CONSTRUCTION OF SUBTRACTED OSTEOBLAST cDNA LIBRARY WITH LASER-IRRADIATION-ENHANCED TRANSCRIPTION

Shiki Hosoya, Kazutoshi Tamura, Kazushi Nomura, and Yoshimitsu Abiko

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

We examined an approach in the cloning of the vast majority of those osteoblast mRNA whose transcription was highly induced when the osteoblasts were irradiated with a GaAlAs diode laser. To identify the genes induced by laser irradiation, a stepwise subtraction procedure was carried out between laser-irradiated and non-irradiated MC3T3-E1 cells. Several clones exhibited high homology with mitochondrial protein- and DNA replication related-genes. Our strategy of subtraction cloning is a powerful tool for isolating the specific laser-irradiated MC3T3-E1 cells or highly induced cDNAs. This approach is useful for studying the mechanisms of cell response following bioactivation with low incident levels of laser irradiation.

Key words: laser irradiation, cell culture, GaAlAs diode laser, gene expression, MC3T3-E1 cell

Introduction

A large number of studies have appeared in the recent literature showing the bioactivative effects of low-power laser irradiation in vivo and in vitro, such as stimulation of wound healing,(1-3) fibroblast proliferation,(4-6) chondral proliferation,(7) collagen synthesis,(8-11) and nerve regeneration.(12) These effects are all related to tissue regeneration which includes bone. However, the possible mechanisms of laser bioactivation of osseous tissue are still not fully understood. This lack of knowledge in the area of laser therapy for stimulation of bone growth, particularly at the level of molecular biology and associated research, may well bring about delays in the application of laser therapy in clinical medicine due to a lack of understanding.

The isolation and identification of differentially expressed genes are important in studying the cell cycle, differentiation and bone calcification. The accumulation of knowledge obtained from an approach at the molecular level could be very useful for more development of the therapeutic benefits following laser therapy due to a better understanding of the basic principles by which laser activates osteoblast activity.

In this study, we therefore examined an approach to clone almost all mRNAs whose transcription was highly induced in a mouse osteoblast-like cell line, MC3T3-E1 cells, irradiated with low incident levels of GaAlAs diode laser. To identify the genes induced by laser irradiation, we carried out a stepwise subtraction between the cDNA libraries made from laser-irradiated MC3T3-E1 cells and mRNA derived from non-irradiated cells. The subtractive screening was performed by hybridization between single-stranded DNA (laser irradiated) and photobiotinylated RNA (non-irradiated). After treatment with streptavidin and phenol, hybrids and single-stranded biotinylated materials were sequestered to the aqueous/organic interface, while unhybridized material remained in the aqueous supernatant. The subtracted cDNA library was generated following transformation into Escherichia coli (E. coli) with the unhybridized cDNA by electroporation. DNA sequences were determined from the 5' end of 40 cDNA clones obtained from the subtracted cDNA library. Homology to known sequences was assessed in the...
Fig. 1: A schematic presentation of the strategy used to construct cDNA library of MC3T3-E1 cells.
GenBank and EMBL nucleic acid databases by using the FASTA program. As a result, several clones exhibited high homology with mitochondrial protein genes, signal transduction genes, and DNA replication-related genes.

**Materials and Methods**

**Cell culture procedure**

MC3T3-E1 cells, established from newborn mouse calvaria by Kodama et al.,<sup>13</sup> were cultured in minimal essential medium (αMEM; GIBCO) containing 10% fetal calf serum and antibiotics comprising 100 μg/ml penicillin G (Sigma Chemical Co.) and 50 μg/ml gentamicin sulfate (Sigma).

**Laser irradiation**

The laser source used was a gallium aluminium arsenide (GaAlAs) diode laser system ( Matsushita Industrial Equipment Inc., Osaka, Japan: Panalas<sup>®</sup> 1000). The technical specifications of this laser therapy system are as follows: wavelength, 830 nm; output power: 100~700 mW, variable in continuous wave (c/w). In the present study, an output power in c/w of 500 mW was selected. The probe was fixed 50 mm from the cells to be irradiated, giving a spot size of 78.5 cm<sup>2</sup> and an incident power density of approximately 6.4 mW/cm<sup>2</sup>. The irradiation time was 20 minutes, delivering an incident energy density of 7.6 J/cm<sup>2</sup>. Specimens of mRNA were harvested after 1 h, 6 h, 12 h, and 24 h both with and without GaAlAs diode laser irradiation.

**Preparation of cDNA libraries carrying directional inserts**

The strategy for preparation of the cDNA library is illustrated schematically in Figure 1. Total RNA followed by purification of poly (A)<sup>+</sup> RNA was extracted from the laser-treated MC3T3-E1 cells by the guanidine thiocyanate/GTFA method.<sup>14</sup> The cDNA libraries were prepared as described by Gulber and Hoffman<sup>15</sup> with some modifications. Briefly, 2-5 μg of Poly (A)<sup>+</sup> RNA isolated from MC3T3-E1 cells was employed as a template and cDNA was synthesized by reaction with reverse transcriptase (Superscript II) and 1.6 μg of oligo (dT) primer carrying a Not I site in the presence of 5′Methylated-dCTP. The reaction mixture was treated with RNase H, followed by reaction with DNA polymerase I. Each end was blunt-ended with T4 DNA polymerase and ligated to an unphosphorylated Bgl II-Sma I adapter. After digestion with Not I, small DNA fragments of less than 300 bp were removed by a CROMA spin-400 column (Clontech, USA). The cDNA fragments were directionally inserted between the Not I (dephosphorylated) and Bgl II sites of vector pAP3neo (H. Nojima, unpublished data). The ligation mixture was electroporated into MC1061A cells as previously described.<sup>16</sup> The complexities of the cDNA libraries of cDNA used were 1.2 x 10<sup>6</sup> colony forming units (cfu) for the laser treated MC3T3-E1 cells.

**Stepwise subtraction procedure**

The strategy for preparation of the subtracted cDNA library is depicted in Figure 2. To prepare the single-stranded plasmid DNA, the plasmid DNA prepared from the cDNA library of laser-treated MC3T3-E1 cells was transformed into E. coli DH5αF′TQ cells by electroporation. After 1 h of culture in rich medium (2x YT medium), transformed cells were infected with R408 helper phages.<sup>17</sup> Single-stranded DNA was then purified from the supernatant of the overnight culture. Biotinylated poly (A)<sup>+</sup> RNA drivers were isolated from the non-treated MC3T3-E1 cells by the guanidine thio-
HEPES (pH 7.5), 1 mM EDTA, 0.1% SDS and 1 µg of oligo-poly (tA). After hybridization, the mixture was added to 400 µl of the hybridization buffer without SDS, and a further 10 µg of streptavidin was added. The mixture was incubated at room temperature for 5 min and extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The organic phase was re-extracted with 100 µl TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The aqueous phase was pooled, and the recovered DNA was subtracted once more with biotinylated RNA (1.4 µg). The single-stranded DNA obtained was subjected to BcaBEST™ DNA polymerase (Takara Shuzo, Japan) reaction at 65°C for 30 min to obtain the double-stranded cDNA. The DNA was dissolved in 10 µl TE buffer, and 1 µl aliquots were introduced into E. coli MC1061A cells by electroporation.15

DNA sequencing
Plasmid DNA of each clone randomly selected from subtracted cDNA library was isolated by PI-100 (Kurabo, Japan).

Dideoxy-chain termination sequencing reaction18 were performed with fluorescent dye-labeled primers and SequiT Therm™ Long-Read™ cycle sequencing kits for LI-COR® Sequencing (Epicentre Technologies, USA). The reaction products were analyzed by a 4000LS Long ReadIR™ DNA sequencing system (LI-COR, USA).

Results

Fig. 4: Agarose gel electrophoresis of Mla I and Nsi I digested cDNA clones. Arrow indicates the linearized cloning plasmid, pAP3000.
Table 1: Homology search. 5' partial end sequences from 40 cDNA clones obtained from the subtracted library were compared with the GenBank and EMBL nucleic acid databases using the FASTA program.

<table>
<thead>
<tr>
<th>Clone No</th>
<th>Homologous gene (species)</th>
<th>Similarity</th>
<th>(base)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCL-01</td>
<td>yw96f09.s1 Homo sapiens cDNA clone 260105 3' (human)</td>
<td>72.4%</td>
<td>(450)</td>
</tr>
<tr>
<td>MCL-10</td>
<td>aorta cDNA 5'-end GEN-306D11 (human)</td>
<td>73.5%</td>
<td>(279)</td>
</tr>
<tr>
<td>MCL-11</td>
<td>yd75h05.r1 Homo sapiens cDNA clone 114105 5' (human)</td>
<td>61.8%</td>
<td>(296)</td>
</tr>
<tr>
<td>MCL-15</td>
<td>x60f10.r1 Homo sapiens cDNA clone 266155 5' (human)</td>
<td>63.1%</td>
<td>(396)</td>
</tr>
<tr>
<td>MCL-16</td>
<td>yx75d04.r1 Homo sapiens cDNA clone 267559 5' (human)</td>
<td>73.1%</td>
<td>(443)</td>
</tr>
<tr>
<td>MCL-23</td>
<td>mitogen inducible gene mig-2 (human)</td>
<td>89.0%</td>
<td>(477)</td>
</tr>
<tr>
<td>MCL-31</td>
<td>musculus domesticus mRNA for membrane glycoprotein (mouse)</td>
<td>71.0%</td>
<td>(192)</td>
</tr>
<tr>
<td>MCL-32</td>
<td>musculus mCIF1 protein mRNA (mouse)</td>
<td>69.5%</td>
<td>(843)</td>
</tr>
<tr>
<td>MCL-33</td>
<td>alpha-tubulin isotope M-alpha-4 mRNA (mouse)</td>
<td>91.0%</td>
<td>(958)</td>
</tr>
<tr>
<td>MCL-42</td>
<td>musculus G/T-mismatch binding protein (Gralbp) mRNA (mouse)</td>
<td>97.2%</td>
<td>(1003)</td>
</tr>
<tr>
<td>MCL-60</td>
<td>mRNA for CDC46 (MCM 5) homologue (mouse)</td>
<td>97.0%</td>
<td>(801)</td>
</tr>
<tr>
<td>MCL-68</td>
<td>EST12192 cDNA 5' end similar to CD9 antigen (human)</td>
<td>86.5%</td>
<td>(185)</td>
</tr>
<tr>
<td>MCL-76</td>
<td>yl49b01.r1 Homo sapiens cDNA clone 161545 (human)</td>
<td>62.3%</td>
<td>(302)</td>
</tr>
<tr>
<td>MCL-78</td>
<td>yn52b05.r1 Homo sapiens cDNA clone 172017 5' (human)</td>
<td>84.0%</td>
<td>(400)</td>
</tr>
<tr>
<td>MCL-126</td>
<td>mRNA for U2 snRNP-specific (human)</td>
<td>88.5%</td>
<td>(826)</td>
</tr>
<tr>
<td>MCL-127</td>
<td>human (chromosome 3p25) membrane protein mRNA (human)</td>
<td>86.1%</td>
<td>(735)</td>
</tr>
<tr>
<td>MCL-140</td>
<td>musculus mRNA for P1 protein (MCM 5) (mouse)</td>
<td>96.2%</td>
<td>(240)</td>
</tr>
<tr>
<td>MCL-201</td>
<td>FoF1-ATPase, Fo-subunit-b (rat)</td>
<td>94.9%</td>
<td>(771)</td>
</tr>
<tr>
<td>Unknown</td>
<td>other 22 clones</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Histogram of cDNA insert size

Figure 3 shows the size of the cDNA insert of clones obtained from each cDNA library before and after subtraction. The DNAs were cleaved with Mla I and Not I and electrophoresed in agarose gels. After ethidium bromide staining, the size of the DNA fragments was measured. The average size of inserted DNAs was 1.8 kbp and 0.8 kbp for the original cDNA library and subtracted cDNA library, respectively. Figure 4 shows a representative electrophoresed pattern of the subtracted cDNA library indicating that each fragment had a different size.

Homology search

Forty representative clones were randomly selected from the subtracted cDNA library and a 5'-portion of each insert was sequenced. The homology of each clone was examined using the GenBank and EMBL nucleic acid databases and results are listed in Table 1. Several clones exhibited high homology with sequences for membrane protein, mitochondrial protein, cytoskeleton protein, licensing factor, and transcription factor. Eleven clones were unclassified, and twenty-two clones showed no similarity to those in the data bases.

Discussion

The mechanism by which laser irradiation promotes bone formation is not fully understood. Ozawa et al. reported that a number of calcified bone nodules were formed following low incident levels of laser irradiation, also known as low level laser therapy or LLLT, and concluded that exposure to LLLT early in the cell culture period was more effective in inducing nodule formation due to the differentiation of osteoblasts and that osteoblasts might have a specific optimal dosage of irradiation for acceleration of fibroblast to myofibroblast transformation. There have also been some studies of the stimulation of collagen synthesis, including that by Saperia et al., who clearly demonstrated that the mRNA level of type I collagen was increased following application of LLLT during wound healing in the skin. Since type I collagen is a major matrix protein in bone, these observations are supportive data for a photobioactivative effect of LLLT on bone formation.

Osteoblast-rich cells from foetal rat calvaria are frequently used for many in vitro experiments, including those involving laser irradiation. However, this cell population consisted of heterogeneous, unidentified
and undifferentiated cells, with possible complex cell-to-cell interactions. To evaluate whether the same phenomenon occurs in a clonal osteoblast-like cell line, we used the mouse osteoblast-like cell line MC3T3-E1 established by Kodama et al.,(13) which is capable of exhibiting in vitro various characteristics of osteoblasts including mineralization.

The aim of this study was systematically to isolate and characterize the cDNA clones specifically expressed in laser-irradiated cells. It has been shown that 90% of randomly selected cDNA clones from an ordinal cDNA library turned out to be previously known housekeeping genes.(22) In contrast, the present study clearly indicates that our strategy of subtraction cloning of the laser-irradiated cDNA library by the non-irradiated is a powerful tool for isolating the laser-irradiated MC3T3-E1 cell-specific or highly induced cDNAs.

It has been shown that laser irradiation stimulates cell proliferation.(11–16) We isolated several clones with high homology to genes coding for licensing and transcription factors. No studies concerning the enhanced expression of these genes after laser irradiation have been reported to date. These proteins are all involved in controlling cell proliferation. Furthermore, we successfully isolated twenty-two unknown and eleven unclassified (uncharacterized) genes. On the other hand, several clones exhibited high homology with mitochondrial protein (MCL-201). Greco et al.(23) reported that LLLT stimulated the synthesis of many mitochondrial proteins associated with transcription and translation. Passarella et al.(24) noted an increase in the activity of ADP/ATP exchange in mitochondria following doses of LLLT. These findings may support our results that the subtracted cDNA library constructed in this study contained cDNA clones whose transcription was highly induced when cells were irradiated by low incident levels of laser energy.

Having proved these preliminary results of LLLT on an osteoblast-like cell model at the parameters reported above, we intend in future studies to examine the effects of different irradiation doses, repeated laser irradiation, different beam types (i.e. pulsed versus c/w) and different wavelengths on gene expression, and to clarify the function of the unknown genes illustrated above. In addition, the cDNA clones highly expressed in a particular stage of MC3T3-E1 cell differentiation would be useful in elucidating the molecular mechanisms of calcification and proliferation. The molecular approach described here will open the gate to develop further the basic science of laser therapy associated reactions and thus help to advance the clinical application of low level laser therapy in medical practice.

Acknowledgments

The authors wish to thank professor Hiroshi Nojima for supplying the pAP3neo vector and Mr. Akira Kaneda, senior engineer of Matsushita Industry Equipment Company Limited for providing the laser therapy apparatus. This study was supported in part by a grant for Comprehensive Research on Aging and Health from the Ministry of Public Welfare of Japan (96-A2303).

References


S. HOSOYA ET AL.


