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眼睛翳狀贅肉結膜下彈性素堆積之基因轉殖鼠模式之建立

(2/3)

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Introduction

Pterygium is a worldwide ocular surface disease, an ocular disorder that occurs more often in the population of tropical and subtropical region [1]. It often causes irritating or inflamed ocular symptoms, and also accompanies with local drying of corneas and conjunctivas.

Elastin is a major component of connective tissue in large arteries, lung, skin, and ligmenetum, and provides elasticity in these tissues [2]. In normal skin, elastic fibers are found in the dermal connective tissue in only small amounts. In photodamaged skin, on the other hand, elastic fibers are the most prominent component of extracellular matrix and are located in the superficial to mid-dermis as an amorphous blue staining area on routine hematoxylin-eosin staining [3,4]

Accumulation of elastin in solar elastosis of photodamaged skin has also been demonstrated in clinical situation and experimental animals, by both immunohistochemistry and molecular biology techniques[5,6]. It is likely that the pathologic changes in conjunctiva in response to chronic UV irradiation are similar to those in chronically sun-damaged skin. For example, elastodysplasia and elastodystrophy are known to be present in the subepithelial connective tissue of pinguecular part in pterygium, and similar pathologic elastin accumulation is found in chronically sun-damaged skin [7]. Therefore,we speculate that the pathogenesis of pterygia may be the same as photodamaged skin.

The increase in elastin mRNA levels has been demonstrated in fibroblasts of photoaged skin, as compared with those from skin of normal subjects. This increased level of elastin mRNA in sun-damaged skin is resulted from enhanced elastin promoter activity. Although we

found that post-transcriptional regulation of elastin promoter plays an important role in the formation of elastoid degeneration of pterygium, we still want to investigate the possibility of transcriptional regulation of elastin promoter. We will try to use transgenic model to study the elastin expression in pterygium.[8]

In the present study, we tried to generate a transgenic mice carry a 2.8 kb human elastin promoter with a GFP reporter gene, and apply this mice to the model of pterygial formation. We also try to generate an ablation model of conjunctival fibroblasts by using DTA constructs.

Material and method

＊ Isolate genomic DNA from human blood and Hela cell line：

Genomic DNA Isolation Kit （GENTRA）was used to isolate human genomic DNA from human blood and Hela cell line.

＊ Cloning and Sequence Analysis of the Human Elastin Promoter：

A 2.8 kb Sac I/Hind III human genomic DNA fragment containing the 5’ portion of the human elastin gene is derived from PCR/linker (Forward primer, 5’-(-2260)-Sac I----3’, 5’-cgagctcggatcctccagcctaagcctg-3’; Reverse Primer, 3’--(+566) Hind III-5’, 5’-cccaagcttgggggatcccgcttcccaggggt-3’) and subcloned into the pBluescript SK vector (Stratagene, La Jolla, CA). The complete nucleotide sequence of the insert is determined, using T3, T7 primers and walk-in primers. The sequence alignment is performed using the “CLUSTALW” program (http://www.genome.ad.jp/SIT/CLUSTALW.html). Putative transcription factor binding sites is searched using the “TFSEARCH” program (ver 1.3) (http://www.blast.genome.ad.jp /SIT/ TFSEARCH.html) with threshold score (default: 90.0)

＊ Transgenic Mice

The minigene construct is generated by inserting a 2.8 kb Sac I/Hind III fragment containing a splice acceptor (SA) site and GFP from the pEGFP-1 plasmid (Clontech, Palo Alto, CA) at a unique Xho I site in intron 1 of the human elastin gene (HEL). The HEL2.8-GFP-SV40 poly A insert (4.3 kb) is excised from the vector by Sac I and Afl II digestion,

purified by agarose gel electrophoresis. Purified DNA fragment is microinjected into the male fertilized pronucleus of C57BL/6 X BALB/c hybrid strain zygotes by the “transgenic core facility” at the Insitute of Biomedical Sciences. Academia Sinica, Taipei.

∗ Identification of Transgenic mice by Southern blotting hybridization and polymerase chain reaction (PCR)

To determine the copy number of the transgene, Southern blotting hybridization is performed as follows. Tail DNA (10 μg) is digested with Hind III that is a unique site within the entire transgene. Three standard samples containing 50, 10, and 1 copy of the transgene, respectively, are mixed with Hind III digested DNA samples from wild-type C57BL/6 mouse and run side by side with the samples to be tested. The copy number of the transgene is determined by the intensity of an 4.3 kb band hybridized with a 32^{P}-labeled GFP DNA probe (4.3 kb). Routine genotyping of the transgenic mice is done by PCR using primers as described above. Toes are cut and lysed at 55°C in 40 μl of lysis buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20, and 60

1 hr. Samples are then boiled for 10 min, and cooled on ice. An aliquot of 7 μl of proteinase K digested tissue lysate is mixed with 18 μl of PCR cocktail solution containing 50 mM KCl, 1.5 mM MgCl2 10 mM Tris-HCl (pH 8.3), 0.001% gelatin, 200 M in each of the four dNTPs, 0.4 M of each primer, and 0.5 units of recombinant Taq polymerase (AmpliTaq; Perkin-Elmer, Norwalk, CT). The mixture is then overlaid with 20 μl of light mineral oil. PCR is performed for 35 cycles with a

DNA thermal cycler (Perkin-Elmer, Norwalk, CT) at each cycle of denaturing temperature of 94°C for 30 sec, annealing temperature of 65°C for 1 min, and elongation temperature of 72°C for 1 min. The resulting PCR products (285 bp) are resolved on 4% NuSieve:SeaKem (3:1, FMC) composite gel in TAE buffer at 80 V for 30 min and visualized with UV light after staining with ethidium bromide

∗ **Cell Culture**

To establish the conjunctival and pterygial fibroblast cultures, which the method is previously descrivbed [9,8], the subepithelial connective tissues are diced into small pieces and treated with 0.05% Trypsin-EDTA solution (Sigma type XI), in phosphate balanced saline [PBS, 8 g/l NaCl, 0.4 g/l KCl, 1 g/l Glucose, 0.4 g/l NaHCO3, 0.22 g/l Na2-EDTA (0.6 mM), pH 7.0] at 37°C for 30 min. The matrix-free fibroblasts are collected by centrifugation and are cultured in DMEM plus 10% fetal bovine serum. To culture mouse embryonic fibroblasts (MEF), embryos (14-days-old) are rinsed in PBS to remove as much blood as possible after removal of liver and heart, and then teased with forceps. To each embryo, 3-5 ml of trypsin solution are added and incubated at 4°C overnight. The trypsin solution is removed by aspiration, the specimens is further incubated at 37°C for 30 min. 10 ml of tissue culture medium (10% fetal bovine serum in high glucose containing DMEM and 10-4 M β-mercaptoethanol) are added and pipetted vigorously to break up the tissue. The cells are then cultured in the same medium [10]. Cells between passage 1 to 4 are used for the experiment.

∗ **UV Source**

Ultraviolet A and B (UVA, 350 nm; UVB, 312 nm) light was supplied by a Westinghouse Fs-40 Sunlamp, which delivers uniform irradiation at a distance of 38 cm. The output of the Fs-40 Sunlamp is 23.4 units UVB light/hour UVB at a distance of 38 cm, where each unit is equivalent to 21 mJ/cm^2 of erythema-effective energy. Irradiation of mice with a single dose of ultraviolet B radiation (400-600 mJ/cm^2) or ultraviolet A radiation (30-50 J/cm^2) will perform on the conjunctiva of transgenic animals. In addition, *in vitro* studies in elastin promoter activity in response to ultraviolet B radiation (3-6 mJ/cm^2), and ultraviolet A radiation (1-3 J/cm^2) will also performed and tested in cultured conjunctival fibroblasts from transgenic mice for our hypothesis. We hope that we can apply this system to serve as a useful *in vivo* and *in vitro* model to study elastodysplasia and elastodystrophy in pterygium.

Cells were irradiated after immediately reaching confluence, and culture medium was replaced with phosphate buffered saline. After irradiation, phosphate buffered saline was substituted with fresh culture medium.

∗ Northern blotting hybridization

Total RNA is extracted from cultured cells or transgenic mouse embryo eyes using TRI-reagent® (Molecular Research Center, Cincinnati, OH) following manufacturer instructions. Ten μg of total RNA is electrophoresed in 1.3% agarose containing 2 M formaldehyde buffered with TAE (Tris/Acetate/EDTA). The RNAs are then transferred to Magna-Charge® membranes (Osmonics, Inc, Westborough, MA) and hybridized with 32^P-labeled human elastin, and gapdh (glyceraldehyde 3-phosphate dehydrogenase) cDNA, respectively, in a hybridization

solution containing 50% formamide at 42°C overnight as previously described ultures, which the method is previously descrivbed [9]. The excess 32^{P}-probes are removed by stringent washing three times with 0.1X SSC and 1% SDS (sodium dodecyl sulfate) at 65°C for 30 min each. Hybridization signals are detected with a phosphoimager (Molecular Dynamic, Sunnyvale, CA).

Result and discussion

1. We had prepare genomic DNA from human blood and Hela cell line.
2. Cloning and Sequence Analysis of Human Elastin Promoter：

PCR (polymerase chain reaction) was used to amplify the human elastin promoter from human genomic DNA. Try to integrate PCR product into pEGFP-1 plasmid, which carry green fluorescent protein (EGFP) reporter gene and transform the recombinant plasmid into DH5 α E.coli strain. We had screening and sequence many PCR products, but these are all not our target gene (human elastin promoter). Because the 2.8 kb human elastin 5' flanking promoter is locate on human chromosome number seven, so we according human chromosome seven sequence in NCBI gene bank to design forward and reverse primer again, and then do PCR to hope cloning human elastin promoter success. Because the human elastin 5' flanking promoter fragment is so long (2.8 kb) , we use special Taq (super Taq XL to purchase from PROTECH), which can do long chain elongation and has proofreading function to confirm elongation correct. Except the above-mentioned, we also adopt Touch Down PCR method、Nest PCR method, to get many likely PCR products and to do screening.

In order to easy insert PCR product into p-EGFP plasmid, we according p-EGFP vector MCS（multiple cloning site）sequence, design forward and reverse primer with restriction endonuclease sequence linker. Some wrong PCR products in gel electrophoresis had correct ban （2.8kb）, but after restriction enzyme digestion, PCR product can not

ligate to p-EGFP plasmid. We suggest these wrong PCR products may be a elastin pseudo gene.

From our primary results, it is very difficult to clone human elastin promoter by our PCR results. The reasons for these difficulties include the incorrect DNA sequence published in Genebank, the high GC ratio in the published DNA sequence, and the incorrect PCR methodology in the original designs. In the study of Uitto et at, they used a 5.3 kb human elastin promoter to generate a transgenic mouse model. But we could not find the whole human elastin promoter DNA sequence from Genebank. Therefore, we doubt the fidelity of DNA sequence published in Genebank.

We also test the fidelity for human elastin promoter DNA sequence, but the result was inconsistent. In the same time, we can correctly generate other transgenic mice by using PCR cloning methods. We strongly doubt the DNA sequence.

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