TELOPEPTIDES OF TYPE II COLLAGEN BINDING TO HUMAN ANKLE CHONDROCYTES

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**INTRODUCTION:** Type II collagen, a major component of cartilage, consists of a triple helical domain along with N and C non-helical domains. Various enzymes can generate a variety of naturally occurring fragments. Matrix metalloproteinase – 3 is known to cleave type II collagen within the N-telopeptide; this cleavage releases N-telopeptide in the process disturbs the fibrillar network. Annexin-V, a 34-kDa protein, can bind intact type II collagen and the N-telopeptide. Unique genes encode Annexin family and exhibit a wide range of functions including cell-matrix interaction, calcium ion channeling and signal transduction. Protein Kinase C (PKC) is one of the kinases that is involved in the cell-matrix interaction as well as signal transduction. The activation of PKC by influx of calcium is also linked to the reorganization of actin cytoskeleton, cell mobility and adhesion. Studies indicate that binding of integrins to the extracellular matrix proteins leads to PKC activation. In order to further investigate cell-matrix interactions (initial events of peptide binding to the annexin-V), PKC activation was studied. It is also unclear which is the initial domain of type II collagen that is involved in (N, C, and helical peptides) binding of the protein to the cell surface. The objective of this work was to characterize peptide interactions and its subsequent effects on the intracellular calcium levels and PKC activation.

**METHODS:** In order to define the effect that collagen telopeptides have on surface binding, synthetic peptides (synthesized by Dr. Lee (University of Illinois at Chicago) ) were generated to correspond to the N, C, and helical-peptides of human type II collagen. The sequences were obtained through the NIH molecular biology database. Intact type II collagen was used as a control. Collagen II fragments were prepared from adult bovine cartilage. The fragments were generated by digestion with Collagenase (type V) and enzyme/20 mg collagen at 37°C for 48h). The digested sample was filtered through an Amicon filter (p10, allows <10 kDa to pass) to remove bacterial collagenase and large fragments. The type II collagen fragments (<10 kDa) are enriched with N- and C- terminal telopeptides.

**Cell culture:** For cell suspension cultures, the chondrocytes were obtained from human ankle joints from Regional Organ Bank of Illinois (ROBI) and released by pronase and collagenase-P.

**Flow cytometry:** Chondrocytes were suspended for 2h at 37°C in DMEM / Ham’s F12 and 5% Fetal Bovine Serum (FBS) and then incubated with calcium indicator fluo-3, AM cell permeant probe (Molecular Probes) for 30 min at room temperature. The above probe is nonfluorescent unless bound to Ca++ . Fluo-3 has been used for experiments involving phototoxicity of “caged” chelators and second messengers. The cells were subsequently washed and incubated with either 1 mg/ml synthetic N-terminal peptide, 1 mg/ml synthetic C-terminal peptide, 1 mg/ml synthetic helical peptide or 1mg/ml type II collagen for 1h at 37°C. The cells were analyzed by FACScan.

**Immunofluorescence:** Nikon Eclipse TE200 confocal microscope with a Meilis Griot 43 series ion laser along with a metaMorph program was used to capture the images.

**Western Blot:** Chondrocytes were suspended for 2h at 37°C in DMEM / Ham’s F12 and 5% Fetal Bovine Serum (FBS) and then incubated with either 1 mg/ml synthetic N-terminal peptide, 1 mg/ml synthetic C-terminal peptide, 1 mg/ml helical peptide or 1mg/ml type II collagen for intervals from 5 minutes to 1hr at 4°C. Phospho-Protein Kinase C (PKC pan) antibody was used to detect the endogenous levels of phosphorylated PKC in the presence of phospholipids and Ca++. Activation of PKC leads to variety of cellular responses such as gene expression, proliferation and secretion.

**RESULTS**

**Binding of the peptides to the cell surface results in the release of Calcium**++. Flow cytometry analyses showed that the addition of intact type II collagen to the cell suspension resulted in a calcium release within the first 20min of incubation, which was followed, by a gradual release of calcium over time (data not shown). Two different levels of calcium influx were observed with N and C-telopeptide. Incubation of cells with N-telopeptide demonstrated a rapid calcium release within the first 5-min which was followed by a decrease and a second increase at 20 min. A similar pattern was observed with the C-telopeptide as for the N-telopeptide. These rapid increases were not seen with the helical peptide, but rather a gradual increase that remained at the same level over one hour of incubation.

Confocal microscopy confirmed the flow cytometry results. When the N-telopeptide was added to the cell suspensions, we observed calcium release within the first five minutes of telopeptide addition. The C-telopeptide had a similar pattern of release, however the fluorescence intensity of both C-telopeptide and helical peptide was less intense than that of the N-telopeptide. Control background fluorescence did not alter over time.

**Addition of N-telopeptide, C-telopeptide, helical peptide and type II collagen resulted in the release of calcium and thus activation of PKC.**

In order to determine whether influx of calcium resulted in the activation of the signal transduction pathway western blot analyses were used to detect the activation of PKC. The profile of PKC-pan demonstrated an increase over time (30min to 60min) in activated PKC with mixture of fragments, while incubation with helical peptide, intact type II collagen and C-telopeptide showed no difference over 1hr incubation. Also, incubation of cells with N-telopeptide showed a decrease (30min to 60min) in calcium release and thus PKC.

**DISCUSSION**

From our previous work we know that the N-telopeptide of type II collagen binds via annexin-V. Flow cytometry data demonstrate that upon N-telopeptide binding to the cell surface calcium is rapidly released. The release of calcium was also observed with other peptides, C-telopeptide and helical peptides, but to the lesser extent. Further investigation with the PKC antibody demonstrated a gradual decrease over time in the PKC activation with the N-telopeptide. The rapid release of calcium with N-telopeptide might suggest that this is the initial region where type II collagen binds to the cell surface and potentially controls the short-term metabolic activities towards matrix homeostasis.

**REFERENCES**


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