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PROMOTER COMPETITION ASSAY FOR ANALYZING GENE REGULATION IN JOINT TISSUE ENGINEERING

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1. ABSTRACT

We describe a new biochemical technique, “promoter competition assay,” for examining the role of cis-acting DNA elements in tissue cultures. Recent advances in tissue engineering permit the culture of a variety of cells. Many tissues are engineered, however, without an appropriate understanding of molecular machinery that regulates gene expression and cellular growth. For elucidating the role of cis-acting regulatory elements in cellular differentiation and growth, we developed the promoter competition assay. This assay uses a transient transfer into cells of double-stranded DNA fragments consisting of cis-acting regulatory elements. The transferred DNA fragments act as a competitor and titrate the function of their genomic counterparts. Using synovial cells derived from a rheumatoid arthritis patient, we examined a role of NF-κB binding sites in the regulation of the expression of matrix metalloproteinase (MMP) genes. The results support a stimulatory role of NF-κB in transcriptional regulation of MMP-1 and MMP-13.

2. INTRODUCTION

The availability of a complete set of human genome sequences provides an exciting, challenging opportunity for tissue engineers (1-3). For engineering a specific type of tissue such as cartilage (4, 5), genomic DNA sequences are a rich resource useful in elucidating molecular mechanisms underlying tissue growth and differentiation. In testing for the role of regulatory DNA sequences, a conventional assay such as a reporter gene assay or an electrophoretic mobility shift assay is, however, not well suited to deal with a large volume of databases in the post human genome project era (6). There is a demand for a new assay which provides an efficient functional test of regulatory DNA elements.

In order to efficiently test for the role of genomic promoter sequences, we developed a new biochemical assay named the “promoter competitor assay.” In this assay DNA fragments consisting of a specific cis-acting element are transiently transferred into cultured cells. Alterations in the mRNA level of genes of interest are monitored by reverse transcription and PCR (Figure 1). Since exogenous DNA fragments act as a competitor of genomic cis-acting elements, reduction in specific gene transcripts in the assay would suggest that the transferred cis-acting elements mimic the binding capacity of endogenous cis-acting elements. Since preparation of DNA fragments is straightforward compared to preparation of plasmid vectors or antibodies, a scaled-up systematic assay for the

Table 1. PCR primers used in this study

Gene	Sense Primer	Antisense Primer	cDNA size (bp)
MMP-1	CACAGCTTTCCTCCACTGCTGCTGC	GGCATGGTCCACATCTGCTCTTGGC	396
MMP-8	TAAAGACAGGTACTTCTGGAGAAGG	GCTTCAGCGATATCTACAGTTAAGC	514
MMP-13	TGGTGGTGATGAAGATGATTTGTCT	AGTTACATCGGACCAAACTTTGAAG	376
GAPDH	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC	600

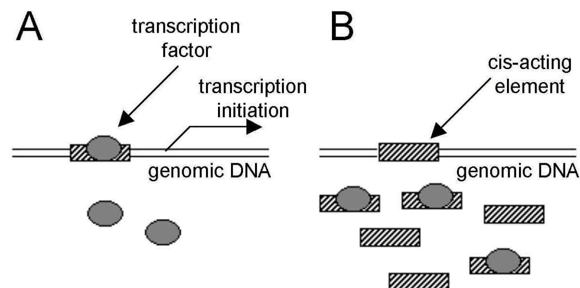

Figure 1. Schematic illustration of the promoter competition assay. (A) Control without competitive cis-acting elements. (B) Transcriptional machinery rendered inactive with competitive cis-acting elements that bind transcription factors.

role of putative cis-acting elements can therefore be designed.

In this study, we examined the promoter competition assay using synovial cells derived from a rheumatoid arthritis patient (7). Rheumatoid arthritis is a chronic joint disease caused by complex interactions with the immune system. Inflammation and degradation of soft joint tissue are major symptoms (8-10). It is imperative for tissue engineers to identify gene regulation mechanisms that are specific to rheumatic auto-inflammatory responses. In that way it should be possible to generate tissue resistant to inflammation or degradation. Suppressing the expression of proteolytic enzymes such as matrix metalloproteinases (MMPs) is one approach to alleviate rheumatic symptoms (11-13). Focusing on a role of NF-κB in the transcriptional regulation of MMPs, we demonstrate here that mRNA levels of MMP-1 and MMP-13 in synovial cells are diminished in the promoter competition assay. NF-κB is shown to stimulate inflammatory responses in rheumatic joints (14). Since synoviocytes are under shear stress in joints, the cells for this study were grown under 0-10 dyn/cm^2 shear stress. The results provided a “proof of concept,” which supports a potential use for the promoter competition assay in identifying roles specific cis-acting elements play in gene regulation. Elucidating such roles is an important challenge for tissue engineering.

3. MATEIRALS AND METHODS

3.1. Cell culture

A human synovial cell line (MH7A, Riken Cell Bank, Japan) derived from the intra-articular soft tissue of the knee joint of a rheumatoid arthritis patient, was used in this study (7). MH7A cells are fibroblast-like synoviocytes immortalized by transfection with SV40 T antigen. The cells were spread on a glass slide coated with type I collagen and grown in the RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics at 37°C. We used cells at ~80% confluency 3-4 days after fresh spreading.

3.2. Promoter competition assay

Cells were incubated with double-stranded DNA fragments consisting of a specific cis-regulatory element. The mRNA level of target genes was determined by reverse transcription and polymerase chain reactions (RT-PCR). In the current study, we used two 18-bp double-stranded DNA fragments to test a role of NF-κB: 5’-TGCAGGGGATYCCCGACT-3’ (including NF-κB binding site) and 5’-TGCAGACTCATGTAGCGT-3’ (random sequence) as a control. Cells were transiently incubated with 0.05 - 5 μM DNA fragments that acted as a competitor of the corresponding genomic cis-acting elements. The DNA fragments with and without phosphorothioate modification (Ana-Gen Technologies, Inc.) were used.

3.3. Mechanical shear

Since joint cells *in vivo* are under shear stress, mechanical shear stress was applied to MH7A cells using a Streamer Gold flow device (Flexcell International Corp.). The device generates a Poiseuille flow within a pair of parallel plates separated by 500 μm and induces uniform fluid shear. Cells were grown in a monolayer in the medium consisting of 10% fetal calf serum. Shear stress at 1, 2, 5, and 10 dyn/cm^2 was applied for 1 hour.

3.4. Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was isolated using an RNeasy mini kit (Qiagen, CA), and the isolated RNA was reverse-transcribed by MMLV reverse transcriptase using random primers. The cDNAs corresponding to MMP-1, MMP-8, MMP-13, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, control) were amplified by PCR (GeneAmp PCR System 2400, Perkin Elmer). The PCR primers are listed in Table 1 (15). The PCR included 25 cycles at 94°C for denaturation (1 min), 54-62°C for annealing (30 sec), and 72°C for extension (30 sec). All experiments were performed three times to demonstrate reproducibility.

3.5. Cellular growth analysis

In order to examine effects of incubated DNA fragments on cellular growth in the promoter competition assay, cell number was monitored after transferring DNA fragments. The synovial cells were incubated with 5 μM of DNA fragments for 1 hour on day #1 and day #2, and harvested for determining the growth rate (cell number) on day #3. The mean number of cells and the standard deviation were determined for the normal control, the control incubated

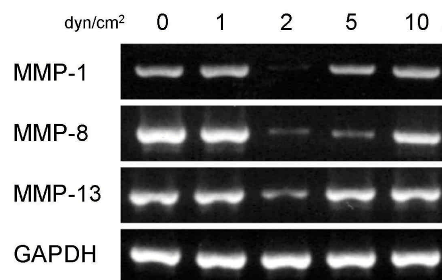

Figure 2. Expression of MMP mRNAs under increasing shear stress. Using MH7A synovial cells, the mRNA level of MMP-1, MMP-8, and MMP-13 was determined by RT-PCR under 1-hr uniform shear stress at 0, 1, 2, 5, and 10 dyn/cm^2. GAPDH served as control for RT-PCR.

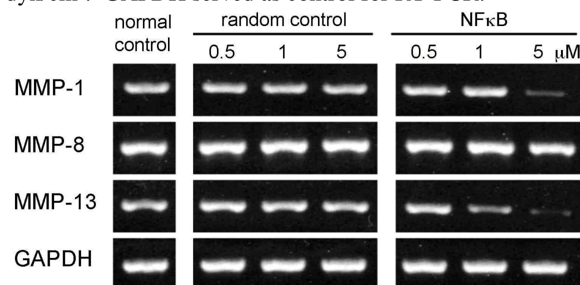

Figure 3. Expression of three MMP mRNAs in an NF-κB promoter competition assay. The level of the MMP mRNAs was determined after 1-hour incubation with DNA fragments consisting of NF-κB cis-acting elements and random DNA sequences. The concentration of the DNA fragments was 0 (normal control), 0.5, 1, and 5 μM. Incubation with random DNA fragments served as negative control. Reduction of the mRNA level by the NF-κB fragments was 82 ± 11% (MMP-1 at 5 μM), 10 ± 5% (MMP-8 at 5 μM), and 55 ± 12% and 91 ± 9% (MMP-13 at 1 and 5 μM, respectively).

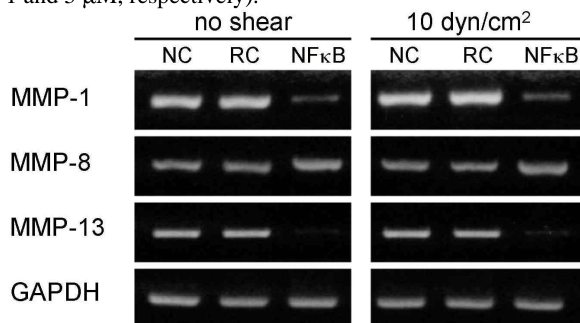

Figure 4. Suppression of MMP-1 mRNA and MMP-13 mRNA under shear stress by NF-κB cis-acting elements. MH7A cells were incubated for 1 hour with 5 μM DNA fragments consisting of NF-κB cis-acting elements or random sequences. The cells were grown under 0 or 10 dyn/cm^2 shear stress for 1 hour, and the levels of mRNAs corresponding to MMP-1, MMP-8, and MMP-13 was determined by RT-PCR. NC: normal control; RC: control incubated with random DNA sequences; and NF-κB: experimental incubated with NF-κB cis-acting elements. In the presence and absence of shear, the mRNA level was reduced to 23 ± 2% (MMP-1) and 11 ± 3% (MMP-13).

with random DNA sequences, and the experimental cells incubated with DNA fragments consisting of NF-κB cis-acting elements.

4. RESULTS

4.1. Response to mechanical shear

Prior to employing the promoter competition assay we first determined basal mRNA levels for three MMPs. Since synovial cells *in vivo* are under mechanical stress, we applied flow shear to cells for 1 hour at varying intensities of 0-10 dyn/cm^2. RT-PCR revealed that under gentle shear at 2 dyn/cm^2 the mRNA level of MMP-1, MMP-8, and MMP-13 was decreased (Figure 2). However, under high shear at 10 dyn/cm^2, the mRNA level of three MMPs was elevated up to the basal control level (Figure 2). The results support the previous finding that the level of MMP mRNA is shear-stress dependent. The optimal stress to minimize the mRNA expression of MMPs exists at a few dyn/cm^2 (16).

4.2. Promoter competition assay using NF-κB cis-acting elements

In synovial tissue derived from a rheumatoid arthritis patient, suppression of proteolytic enzymes such as MMPs is thought to contribute to preventing tissue degradation. In employing the promoter competition assay we focused on the cis-acting element consisting of the NF-κB binding sites. Synovial cells were incubated with 0.05 – 5 μM double-stranded DNA fragments for 1 hour. We used two different DNA fragments, one consisting of NF-κB binding sites and the other containing random DNA sequences. The cells were harvested immediately after incubation with exogenous DNA fragments. RT-PCR was then conducted to determine the level of MMP mRNAs (Figure 3). When cells were incubated with 5 μM of DNA fragments, the mRNA level of MMP-1 and MMP-13 was reproducibly reduced. However, incubation with the same amount of random DNA fragments did not alter MMP expression. The expression of MMP-8 was not affected in the assay containing any of the DNA fragments.

4.3. Promoter competition assay under mechanical shear

We next employed the promoter competition assay to examine the cells under mechanical shear, since joint tissue *in vivo* is under constant shear. MH7A cells were grown for 1 hour under 0 dyn/cm^2 (control) or 10 dyn/cm^2 shear after 1-hr incubation with 5 μM of double-stranded DNA fragments. The RT-PCR results showed that the DNA fragments consisting of NF-κB binding sites reduced mRNA expression of the MMP-1 and MMP-13 genes with and without mechanical shear (Figure 4). The incubation with the random DNA sequences did not alter any mRNA expression. The expression of MMP-8 was unaltered in a promoter competition assay with either DNA element.

In antisense DNA applications modified DNA oligonucleotides are often used to increase efficiency of a DNA transfer and to enhance stability of transferred molecules. We used the DNA fragments with and without

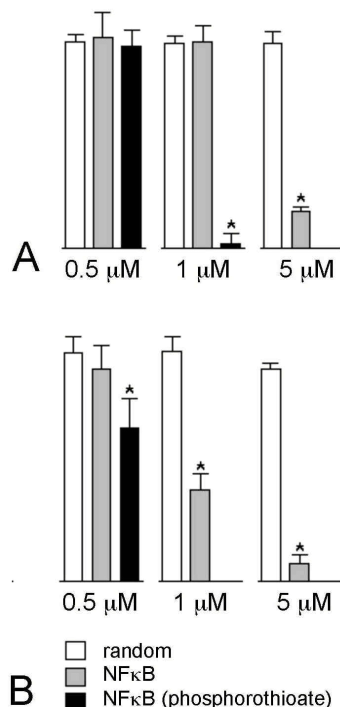

Figure 5. Comparison of DNA fragments with and without phosphorothioate modification. The mRNA level is normalized by the basal control level, and a standard deviation is indicated by a bar. The white, gray, and black columns correspond to the random DNA fragments, NF-κB fragments without modification, and NF-κB fragments with phosphorothioate modification. The asterisk indicates statistically significance difference from control without DNA transfer ($p < 0.05$). (A) Level of MMP-1 mRNA. (B) Level of MMP-13 mRNA.

phosphorothioate modification and compared effectiveness in suppression of MMP transcripts. The DNA fragments with phosphorothioate modification were more effective in reducing the mRNA expression of MMP-1 and MMP-13 than the fragments without modification (Figure 5). For instance, the modified fragments at 1 μM nearly abolished the expression of MMP-1 mRNA, but the unmodified counterpart at 1 μM did not alter it expression. The expression of MMP-8 mRNA was not altered by either DNA construct.

4.4. Effects of NF-κB cis-acting elements on cellular growth

Realizing that exogenous NF-κB cis-acting elements induce a reduction in mRNA expression of the MMP-1 and MMP-13 genes, we further examined effects of this competitor on cellular growth. During a 3-day culturing period, cells were exposed twice to 5 μM DNA fragments for 1 hour in day #1 and day #2. The number of cells was determined as 13.5 ± 2.8 (mean ± standard deviation in an arbitrary unit; normal control), 11.9 ± 2.2 (control with random DNA fragments), and 9.3 ± 1.5 (experimental with NF-κB DNA fragments). The reduction in the number of cells in the experimental sample was statistically significant compared to the normal control ($p < 0.0001$ in t-test). No statistical significance was detected between the normal control and the control with random DNA fragments ($p = 0.09$). Since rheumatic cells proliferate at a higher growth rate than normal synovial cells, a slower growth rate by NF-κB elements can be considered a favorable phenotype.

5. DISCUSSION

The described promoter competition assay is a new technique for testing the functional significance of cis-acting elements in cultured cells. Using synovial cells derived from a rheumatoid arthritis patient as a model system, we tested a role of NF-κB binding sites in the mRNA expression of three MMPs. The results showed that (i) the expression of three MMPs was sensitive to flow shear and the response was stress-intensity dependent; (ii) the mRNA expression of MMP-1 and MMP-13, but not MMP-8, was suppressed in the promoter competition assay using NF-κB cis-acting elements; and (iii) cellular growth was retarded by the competitive NF-κB elements. Since the assay using random DNA fragments did not affect either MMP expression or cellular growth, the results in this study support a stimulatory role of NF-κB for the expression of MMP-1 and MMP-13 as well as cellular growth.

In rheumatoid arthritis, NF-κB plays an essential role in transcriptional activation induced by TNF-α and IL-1 (14). The promoter competition assay revealed a differential role of NF-κB binding sites among three MMP genes tested here. In the 5'-end regulatory sequences of many MMPs, putative NF-κB binding sites have been identified. There are 1, 2, and 1 site in an 800-bp promoter of MMP-1, MMP-8, and MMP-13, respectively. However, a consensus binding site for NF-κB (GGGRC/A/TTYYCC) consists of degenerative sequences and it is difficult to identify functional NF-κB binding sites from DNA sequences alone. In the current assay using NF-κB cis-acting elements, the mRNA levels of MMP-1 and MMP-13 were reduced but the level of MMP-8 mRNA was unchanged. Since most MMP genes are co-regulated by AP-1, AP-2, and PEA-3 sites, multiple cis-acting elements besides NF-κB appear to regulate the expression of MMPs (17-19). In separate experiments the DNA fragments consisting of AP-1 site (TGACGTNTGASTCAGCATGC) partially reduced mRNA expression of MMP-1 but the fragments containing AP-2 site (TGCAMKCCCSCNGGCGGACT) did not alter expression (data not shown). It will be possible to test a combinatorial effect of multiple cis-acting elements in this assay.

Although the current study provides "proof of concept," the efficiency of transferring and stability of DNA fragments need to be further analyzed. In this study, 0.05 - 5 μM DNA fragments without any modification were primarily used and 1-hour incubation with 5 μM DNA fragments yielded significant effects on gene regulation and cellular growth. The response was dosage dependent in the concentration range of 0.5 to 5 μM, and the higher

concentration at 5 to 10 μM showed the same results obtained at 5 μM. Phosphorothioate-modified nucleotides were a more potent suppressor of the mRNA expression of MMP-1 and MMP-13, and other DNA modifications used for antisense DNA may further enhance the effectiveness of transferred DNA fragments (20, 21).

6. PROSPECTS

Like the impact of modern molecular biology on research projects in embryology, elucidation of a complex regulatory network is indispensable for engineering tissue differentiation and growth. This assay has the potential for identifying a role of a cis-acting element with reference to a complete set of human genomic sequences. The described assay is suited for a systematic examination of multiple cis-acting elements. Indeed, a micro-fabricated cell plate can be designed. In the future it should be possible to culture cells on a micro-DNA array that provides a source for testable cis-acting elements (22).

Tissue engineering strategies are multifaceted, and are comprised of several components and features. Included is the preparation and culture of appropriate starter cells for differentiation; design of functional matrices for physically supporting those cells while they multiply and specialize; surgical insertion methods; and the design and administration of drugs/growth factors to regulate the transition of the starter cells from a dissociated and/or cultured state to an integrated/interconnected functional cell mass (23). Substantial progress has been made for several aspects of those strategies. The most refractory aspect will likely continue to be in the area of regulating gene expression, both qualitatively and quantitatively, in cells that comprise the engineered tissue.

The promoter competition assay described herein represents a model system for the design of future experiments. It should be possible, for example, to extend the principle of this assay for elucidating other cis-acting regulatory components, such as silencers and enhancers (24, 25). As well, it should be possible to use this same principle for assessing the extent to which a cis-acting nucleotide sequence is involved in the coordinate regulation of other--perhaps presently unrecognized--transcription events. Using either differential hybridization or gene microarrays for elucidating complex gene expression patterns, accurate profiles of regulation of gene expression in cells targeted for tissue engineering will likely be achieved. Once those profiles are available, rational drug design and educated searches for appropriate growth factors for enhancing proper differentiation in starter cells for tissue engineering can proceed with higher expectations for success (26).

Human genome sequence data will likely provide the driving force for patterning large-scale screens in which various cell types which are candidates for tissue engineering are cultured directly on micro-DNA array plates which contain putative cis-acting sequences. With appropriate gene expression markers the regulatory circuitry that controls phenotypic expression will be elucidated. Then, more accurate choices of cell type and/or sub-populations of heterogeneous tissues for replacement therapy can be made.

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