

Chapter 2.

Detection Techniques Used to Identify Meniscal Cell Production of Collagen Type I.

Introduction

Collagen is by far the most abundant protein in mammals constituting 25% of their protein mass.¹ The majority of skeletal tissues including the organic phase of bone (89%), and the dry weight of tendon (75%), ligaments (75%), cartilage (55%), and the meniscus (70%) are composed of collagen fibers.^{22,23} Collagen is composed of three α polypeptide chains that hydrogen bond to one another in such a way as to create a triple helix structure called the α -helix.

Collagen formation begins with each polypeptide chain being formed by membrane bound ribosomes and fed into the lumen of the endoplasmic reticulum (ER) within the cell. The polypeptide chain consists of three amino acid residues linked together with glycine as every third residue in the series and two other amino acids, most commonly proline and hydroxyproline (H-proline), claiming the remaining positions. It is in the ER that the amino acid residues, lysine and proline, are hydroxylated and cause hydrogen bonding to take place between the individual α -polypeptides to form the α -helix. In this stage the molecule is referred to as procollagen, termed as such to indicate that the molecule is not yet functional because of propeptides located at the molecules terminal ends. Vesicular transport carries the procollagen molecule to the cell membrane to which it fuses, releasing the procollagen molecule into the extracellular space.

Once outside the cell the propeptide ends are cleaved by enzymatic action allowing the collagen α -helix to spontaneously bond with other collagen α -helices to form a collagen fibril, to which the fibrils then bond together to form a strong cable like collagen fiber that compose the extracellular matrix of the tissue. Collagen fibers are resistant to tensile strain in the longitudinal or lengthwise direction of the fiber. The

structure of the collagen fiber gives flexibility and tensile strength to the tissues that it composes.

Type I collagen is the predominant collagen type in the meniscus accounting for approximately 90% of the collagen content.^{6,23} Collagen type I fibrils are composed of two α_1 and one α_2 chains that form the helix. The orientation of the fibers in the meniscus are predominantly in the circumferential direction, spanning from the posterior to the anterior attachment, to restrain hoop stresses developed within the tissue due to compressive mechanical loading.²³

Mechanical loading has been found to perform a maintenance function in musculoskeletal tissues including the meniscus by regulating the biochemical pathways responsible for tissue matrix protein production.^{7,8,10,18,19,26-28,30,33} Within meniscal tissue, the cells responsible for protein synthesis reside in the interstitial spaces while attached to the fiber matrix. Since the meniscus is a biphasic material with the majority of its overall composition being water, it is hypothesized that during compressive loading the fluid contained in the tissue moves within the tissue matrix causing fluid flow to occur in the porous spaces. Repetitive loading, such as caused by walking, creates a back and forth fluid flow referred to as oscillatory fluid flow (OFF). Shear stresses resulting from OFF are then thought to be transduced to the cell, stimulating protein production.^{2,9,12,13,15,25} Detection methods have been employed at various stages of collagen synthesis including measuring DNA (gene expression), mRNA(transcription), H-proline (formation in ER; detection after protein production) incorporation, and protein (cell secretion into extracellular space) levels. The most popular technique in the literature is H-proline detection by radioactive incorporation of [3H]-proline by the cells analyzed by

scintillation counting^{20,27,30,31} and sometimes includes separation through electrophoresis.^{3,4,24} Detection of H-proline is actually a measure of total protein produced by the cell since there are other cellular proteins produced that contain H-proline amino acid residues. However, because proteins containing H-proline other than collagen are so infrequent, and since collagen is the most abundant protein produced by musculoskeletal cells, this detection method has been used as an estimate of collagen synthesis.

Measures of mRNA by the reverse transcriptase polymerase-chain reaction (RT-PCR) technique are also used for collagen detection.^{5,14,29,32} RNA must first be extracted from the cell of which there are many procedures and kits available,^{5,14,29,32} then by use of a specified primer the PCR is performed creating DNA copies of the gene of interest essentially amplifying the signal for detection. Northern blotting, which detects extracted mRNA directly can also be used,⁴ but this techniques is rarely performed. However, because posttranslational modifications occur before a protein can become functional, mRNA analysis may not be an accurate measure of the amount of collagen actually secreted from the cell essentially forming the extracellular matrix.

Western blotting is a method that is used to detect functional proteins after they are produced by the cell. Both procollagen and collagen can be detected using this method.^{4,16,21} A mixture of proteins are separated using electrophoresis based on their electrical charge, which corresponds to the molecules weight or size, and then transferred to a membrane to which the proteins become affixed. Antibodies are applied to the membrane that are specific to react or bind with the protein of interest, and these complexes are then detected by chemiluminescence and film development.

The focus of this research is to determine if collagen type I production by meniscal cells is promoted by OFF induced shear stress measured through Western blotting detection techniques. The implications of this research will be used in tissue engineering efforts to create tissue constructs, as well as to aid in determining the mechanisms of tissue creation and degradation.

Methods

Cell Culture and Protein Studies were performed as described previously in Chapter 1.

mRNA Cell Isolation and Storage

Samples were prepared and tested exactly the same as described for protein studies. After the termination of OFF glass slides containing meniscal cells were transferred from the multichannel flow chamber to a new Petri dish with 8 ml of fresh post flow media. After a 6 or 24 hour incubation period to allow mRNA type I collagen synthesis,^{17,29} the media was aspirated from the slide and 1 ml of trypsin was placed on the slide for 10 min to detach the cells from the slide. The cell mixture was then pooled and collected in a 1 ml pipette and transferred to a cryogenic vial and flash frozen in liquid nitrogen²⁸ for later mRNA analysis using RT-PCR by Eric Blough's lab at Marshall University, Huntington, WV.

Total Protein Isolation

Total cell protein content was used to determine the volume of cell lysate and media to add to the gels necessary to yield an estimated amount of type I collagen. Total protein for lysate and media samples were performed as stated previously in Chapter 1.

Concentration of Samples

Preliminary data proved that there was not enough protein in the maximum volume (10 μ l) that could be applied to the 30 μ l wells of the 7.5% SDS-polyacrylamide gels without sample concentration. Varying amounts of sample were placed into 15 ml conical tubes containing concentration filters (Millipore Amicon Ultra 10,000 MW with a 4 ml sample volume) and centrifuged at 4500 rpm (5000 g) for 20 min at room temperature to yield a 50 μ l volume of the concentrated sample.

Western Blotting

Western blotting^{11,21} was used to quantify the amount of collagen type I proteins produced by meniscal cells after OFF stimulation. Total protein was quantified using the Lowry Method (Bio-Rad DC Protein Assay II, Hercules, CA) with albumin as a standard for the concentrated lysate and media from each sample. Twice the amount of Lamelli Sample buffer (BioRad) containing 0.5% β -Mercaptoethanol (BioRad) was added to each sample, and the mixture was heated to 100 °C for 3-5 min to denature existing proteins. The sample mixtures, containing equivalent amounts of protein, were loaded onto 7.5% SDS-polyacrylamide gels (BioRad), resolved by electrophoresis and transferred overnight in transfer buffer (18 hrs) to an Immun-BlotTM polyvinylidene difluoride (PVDF) membrane (BioRad). A representative amount of collagen type I human placenta (Calbiochem) was used as a positive control. The membranes were blocked in 5% non-fat milk in TBST (1X Tris Buffered Saline (Bio-Rad) with 0.5% Tween-20) for 2 hours at room temperature, incubated in a 1:20 dilution of rabbit anti-collagen type I polyclonal antibody (Oncogen Research Products, San Diego, CA) in blotto (5 % non-fat milk in TBST) overnight (18 hrs) at 4°C with rocking, washed three times in TBST, incubated with Goat anti-rabbit IgG linked to horseradish peroxidase (Calbiochem) for 1

hour in blotto diluted 1:2500, and washed five times in TBST.²¹ The proteins were visualized through enhanced chemiluminescence by covering the membranes in ECL detection reagents for one minute (Amersham, UK). The membranes were then immediately exposed to X-ray film for 5-10 min, after which the film was placed in developer and fixer for 5 min, washed with water, and viewed.

Results

The use of western blotting techniques could not detect any collagen type I in the samples regardless of condition, be it flow or the no flow control. The fact that the collagen type I human placenta positive control was visualized at varying concentrations from 1 to 10 μg suggests that the Western technique and all associated chemicals and equipments were working properly. Smear bands appeared near the top of the gel in the concentrated media samples from rabbit specimens 10, 11, 13, and 14 that most likely pertain to aggregate molecules that precipitated out of solution during electrophoresis (Oncogene). Specimen 10 and 11 concentrated media trials contained 56.8 μg of total protein, and specimen 13 and 14 concentrated media trials contained 85.5 μg of total protein in 10 μl of concentrated samples.

Specimens 13 and 14 contained bands that appeared to coincide with the bands produced by the positive control, and having molecular weights comparable to the α_1 (210 kDa) and α_2 (95 kDa) chains as compared to the rainbow marker. The results were found to be similar to those obtained by Goncalves-Neto et al., 2002 and Lim et al., 2002 who also used Western blotting techniques to detect collagen type I.^{11,21} However, the 10 min exposure time necessary to visualize the characteristic Western bands created a very dark image and background that made it difficult to distinguish the individual bands and

impossible to effectively analyze the films using densitometry. A problem also existed in the uniformity of the individual lanes as the samples were run on the gels as shown in Figure 2. The run line had a tendency to frown as it was descending. Unsatisfactory results and a depletion of resources led to the termination of further testing using Western blotting by this group to detect collagen type I. Figure 1 contains the concentrated media sample films from Specimens 10 and 11, and Figure 2 contains sample films from specimens 13 and 14. Table 1 gives a representative summary of the Western blotting procedures used and results gained from each Western blot performed by this group to date.

Discussion

Western blotting techniques were employed to detect whether or not functional type I collagen used to compose the extra-cellular matrix was increased due to OFF induced shear stress in cells isolated from the meniscus. A parallel plate flow chamber was used to apply OFF to the monolayer of cells cultured onto glass slides. Collagen type I was unable to be detected by Western blotting techniques as performed in this study for either the flow or control condition. Findings of this study suggest that meniscal cells cultured in monolayer are not able to produce significant amounts of collagen type I.

The sensitivity of the primary antibody was limited to 1 μg of collagen type I as indicated by trials where the positive control was considered visibly adequate. The results of this study suggest that there was not enough collagen in the concentrated media samples to be visualized. However, Lim et al., 2002 was able to adequately detect collagen type I bands using only 50 μg of cell lysate protein or 100 μl of conditioned

media, suggesting that there was more than enough protein in specimens 10-14 for detection using this method.²¹

Primary antibody dilution was maximized at 1:20 with an incubation time of 18 hrs at 4°C. A secondary dilution of 1:2500 with an incubation time of 1 hour was sufficient in detecting the positive control. Modification of the blotting technique used as stated in this study is not suggested, and is not believed to be the reason for the negative results.

The primary antibody itself is specific to human, and has been found to be cross-reactive with rabbit. The sensitivity of the primary antibody to rabbit may not be sufficient to detect the small quantities of collagen that exist in the samples. The secondary antibody used in this study is specific to human. It would be beneficial to find a primary antibody that is specific to rabbit type I collagen as well as a secondary antibody that is reactive with rabbit species. To this date there is not a primary or secondary antibody of this kind.

The media used in the samples contained DMEM/Ham's F12 with phenol red supplemented with 2% FBS and 1%P/S. It was evident during sample concentration that the phenol red in the media also became quite concentrated giving the samples a dark red appearance. During electrophoresis the run line separated into a red line represented by the phenol red in the media of the samples, and a blue line (normal) caused by the Lamelli sample buffer used to reduce the samples. Further investigation may be needed to determine if phenol red might interfere with the electrophoretic process in Western blotting, at least when samples using media containing phenol red are concentrated.

The 7.5% SDS polyacrylamide gels used during the last trial to test specimens 13 and 14 (S/N: 681 and 682; Lot: L080403AS; Ex. Date: 19-Sep-03) did not appear to be in very good condition. The well walls in both of the gels were hanging and in one case the well openings were blocked by a hanging piece of gel that spanned at least 5 of the 10 lanes. It was obvious during sample loading that the well volume may have been compromised because of the tilted well walls. This may have effected the trial during electrophoresis causing the gel to run unevenly creating the frowned appearance. The results of this trial were not used or recorded due to the impact of the imperfect gels.

Varying the incubation time of the membrane with ECL reagents, exposure time, and incubations of the film in developer may provide a way to reduce the background noise (darkened appearance) and allow the bands to become more pronounced. An automatic developer would aid in this process, but the only way to verify this is through trial and error.

If indeed more protein is needed to receive an adequate amount of collagen type I for visualization, a greater amount of media could be concentrated and cell lysate volumes could be pooled and concentrated from multiple samples. Media samples for specimens 13 and 14 were concentrated from 4 ml down to 50 μ l to give a total protein yield of 85.5 μ g in 10 μ l that was then loaded onto the gel after reduction. The concentration filter tubes used in this study were only able to hold a maximum volume of 4 ml.

It may be beneficial to employ another means to detect collagen synthesis by meniscal cells. The most favorable technique at this time would be RT-PCR to measure mRNA levels of type I collagen. This process may amplify the type I collagen signal

enough to allow a comparison to be made between the flow and no flow groups. Additional samples have been collected for no flow and flow conditions at 4 Pa and 1 Hz frequency for a 1 hour duration with 6 and 24 hr incubations to be used for mRNA analysis using RT-PCR. Samples will be transferred, and mRNA analysis will be performed, by Dr. Blough's laboratory at Marshall University, Huntington, WV. Alternative methods used to measure H-proline total protein content without radiolabeling may also be of use to discover the relative amount of collagen between conditions.

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Figure Legend

Figure 1. Western blotting results from specimens 10 and 11 using media concentrated from 300 μl to 50 μl yielding a total protein concentration in the media of 56.8 μg in 10 μl of sample. Gel loading sequence given in Table 1. Exposure times and date performed are indicated on film.

Figure 2. (A) Western blotting results from specimens 13 and (B) 14 using media concentrated from 4 ml to 50 μl yielding a total protein concentration in the media of 85.5 μg in 10 μl of sample. 10 min exposure time was used directly after application of ECL reagents followed by a soak in developer and fixer for 5 min. Gel loading sequence given in Table 1.

Figure 1.

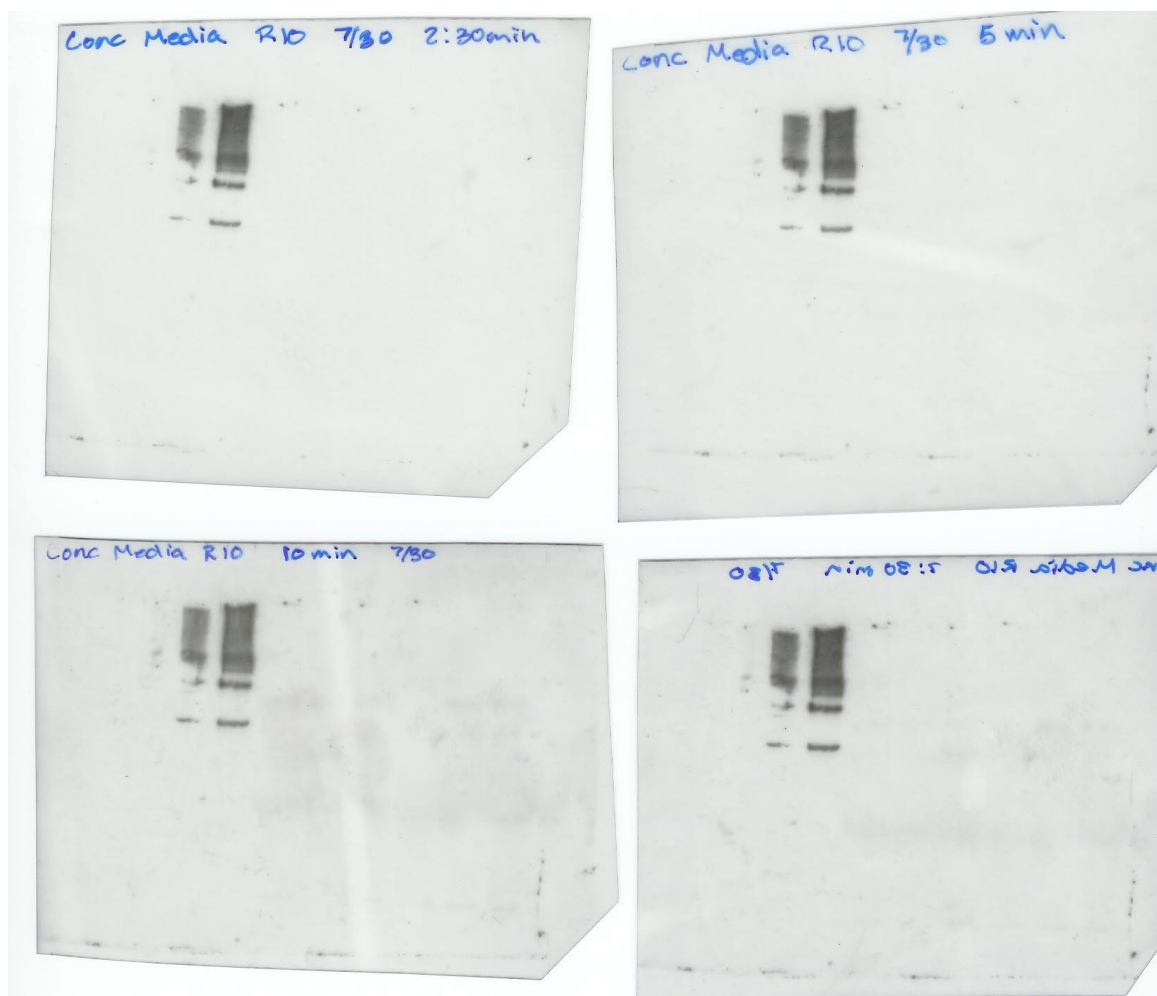


Figure 2. (A)

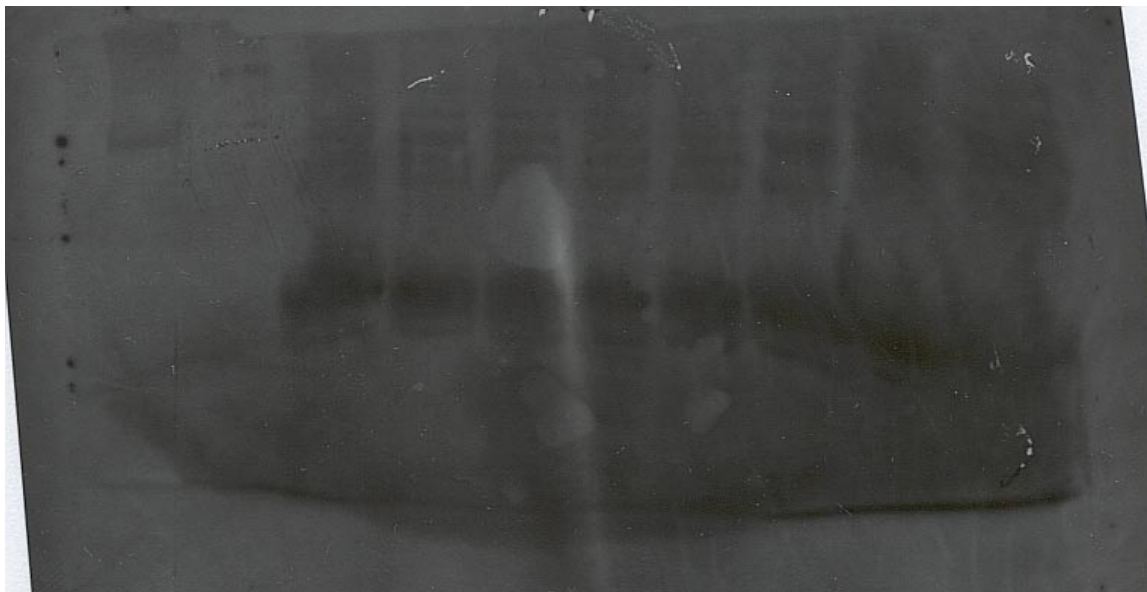


Figure 2. (B)

