such as anti-inflammatory results have been reported, the physiological mechanism is not yet clarified. The purpose of the present study was to determine the effect of laser irradiation on PGE_2 production and cyclooxygenase (COX)-1 and COX-2 gene expression in LPS-challenged human gingival fibroblast (hGF) cells *in vitro*. hGF cells were prepared from healthy gingival tissues and challenged with LPS, and Ga-Al-As diode laser was irradiated to the hGF cells. The amount of PGE_2 released in the culture medium was measured by radioimmunoassay, and mRNA levels were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Irradiation with Ga-Al-As diode low-level laser significantly inhibited PGE_2 production in a dose-dependent manner, which led to a reduction of COX-2 mRNA levels. In conclusion, low-level laser irradiation inhibited PGE_2 by LPS in hGF cells through a reduction of COX-2 mRNA level. The findings suggest that low-level laser irradiation may be of therapeutic benefit against the aggravation of gingivitis and periodontitis by bacterial infection.

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There is a possibility that prostaglandins (PGs) play a role in bone metabolism (1) and inflammation (2). Klein & Raisz (3) found that PGE_2 is a potent stimulator of bone resorption. The relationship between PGE_2 and the progression of periodontal diseases has been intensively studied. PGE_2 concentration increases in the periodontal connective tissues of adult periodontitis lesions (4–7). Further, PGE_2 levels in the crevicular fluid can serve as a static assessment of ongoing disease activity (8, 9).

The bacterial products themselves as well as the by-products of the host defense system both contribute to alveolar bone loss. Gram-negative species possess lipopolysaccharide (LPS) as a cell wall constituent, which is generally believed to be one of the virulence factors. In fact, bacterial LPS possesses significant bone resorptive-activity in the pathogenesis of several forms of periodontitis (10). Early studies indicated that LPS can penetrate gingival tissues (11, 12). Moreover, the LPS levels in gingival crevicular fluid have been positively correlated with clinical and histologic signs of gingival inflammation (13, 14). Unstimulated human gingival fibroblasts (hGF) were found to produce low levels of PGE_2 (15), while LPS from periodontal pathogens stimulated PGE_2 production

from hGF (16). *Campylobacter rectus* (formerly *Wolinella recta*) is a Gram-negative, strictly anaerobic rod that is associated with adult periodontitis (17), and it was demonstrated to be the most potent enhancer of PGE_2 production by hGF (18).

Among the many physiological effects of low-level laser irradiation, anti-inflammation and stimulation of wound healing have been reported (19). However, little is known about the biological mechanism of the anti-inflammatory effect of laser irradiation in oral diseases. This lack of knowledge may cause a delay in the active application of laser therapy. Therefore, the purpose of the present study was to examine the effects of low-level laser irradiation on the PGE_2 production from *C. rectus* LPS-challenged hGF *in vitro*.

Cyclooxygenase (COX) is known as a key enzyme of PGE_2 production (20). Two forms of COX exist, a constitutively expressed COX-1 (21) and an inducible COX-2 (22). COX-2 is induced by lipopolysaccaride (23) and cytokine (24). The effectiveness of cyclooxygenase-2 inhibitors in the treatment of rheumatoid arthritis has been reported (25). We also reported that selective COX-2 inhibitors, NS-398, inhibited the PGE_2 production from periodontal ligament cells by tension force (26). Since the prevention of COX-2 would provide a rationale for the effectiveness of laser irradiation on the reduction of PGE_2 production, effect of laser irradiation on COX-2 gene expression was also examined.

Material and methods

Bacterial culture and LPS preparation

C. rectus ATCC 33238 was grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (BBL; Becton Dickinson Microbiology Systems, Cockeysville, MD, USA), 0.2% ammonium formate, and 0.3% sodium fumarate at pH 7.6. The bacterium was grown at 37°C in an anaerobic chamber containing 80% N_2, 10% H_2, and 10% CO_2. LPS was extracted and partially purified from *C. rectus* as described by KOGA *et al.* (27).

hGF separation and cell culture

hGF cells were established by cellular outgrowth from explants of healthy papillary gingival tissues removed from patients aged 10–12 yr who were undergoing routine dental surgery according to the procedure described by SOMERMAN *et al.* (28). Informed consent was obtained from all patients. The gingival epithelium was removed with a surgical scalpel, and the tissue was washed twice in phosphate-buffered saline (PBS). It was dissected into approximately 2- to 3-mm cubes and transferred to 35-mm tissue-culture dishes containing α-MEM (Gibco, Grand Island, NY, USA) supplemented with 100 μg/ml penicillin G (Sigma, St. Louis, MO, USA), 50 μg/ml gentamicin sulphate (Sigma), 0.3 μg/ml amphotericin B (Flow Laboratories, Mclean, VA, USA), and 10% fetal calf serum (FCS; Cell Culture Laboratories, Cleveland, OH, USA). The cultures were kept at 37°C in a humidified incubator (Forma CO_2 incubator MIP-3326; Sanyo Electric Medica System, Tokyo, Japan) in the presence of 95% air and 5% CO_2. When the cells that had grown out from the explants reached confluence, they were detached with 0.05% trypsin (580 BAEE U/mg, Gibco) in PBS and then subcultured in flasks. We prepared hGF cells from 5 donors [A (cell A), age 12, male; B (cell B), age 10, male; C (cell C), age 11, female; D (cell D), age 11, male; E (cell E), age 12, female].

hGFs were seeded at 5×10^3 per well in 24-well culture plates (Corning Glass Works, Corning, NY, USA) and cultured for 3 d until reaching confluence. The confluent stage cells were incubated for 24 h in medium containing 2% FCS, followed by treatment with the LPS (1.0 μg/ml), and then incubated at 37°C for 8 h in an atmosphere of 5% CO_2 and 95% air.

Laser irradiation

A Ga-Al-As diode laser (model Panalas-1000; Matsushita, Tokyo, Japan), which has a continuous wavelength of 830 nm and a maximum power output of 700 mW, was used in this study. The laser beam was delivered by an optical fiber 0.6 mm in diameter that was expanded at the tip of the fiber and irradiated a circular area 130 mm in diameter at the cell layer level. The power density of the laser beam in the center area (65 mm in diameter) of the irradiation circle was uniform as measured by a laser power meter. Laser irradiation was applied just after the addition of LPS in the culture medium. In this manner, 6 wells out of 24 were simultaneously and uniformly irradiated within the center area of the 24-well plates. The time of exposure was 3–20 min. The total energy corresponding to 3–20 min exposures varied from 0.95–6.32 J/cm^2 (1.9–12.6 J/well).

Assay of PGE_2

The amount of PGE_2 released from the hGF cells into the culture medium was measured by radioimmunoassay, using a commercially available kit containing [^{125}I]-PGE_2 as a tracer (Amersham, Arlington Heights, IL, USA). Radioactivity levels were determined with a gamma-well counter

(Aloka, Tokyo, Japan). The amount of PGE_2 production in the absence of LPS was subtracted from that in the presence of LPS to give the amount of LPS-stimulated PGE_2 production.

Cell number

hGF cells were detached with 0.025% (w/v) trypsin (Gibco) containing 0.02% (w/v) EDTA, and the number of cells per well was counted with a coulter counter (Model 2M; Electronics, Luton, Beds, UK).

RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The total RNA of hGF cells subjected to LPS challenge with or without laser irradiation was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (29). The final RNA precipitate was stored in ethanol at −135°C. cDNA synthesis and amplification by RT-PCR were carried out using a GeneAmp RNA kit (Perkin-Elmer, NJ, USA). The PCR mixture was subjected to amplification using a Gene Amp PCR system 9600 (Perkin-Elmer) set at 94°C for 1 min, 55°C for 2 min and 72°C for 2 min.

The primers for PCR amplification were designed referring to the sequences of cDNA reported for COX-1 (30), COX-2 (31) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (32). The primers designed are shown in Table 1. PCR fragments were resolved by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. To evaluate saturated or incomplete reaction of PCR, three gene fragments were amplified simultaneously every 3 cycles from the 21st to 33rd cycle.

Statistical methods

Values were calculated as the mean ± standard deviation (SD) from 6 cultures. Where comparisons were made between two groups, Student's *t*-test was used. The data were subjected to a one-way analysis of variance (ANOVA). Differences were accepted as significant when $P<0.05$.

Results

The production of PGE_2 from hGF A cells in response to LPS and the inhibitory effects of laser irradiation are shown in Fig. 1. Control hGF cells constitutively synthesized a low amount of PGE_2, while LPS significantly increased PGE_2 production. Laser irradiation significantly inhibited PGE_2 production in a dose-dependent manner; 10 min of irradiation inhibited 90% of the LPS-stimulated PGE_2 production, and 20 min of irradiation reduced PGE_2 production to control level.

Since we examined the inhibitory effect of laser irradiation on LPS-stimulated PGE_2 production using only one hGF cell sample (cell A), as shown in Fig. 1, we attempted to confirm the reproducibility in other hGF cells from different donors. As shown in Fig. 2, a 10-min laser irradiation inhibited

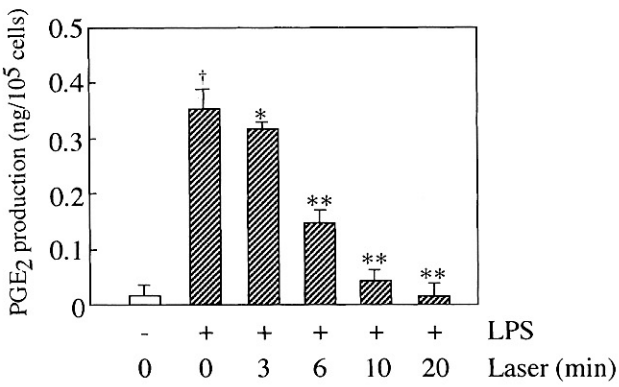

Fig. 1. Effects of LPS and laser irradiation time on PGE_2 production in hGF cells. hGF from donor A was challenged by 1.0 μg/ml LPS for 8 h with laser irradiation for different time. Values are mean ± SD for 6 cultures. Significantly different from corresponding without LPS challenge (†$P<0.001$), non-laser irradiation as control (*$P<0.05$, **$P<0.001$). Laser irradiation inhibited LPS-stimulated PGE_2 production in a laser-irradiated time-dependent manner (one-way ANOVA, $P<0.01$).

Table 1

DNA primers used in PCR analysis

Target gene	Direction	Sequence	Amplified fragment size
COX-1	Forward	5′-TGC CCA GCT CCT GGC CCG CCG CTT-3′	303 bp
	Reverse	5′-GTG CAT CAA CAC AGG CGC CTC TTC-3′	
COX-2	Forward	5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′	305 bp
	Reverse	5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′	
GAPDH	Forward	5′-ATC ACC ATC TTC CAG GAG-3′	318 bp
	Reverse	5′-ATG GAC TGT GGT CAT GAG-3′	

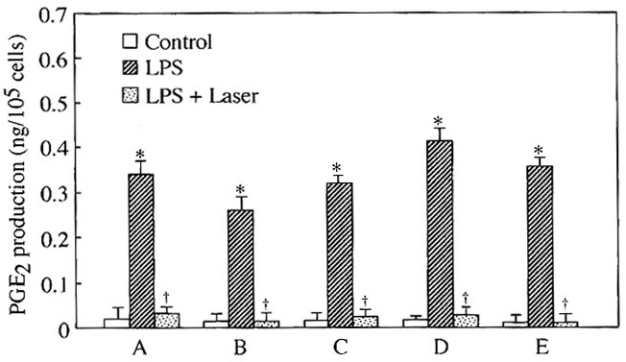

Fig. 2. Reproducibility of the inhibitory effect of PGE_2 production by laser irradiation in hGF cells . Five different hGF samples from individual donors (A–E) were challenged by 1.0 μg/ml LPS for 8 h with laser irradiation (10 min). Values are mean ± SD for 6 cultures. Significantly different from corresponding without LPS challenge (*$P<0.001$) and non-laser irradiation as control (†$P<0.001$).

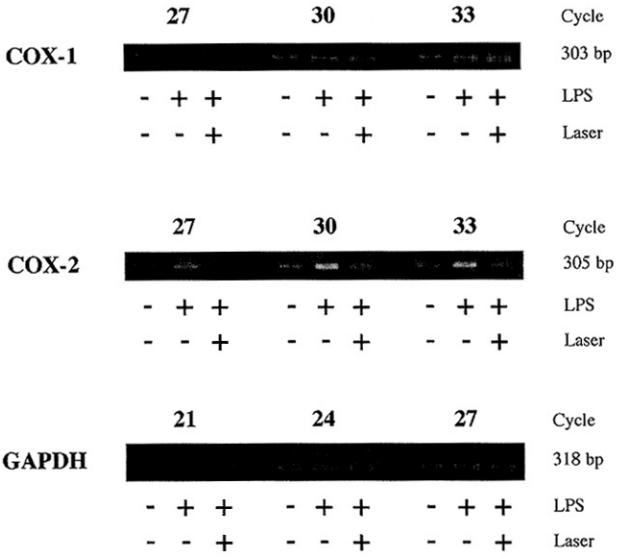

Fig. 3. Semiquantitative analysis of COX-1, COX-2 and GAPDH mRNA levels by RT-PCR. hGF from donor A was challenged by 1.0 μg/ml LPS for 8 h with laser irradiation (10 min). Total RNA was extracted from each cultured cells, and 2 μg of total RNA was reverse transcripted and cDNA obtained was amplified by PCR. Each mRNA to obtain DNA fragments of the size indicated as bp.

LPS-stimulated PGE_2 production in all five hGF cell samples obtained from 5 different donors.

To elucidate the mechanism by which PGE_2 production is altered by laser irradiation, COX-1 and COX-2 mRNA levels in both the control and laser-irradiated cells were examined by RT-PCR analysis. As shown in Fig. 3, each DNA band of the three gene fragments was amplified simultaneously every 3 cycles, constantly increasing as the number of cycles increased, suggesting that the PCR reactions were neither saturated nor incomplete. The figures represent the results from at least two similar experiments from two different hGF cells. Neither LPS challenge nor laser irradiation to the cells affected the bands for COX-1 or the internal standard GAPDH. The DNA bands corresponding to COX-2 mRNA were visible, and the bands of the LPS-challenged hGF cells were clearly more intense than those for the corresponding controls. The COX-2 band was significantly reduced in the hGF cells that had been laser irradiated.

Discussion

In the present study, low-level laser irradiation significantly inhibited the increased PGE_2 production by LPS in hGF cells. The inhibitory effects on PGE_2 production were laser irradiation time dependent with almost complete inhibition found after the 10-min irradiation.

COX is the rate-limiting enzyme which catalyzes the conversion of arachidonic acid to PG endoperoxide and is involved in PGE_2 production. Recent studies have indicated that at least two forms of COX exist, a constitutively expressed COX-1 (21) and an inducible COX-2 (22). Noguchi *et al.* (33) reported that LPS from a periodontopathogenic bacterium, *Porphyromonas gingivalis*, stimulated PGE_2 production through induction of COX-2 gene expression. We previously demonstrated that laser irradiation significantly inhibited PGE_2 production from human periodontal ligament derived cells subjected to mechanical stress (34), however the effect of laser irradiation on COX gene expression was not defined. Therefore, we examined the COX-1 and COX-2 mRNA levels in laser irradiated hGF cells challenged by LPS by RT-PCR. Results showed that the COX-2 mRNA levels in hGF cells were increased by LPS and reduced by laser irradiation, COX-1 mRNA remained unchanged. These results suggest that laser irradiation may enhance gene expression of or stabilize mRNA level of COX-2.

The anti-inflammatory effects of low-level laser irradiation in an *in vivo* experimental model have been previously reported. Honmura *et al.* (35, 36) demonstrated that a Ga-Al-As diode laser, the same as in the present study, inhibitied carrageenin-induced inflammation in rats. Furthermore, low-level laser therapy is widely used clinically for rheumatoid arthritis (37), and PGE_2 in synovial fluid of a patient was found to be decreased after laser treatment (38). These findings suggest that the inhibitory effects of low-energy laser irradiation on PGE_2 production presented here can be considered as an anti-inflammatory action reflecting an *in vivo* event.

In conclusion, low-energy laser irradiation significantly inhibited PGE_2 production that was stimulated by LPS in hGF cells through a reduction of COX-2 gene expression. These results suggest that low-level laser irradiation may be of therapeutic benefit against the aggravation of gingivitis and periodontitis that accompanies the bacterial infection.

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学位論文要旨（博士・歯学）

Effect of Low-Level Laser Irradiation on F0F1-ATPase and COX-2 Gene Expressions

(F0F1-ATPase およびCOX-2 遺伝子発現への低出力レーザー照射の影響)

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要旨：低出力レーザー照射に創傷治癒作用、骨折治癒促進作用、炎症抑制作用、などの生物学的効果があると報告されている。しかしながら、その作用機序は未だ不明な点が多い。レーザー治療をさらに推進するためにはレーザー照射の生物学的効果の機序を解明する必要があり、すでに、レーザー照射によって骨芽細胞での遺伝子発現が促進する遺伝子ライブラリーを構築している。遺伝子ライブラリー中の遺伝子クローンの一つであるF0F1-ATPase subunit-b の遺伝子発現とATP 産生に与えるレーザー照射の影響について検討した。

一方、低出力レーザー照射が歯周病の歯肉炎症を軽減することが報告されているが、その作用機序については明らかになっていない。Prostaglandin E2 (PGE2)は、骨吸収促進因子、炎症のメディエーターとして知られており、歯周病患者の歯肉浸出液中のPGE2 量が健常者のものに比べて有意に増大していること、歯周病治療によって歯肉浸出液中のPGE2 が低下することから歯周病の発症・進展にPGE2 が深く関与していることが示唆されている。細菌の内毒素であるリポ多糖 (lipopolysaccharide; LPS) は、起炎物質、骨吸収因子として歯周病の病原因子となる。すでに、成人性歯周炎の関連菌であると考えられている *Campylobacter rectus* のLPS が、ヒト歯肉由来線維芽細胞のPGE2 産生を促進することが報告されている。そこで本研究では、歯肉由来線維芽細胞に *C. rectus* のLPS を作用させ、低出力レーザー照射によるPGE2 産生の抑制に与える影響を検討した。その結果、以下の知見を得た。

1. マウス骨芽細胞株MC3T3-E1 を用いてレーザー照射によって発現が促進する遺伝子の挿入cDNA の塩基配列はラットのF0F1-ATPase subunit-b 遺伝子と94.9%のホモロジーを有していた。また、Nothern-blot 分析の結果から、mRNA レベルがレーザー照射により増大すること、レーザー照射によりMC3T3-E1 細胞内のATP 量が増大することが判明した。
2. ヒト歯肉由来線維芽細胞に歯周病原菌 *C. rectus* LPS を作用させたところ、PGE2 産生が増大した。
3. ヒト歯肉由来線維芽細胞に歯周病原菌 *C. rectus* LPS を作用させ、レーザー照射したところ、レーザー照射時間に依存してPGE2 産生量が減少した。
4. レーザー照射が遺伝子発現に与える影響を検討した結果、LPS で増大したCOX-2 mRNA レベルがレーザー照射によって減少した。

以上の結果から、レーザー照射の骨形成促進作用、抗炎症作用、創傷治癒作用などの生物学的効果の機序の解明の一助として、レーザー照射は、骨芽細胞のATP 産生能をF0F1-ATPase subunit-b 遺伝子発現の増大を介して促進すること、ヒト歯肉由来線維芽細胞のPGE2 産生能をCOX-2 遺伝子発現の抑制を介して減少させることが明らかとなった。

主論文：Inhibitory effect of low-level laser irradiation on LPS-stimulated prostaglandin E2 production and cyclooxygenase-2 in human gingival fibroblasts. Eur J Oral Sci, 108:29-34, 2000.

副論文：Low level laser irradiation enhances expression of FoF1-ATPase subunit-b gene in osteoblastic cells. *Laser Therapy,* 10: 107-116, 1998.

Effect of hydroxy radical treated fibronectin and laser irradiation on gene expressions in osteoblast

（骨芽細胞の遺伝子発現に与えるヒドロキシラジカル処理フィブロネクチンとレーザー照射の影響）

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要旨：　骨形成機序の解明に向けて、骨芽細胞の情報伝達系、遺伝子発現レベルでの研究が進展している。骨組織の機能発現には骨芽細胞の細胞外マトリックスを介する情報伝達系が関与することが明らかにされ、マトリックス タンパク質であるフィブロネクチンが骨芽細胞の石灰化能に重要な役割を果たしていることが知られている。当教室では、骨芽細胞の石灰化能が老化によって低下する機序の解明の一助として、骨芽細胞をヒドロキシラジカル処理したフィブロネクチン上で培養することを試み、骨結節の形成が著明に抑制されることを見い出している。そこでヒドロキシラジカル処理したフィブロネクチン細胞基質が骨芽細胞の遺伝子発現に与える影響について検索した。

一方、低出力レーザー照射は抗炎症作用や細胞増殖の促進効果など様々な生物学的効果を有することが知られている。特に骨組織においては、骨芽細胞の分化、増殖の促進、骨形成の促進などが注目されている。しかしながら、その作用機序には未だ不明な点が多い。そこで、レーザー照射が骨芽細胞に与える影響を生化学的、分子生物学的手法を用いて解明することを目的として、当教室ではすでにレーザー照射によって骨芽細胞で遺伝子発現が促進される遺伝子の単離、解析を試みている。低出力レーザー (Ga-Al-As diode laser) 照射したマウス骨芽細胞様細胞株(MC3T3-E1)の cDNA ライブラリーを作製し、さらにレーザー照射によって発現が促進した遺伝子クローンをレーザー非照射 mRNA とのサブトラクション法によって単離することを試みた。遺伝子クローンから精製したインサート cDNA の塩基配列の一部を解読した結果、マウス MCM3 遺伝子と高い相同性をもつクローン(MCL-140)が得られた。本研究では MCL-140 クローンについて塩基配列解析、ノーザンブロット分析を行い、さらにレーザー照射の DNA 合成に与える影響について検討し、以下の結果を得た。

1. フィブロネクチンのヒドロキシラジカル処理によって骨芽細胞様細胞 MC3T3-E1 のアルカリホスファターゼ、I 型コラーゲン遺伝子の mRNA レベルが対照群に比べて低下した。
2. レーザー照射 MC3T3-E1 のサブトラクション法ライブラリーから得た MCL-140 クローン cDNA インサートの塩基配列を解読し、ホモロジー検索を行ったところ、既知のマウス MCM3 遺伝子と塩基配列で 99.6%の相同性を認めた。
3. レーザー照射および非照射の MC3T3-E1 細胞から RNA 画分を調製し、cDNA インサートお放射性 DNA プローブを用いてノーザンブロット分析した結果、レーザー照射群で照射後 6 時間後にレーザー非照射群に比較して mRNA レベルの増大が認められた。
4. レーザー照射後 MC3T3-E1 細胞における DNA 合成能を放射性チミジンの取り込み量により検討した結果、レーザー非照射群に比較して取り込み量の増加を認めた。

MCM3 遺伝子は、真核細胞の細胞分裂時に DNA 複製が細胞周期の S 期にただ一度だけ起こることを保証する正の制御因子、いわゆるライセンス因子の一員として注目され、真核細胞の DNA 複製に必須であることが明らかになっている。本研究の結果から、レーザー照射は、MCM3 遺伝子の遺伝子発現を促進することで細胞周期を進行させ、細胞増殖に関与することが示唆された。

主論文：Stimulation of MCM3 gene expression in　osteoblast by low level laser irradiation. *Lasers Med Sci*, 16:

213-217, 2001.
参考論文：H_2O_2-derived free radicals treated fibronectin substratum reduces the bone nodule formation of rat calvarial osteoblast. *Mech Ageing Dev*, 98: 113-125, 1997.

Subtractive Gene Cloning from Osteoblast and Effect of IL-1β Production in Gingival Fibroblasts by Laser Irradiation

(レーザー照射による骨芽細胞の差分化遺伝子クローニングと歯肉由来線維芽細胞のIL-1β産生への影響)

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要旨：低出力レーザー照射が創傷や難治性潰瘍に治癒効果があると言及されて以来、炎症抑制、疼痛減少、創傷治癒促進、骨折治癒促進など広範な臨床効果が報告されている。しかしながら一方では、レーザー照射の効果について心理的なプラセボー効果が大きいであろうとする意見や、生物学的効果は温度上昇効果による単なる血液循環の促進効果であろうとする議論もされている。このような背景からレーザー照射の生物学的効果の作用機序の解明は不十分であり、歯科医学領域にも積極的な応用が期待されているなかで、レーザー医療をさらに推進するためには、有用性の高いレーザー照射の機種、照射法を開発するとともに、生物学的効果を実証科学的に解明していく必要があると思われる。

本研究では、レーザー照射の生物学的効果を実証科学的に証明し、そのメカニズムを解明するために、レーザー照射によって骨芽細胞での遺伝子発現が促進する遺伝子を探索するために差分化遺伝子ライブラリーを構築した。その結果、ミトコンドリアタンパク質、ライセンシング因子、転写因子などの遺伝子クローンがえられた。

一方、低出力レーザー照射が歯周病の歯肉炎症を軽減することが報告されているが、その作用機序については明らかになっていない。IL-1βは、骨吸収促進因子、炎症のメディエーターとして知られており、歯周病患者の歯肉浸出液中のIL-1β量が健常者に比べて有意に増大していることから歯周病の発症・進展に深く関与していることが示唆されている。細菌の内毒素であるリポ多糖 (lipopolysaccharide; LPS) は、起炎物質、骨吸収因子として歯周病の病原因子となる。すでに、成人性歯周炎の関連菌 *Campylobacter rectus* のLPSが、ヒト歯肉由来線維芽細胞のIL-1β産生を促進することが報告されている。そこで本研究では、歯肉由来線維芽細胞に *C. rectus* のLPSを作用させ、低出力レーザー照射によるIL-1β産生の抑制に与える影響を検討した。その結果、*C. rectus* LPSを作用させたヒト歯肉由来線維芽細胞の培養液中IL-1β量 が増大した。そして、レーザー照射時間に依存してこのIL-1β量が減少した。さらに、ヒト歯肉由来線維芽細胞のレーザー照射によるLPS依存性IL-1β産生量の再現性を検討したところ5人のドナーから得られた歯肉由来線維芽細胞で同様の結果が見られた。また、レーザー照射が遺伝子発現に与える影響を検討した結果、LPSで増大したIL-1β mRNAレベルがレーザー照射によって減少した。

以上の結果から、レーザー照射の骨形成促進作用、抗炎症作用、創傷治癒作用などの生物学的効果の機序として、レーザー照射は、骨芽細胞の遺伝子発現レベルを変化させること、ヒト歯肉由来線維芽細胞のIL-1β産生能を遺伝子発現の抑制を介して減少させることが示唆された。

主論文：Inhibition of interleukin 1β production expression in gingival fibroblasts by low-energy laser irradiation. *Lasers Med Sci*,16: 218-223. 2001.

参考論文：Construction of subtracted osteoblast cDNA library with laser-irradiation-enhanced transcription. *Laser Therapy,* 9: 107-114, 1997.

Enhancement of Macrophage Inhibitory Factor Gene Expression in Osteoblasts by Low Level Laser Irradiation

（低出力レーザー照射による骨芽細胞の Macrophage Inhibitory Factor 遺伝子発現の増大）

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要旨：低出力レーザー照射は抗炎症作用や細胞増殖の促進効果など様々な生物学的効果を有することが知られている。特に骨組織においては、骨芽細胞の分化・増殖の促進、骨形成の促進作用が注目されている。近年、低出力レーザー照射の骨形成促進作用に注目して口腔インプラント療法へのレーザー治療の応用に興味がもたれている。しかしながらレーザー照射における骨形成能の促進効果の機序については不明な点が多い。とくに細胞生物学的な分子レベルでの解明は進んでいない。著者らの研究室では、マウス骨芽細胞様株 MC3T3-E1 細胞へのレーザー照射によって遺伝子発現が促進する遺伝子を cDNA マイクロアレイを応用して発現変動遺伝子の解析を試みている。また、非照射の mRNA を用いて差分化した遺伝子ライブラリーを構築して、分子生物学的にレーザー照射によって発現が増大する遺伝子の解明を試みている。

本研究では、差分化遺伝子法により annexin III 遺伝子を、また、cDNA マイクロアレイ法により osteogl y cin 遺伝子が低出力レーザー照射によって mRNA レベルが増大することを見出し、さらに、RT-PCR, real-time PCR 法を用いて確認した。Annexin III は、エナメル芽細胞や象牙芽細胞に見いだされ、細胞カルシウムの調節に関与するといわれている。また、osteogl y cin は従来、骨誘導因子として精製された標品の構成成分であるプロテオグリカである。

また、本研究では新たに差分化遺伝子ライブラリーから MCL-176 遺伝子クローンを選び、組換え遺伝子挿入 DNA の塩基配列を解読し、NCBI DNA データベースを利用して塩基配列のホモロジー解析を行った。その結果、MCL-176 遺伝子はマウスの macrophage inhibitory factor (MIF) 遺伝子群と 99% 以上の高いホモロジーをもつことが明らかになった。次いでレーザー照射、非レーザー照射の MC3T3-E1 細胞から mRNA を回収して cDNA を合成し、reverse transcription-polymerase chain reaction (RT-PCR) 遺伝子増幅法で分析したところ、レーザー照射後、６，２４ 時間で MIF mRNA レベルが増大することを見いだした。さらに real- time PCR 法を用いて確認したところ、低出力レーザー照射は骨芽細胞の MIF 遺伝子 mRNA の発現を増大させることが確認された。MIF は、従来、種々の組織で産生されるサイトカインで、炎症プロセスに関与することが知られていたが、最近、骨芽細胞にも産生されており、骨芽細胞の増殖や骨改造を調節する機能をもつことが示唆されている。

本研究の結果、低出力レーザー照射によって骨芽細胞の annexin III、osteo- gl y cin、MIF 遺伝子の発現が促進されることが明らかになり、これらの遺伝子産物がレーザー照射による骨形成促進メカニズムに関与する可能性が示唆される。

主論文：Enhanced Gene Expression by Low-Level Laser Irradiation in Osteoblast -Identification of Annexin III Gene by Subtractive Gene Cloning. J Jap Soc. Oral Implantol, 17: 3-12, 2004.

参考論文：　Enhanced Gene Expression by Low-Level Laser Irradiation in Osteoblast　- Identification od annexin III Gene by Subtractive Gene cloning-", J Jap Soc. Oral Implantol, 15:323-329, 2002

Effect of Low-Level Laser Irradiation onOsteoglycin Gene Expression in Osteoblasts" Lasers in Medical Science 18:78-82, 2003.

国際会議の予稿集

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Genome Science–Based Gene Expression Monitoring in Osteoblasts Altered by Low-Level Laser Irradiation

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Although the acceleration of bone regeneration by low-level laser irradiation (LLLI) has been reported, the molecular based mechanisms are not well elucidated. Advances in the technology for assaying transcription levels, in a highly parallel fashion coupled with the completion of genome projects, make possible a complete description of gene regulatory systems. The transcription profile analysis such as gene subtraction and DNA microarray technology make possible to discover large number of genes altered their transcription levels by LLLI.

In this study, we have focussed on the characterization of genes altered in osteoblast cells by LLLI and attempted to elucidate the mechanism of biostimulatory effects on bone regeneration using the subtractive gene cloning and cDNA microarray-based expression monitoring system. Subtractive cDNA library of MC3T3-E1, a clonal osteoblast cell line, was constructed using a stepwise subtraction procedure. Nucleotide sequencing of each gene clone was carried out and assessed by homology search in DNA databases. To find transcriptional profiling, we also examined mRNA levels of 3,800 genes using the gene expression cDNA microarray (Atlas^{TM} Glass Mouse 3.8. Clonetech). Total RNAs were isolated and synthesized to cDNAs, and then labeled with fluorescence dye cy3 or cy5. After hybridization with the microarray, signals of fluorescently-labeled cDNAs were followed by computer analysis to derive relative changes in expression level of the genes.

Many genes altered their expression levels by LLLI were identified including ATP synthesis, DNA replication, transcription and translation, growth factor, and other unknown function genes including ESTs. These findings suggest that LLLI may demonstrate biostimulatory effects on osteoblasts through expression of many gene, and cDNA subtraction and DNA microarray technology will provide unprecedented access to elucidate the mechanism of bone regeneration stimulated by LLLI.

Effect of Low-Level Laser Irradiation on IL-8 Production in Synovial Cells from Human Temporomandibular Joint

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Biostimulatory effects of low-level laser irradiation (LLLI), such as anti-inflammation ad relieving pain against temporomandibular joint (TMJ) disorders, have been reported. However, the molecular based mechanisms are not elucidated yet. Synovial cells are believed to play pathological roles in the development and continuation of inflammation via the release of proteases and oxidative products. Infiltrating cells recruited from blood are mediated by chemotactic factors released by activated synovial cells. IL-8, a member of a chemokine superfamily, has been found in synovial fluid as well as IL-1β from patients with TMJ synovitis.

The purpose of this study is examined the effect of LLLI on the IL-8 production stimulated by IL-1β in human synovial fibroblasts. The cells were isolated from TMJ synovial tissues and primary cultured using outgrowth method. The confluent-stage cells were treated with IL-1β,as the same time, LLLI (Linear polarized near infra-red laser; Super Lizer, Tokyo Iken; 645-1,050 nm range) was treated to the cells. The amounts of IL-8 in conditioned medium were measured by ELISA kit. IL-1β induced IL-8 production from synovial fibroblasts in a time- and dose-dependent manner. LLLI significantly reduced IL-8 production in early time. The LLLI did not destroy IL-1βand IL-8 directly in the experimental condition. These findings suggest that LLLI may have anti-inflammatory effect on TMJ disorder through the reduction of IL-8 production.

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