Immuno-SEM characterization of developing bovine cartilage

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Abstract

Collagen is a vital material in the tissues of living organisms. Found almost everywhere in the human body, collagen is important in connective tissues, bone growth, and cartilage. Collagen XI makes up a very small portion of the cartilaginous tissue; however, it plays a key role in cartilaginous tissue. Collagen XI and two collagen XI isoforms, V1b and V2, are critical in the ossification process. The location of collagen XI, V1b, V2, and their specific functions in the ossification process within developing bovine cartilage are not well characterized. In this work, the location of collagens I, II, XI and two collagen XI isoforms, V1b and V2, present in developing bovine cartilage are investigated using the immuno-SEM technique. The results for the locations of collagen I and II indicate a high level of consistency with previous work, thus showing that the technique of immuno-SEM can be used with confidence to determine the location of various collagen types within cartilaginous and mineralized tissue. This work has shown that collagen XI is present in the lower hypertrophic region and also in a pericellular arrangement, within about two microns of cell walls, throughout the cartilaginous tissue. V1b is expressed in the articular surface, mineralized region, resting zone, and the distal edge of the diaphysis. The V2 isoform is most strongly expressed in areas of newly forming cartilage, and disappears with chondrocyte maturation. V2 is present in the distal edge of the epiphysis, as well as in mineralized tissue. Collagen XI and two of its isoforms, V1b and V2, are thought to play a critical role in the ossification process. However, this role is not well understood, and is still being characterized. The detection of collagen XI and two of its isoforms in the osteo-chondral junction as well as at a joint surface further point to collagen XI, V1b, and V2 playing a vital role in the ossification process, and warrants further research as to their specific function within the ossification process.

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1. Introduction

Immuno-SEM refers to the attachment of colloidal gold particles, through antibody interaction, to a surface of interest, followed by imaging of the surface in a scanning electron microscope (SEM). Using gold nanoparticles to label specific proteins within the cartilaginous matrix and then quantifying the labeling is an approach to cartilage characterization that avoids the drawbacks associated with von Kossa staining and transmission electron microscopy (TEM). The limitation associated with von Kossa staining arises from the auto-fluorescence of mineralized tissue, which hinders the ability to distinguish between staining or labeling and mineralized tissue via TEM. The method of immuno-labeling, or attachment, to biological surfaces uses a primary antibody, which binds to specific epitopes on the antigen surface, and a secondary antibody that binds to specific regions on the primary antibody. To attach the colloidal gold particles, streptavidin is conjugated to the colloidal gold particles, and a biotin molecule is bound to the secondary antibody [1]. After immuno-labeling, the surface is imaged via SEM. It follows then that if an antibody specific to a particular antigen of interest is used, the presence of gold particles can prove the existence or absence of the particular antigen [1].

Collagen is the most abundant and vital protein in mammals. It is important in skin, eyes, organs, connective tissues such as tendons and ligaments, blood vessels, bone growth, and even cartilage [2]. Presently, there are more than twenty-eight known different types of collagen [3]. Cartilage can be generalized as the interaction of an extensive network of collagen fibrils,
primarily collagens II, IX, and XI, and a highly hydrated complex of proteoglycans, which are embedded within the collagen matrix [4,5]. Collagen fibrils resist pulling forces, giving them strength in tension, while proteoglycans form an uninterrupted triple helical domain, consisting of not well characterized. Collagen XI contains a 300-nm ossification process, but their location and specific functions are compressive forces.

In this work, collagen XI is of particular interest. Collagen XI and two collagen XI isoforms, V1b and V2, are critical in the ossification process, but their location and specific functions are not well characterized. Collagen XI contains a 300-nm uninterrupted triple helical domain, consisting of α1, α2, and α3 collagen chains [4]. Collagen XI is a constitutive element of the interstitial collagen fibrils of fetal cartilage [6]. Although collagen XI does not constitute the majority of the cartilage matrix, its presence is essential for the regulated assembly, organization, and development of cartilage. Presently six isoforms of collagen XI are known to exist in developing cartilage [7]. Two of these isoforms, V1b and V2, are the primary focus of this work. Immunohistochemistry has shown that staining for both collagen XI isoforms was observed in the longitudinal septa, particularly in the lower hypertrophic and mineralized zones, and staining was also observed in a very restricted pericellular pattern in the resting zone [5]. Staining intensity diminished dramatically in the upper proliferative zone and was restricted exclusively to the perimeter of cells in the resting cartilage.

Immunoelectron microscopy has shown that collagen XI antibodies preferentially labeled thin fibrils with diameters <20 nm [5]. Since these thin fibrils are most concentrated in the hypertrophic and proliferative zones of the growth plate, the frequency of labeled fibrils decreases to zero in areas progressively closer to the resting zone [5]. Immunofluorescence staining and TEM have shown that prior to primary ossification, V1b is detected only in the diaphysis, primarily adjacent to the peristome, and not in the epiphysis. The V2-containing isoforms are most strongly expressed in areas of newly forming cartilage, and disappear as chondrocyte maturation proceeds [6].

In this work, the technique of immuno-SEM was used to verify prior results of immuno-staining and TEM locations of collagen I and collagen II in fetal bovine cartilage, thus establishing immuno-SEM as a technique that can be used to characterize cartilage. This technique is then used to further characterize the locations of collagen XI, V1b, and V2 within developing bovine cartilage. A better understanding of protein organization within the various cartilaginous and mineralized regions of bovine cartilage, particularly in the osteo-chondral junction will result from the use of this technique.

2. Experimental methods

2.1. Gold Conjugate Labeling

The femoral head of a fetal bovine obtained from Gem Meat Packing (Garden City, Idaho, USA) was isolated and fixed in an aqueous formaldehyde fixative containing zinc prior to embedding in paraffin. The samples were subsequently sectioned and affixed to glass slides. The slides were deparaffinized and treated with 0.1 mg/ml of hyaluronidase diluted in 1×TBS for 30 min at room temperature. The slides were then rinsed in buffer containing 10 mM Na2HPO4, 3 mM KH2PO4, 123 mM NaCl and adjusted to pH 7.2, followed by blocking in this buffer containing an additional 2% BSA for 30 min at room temperature. Subsequently, the slides were incubated in primary antibody at the appropriate dilution in the blocking buffer for 1 h at 37 °C. The following primary collagen antibodies were used: monoclonal Type II (Neomarkers, Fremont, CA, USA, 1:100 dilution), monoclonal Type I (Abcam, Cambridge, MA, USA, 1:200), rabbit polyclonal Type XI recognizing the c-telo peptide (1:100), monoclonal Type XI recognizing p6b (not diluted), rabbit polyclonal Type XI recognizing p8 (1:100). After incubation in primary antibody, the slides were washed 3 times for 5 min in the buffer containing 10 mM Na2HPO4, 3 mM KH2PO4, 123 mM NaCl and adjusted to pH 7.2. The appropriate secondary antibody, either anti-mouse or anti-rabbit, conjugated to biotin (Jackson ImmunoResearch, Laboratories, Inc., West Grove, PA, USA) was diluted in buffer (1:200) and incubated on the samples for 1 h at 37 °C. The slides were washed 3 times for 5 min in buffer, prior to incubating the slides for 45 min at 37 °C with 40 nm colloidal gold particles conjugated to streptavidin (Ted Pella Inc., Redding, CA, USA) and diluted in buffer (1:25). The samples were then washed in buffer for 10 min, followed by 4 additional washes for 5 min each in dH2O. After the last wash, the samples were fixed in formalin for 2 h and dehydrated. A control sample was also prepared similarly to the other samples, except that the primary antibody step was omitted. Any labeling present in the control sample is a result of non-specific antibody interaction, and shows the level of labeling that can be expected as background in each sample.

2.2. Scanning electron microscopy

After the labeling with 40 nm colloidal gold particles was completed, samples were sputter-coated with 12 nm of Cr and imaged in a LEO 1430 VP SEM. An accelerating voltage of 30 kV and a spot size of approximately 330 nm were used. Several images of each zone (mineralized, hypertrophic, proliferative, resting, articular surface, and periosteum) were collected. Images of these zones are shown in Fig. 1.

At a magnification of 50,000×, backscatter and secondary electron imaging was used. The number of gold particles or “dots” attached was determined using images collected in backscatter mode. As was observed during sampling, dot counts immediately around and inside a cell were significantly higher than in the surrounding matrix. Thus, the images that included all or part of a cell were separated from the calculations concerning the extracellular matrix.

Due to sample differences, it was difficult to obtain images of the lower hypertrophic region, just below the periosteum,
3. Results/discussion

3.1. Data analysis

In order to analyze the large data set generated here, the method of cumulative fraction was used to analyze the distribution of the collagen types within each region. For each type of collagen, 10 frames were typically collected within each region of the specimen and the number of gold dots in the frame (area = 34 μm²) was counted manually. Next, the measured dot densities were sorted from smallest to largest within each collagen type and region. The cumulative fraction (Fig. 2) was then determined, where the fraction of frames which had at least a given dot density is plotted as a function of dot density. This method allows the large data set to be easily examined and different regions or collagen types compared to one another. For example, steep curves in Fig. 2 indicate a narrow distribution of dot densities and a small standard deviation in the measurements for a given collagen type in a particular region (e.g., collagen II in the proliferative zone, Fig. 2c). In contrast, broad curves indicate that the data covered a large range of dot densities and the standard deviation of the measurements was high (e.g., collagen II in the mineralized zone, Fig. 2c). Curves that are shifted to the left indicate that the dot densities are low, while curves shifted to the right indicate higher dot densities. Finally, the maximum dot density is easily determined as the value where the curve reaches 1 on the y-axis.

3.2. Imaging of mineralized tissue using the technique of immuno-SEM

Using immuno-SEM to locate the specific proteins of collagen XI, V1b, and V2 within fetal bovine cartilage, and then quantifying the labeling, is a new approach to bovine cartilage characterization. One major advantage of using SEM to detect colloidal gold particles is the quantification capabilities that arise from gold labeling[8]. The colloidal gold particles can be counted with high accuracy and confidence due to the almost non-existent background from proteins, which appear almost transparent to an electron beam[9]. This study takes full advantage of the quantification capabilities that arise from the clear gold labeling of proteins, and develops the technique of immuno-gold labeling.

3.3. Control labeling

Control labeling shows background levels of labeling that should be present in each region (Fig. 2a). Low levels of background labeling are expected when examining biological tissues[10]. There are several different sources for background labeling. For example, there may be non-specific interaction between the secondary antibody and the sample surface. Another possibility is a biotin or streptavidin interaction with the sample surface. Some of the proteins present in cartilaginous
and mineralized tissue may have an affinity for biotin or streptavidin. Since these were the two proteins used to bind the gold to the secondary antibody, they would be present in large enough quantities to cause such an interaction. Labeling on the control sample showed relatively low levels in every region except the resting zone and portions of the hypertrophic region. It is possible that these regions contain larger amounts of non-specific binding proteins than other regions. It may also be the case that biotin and streptavidin have a higher affinity for the proteins present in these regions.

3.4. Collagen I labeling

Due to the way in which the sample labeled for collagen I was sectioned, the mineralized, hypertrophic, and proliferative regions were absent from this sample. However, the regions present did show collagen I, see Figs. 2b and 3. We know from previous work that collagen I can be found in regions of fibrous, non-cartilaginous tissues as well as in the tissue surrounding the cartilage matrix, namely the periosteum [6,11–13], and has low levels in the resting zone, which is mostly type II [6,11,12,14–16]. Collagen I has also been shown to be in the articular surface [6,12,13,17]. Upon examination of the regions that were present (periosteum, resting zone, and articular surface), collagen I exhibited the highest labeling in the periosteum and the articular surface, while the resting zone showed low levels of labeling (see Fig. 2b). The continuity between the previous work and the present work for the location of collagen I within cartilaginous tissue provides strong evidence that immuno-SEM is a viable technique for locating proteins within cartilage.

3.5. Collagen II labeling

In the present study, four of the six regions examined showed labeling for collagen II (Fig. 2c). Collagen type II is typical of vertebrates, and should be seen in all regions of cartilage.
3.6. Collagen XI labeling

In the case of collagen XI (Fig. 2d), the amount of labeling in four of the six regions is below the control levels. This result is surprising as fetal cartilage is the most abundant source of collagen XI [23], and its role in cartilage is vitally important [4]. However, collagen XI makes up a relatively minuscule amount of the entire cartilaginous or mineralized matrix [18–20], and has been shown to be restricted to the pericellular region of the cartilaginous matrix, specifically in the thin fibrils of the hypertrophic region and also in mineralized tissue [19,20,24–26]. This work shows limited labeling in both the mineralized and hypertrophic regions. An important point to note here is that the main area of tissue examination consists of the interterritorial matrix and does not include the pericellular matrix. It follows then that since collagen XI has been shown to be more concentrated directly around cells and become less concentrated further from cells, this study would show low levels of labeling, even in the hypertrophic region. Thus, it is possible that the background antibody interactions are more abundant than those of collagen XI within the interterritorial matrix.

3.7. Collagen V1b labeling

The results for the collagen V1b sample, shown in Fig. 2e, showed the highest labeling occurred in the mineralized, resting, and articular surface regions. These regions were followed by the periosteum, and lastly, by the hypertrophic and proliferative zones. The localization of collagen V1b within fetal bovine cartilage is a new area of study that is being developed. Prior studies have shown that V1b should be largely and uniquely concentrated in the periphery of the lower hypertrophic region, just under the periosteum, of fetal rat cartilage [6]. Prior work has also shown a significant amount of V1b in mineralized tissue [27]. The data from this study, showing the relative amounts of V1b in both the mineralized tissue and periosteum, strongly support the presence of V1b in mineralized tissue and in the periosteum. V1b is thought to play a key role in the ossification process [6,27,28]. The periosteum, a region located near the osteo-chondral junction, is likely involved in the ossification process. The abundant labeling of V1b observed within the periosteum further points to a probable involvement of V1b in the ossification process.

The abundant labeling for V1b in both the articular surface and resting zone has not been seen before, and may be unique to fetal bovine cartilage. Examination of cartilage from different species often shows different kinds and concentrations of proteins from the same region of tissue [12,20]. A study of the articular surfaces of various species revealed that rabbits’ do not contain collagen II, while human newborns’ do [12]. The absence of V1b from the articular surface and resting zone in fetal rat cartilage does not necessarily hold for fetal bovine cartilage. Fetal bovine cartilage might simply contain V1b within the articular surface and resting zone. The structural demands of bovine may cause a need for V1b in the articular surface and resting zone.

3.8. Collagen V2 labeling

Distinct labeling for V2, Fig. 2f, was seen in the articular surface, and the mineralized region contained a moderate amount of labeling. These results are consistent with data from previous work [6,27,28]. It has been shown that V2 exists primarily in the distal edge of the epiphysis, which is referred to as the articular surface in this study [6]. V2 has also been shown to be present in mineralized tissue, which corroborates the data collected in this study showing moderate levels of V2 in the mineralized region [27]. In this study, V2 is abundant in the periosteum. This has not been shown before, and may be unique to bovine cartilage. The specific function of V2 in tissue is not well understood. However, the presence of V2 in mineralized tissue, a joint surface, and the osteo-chondral junction suggests that it may play a critical role in the ossification process. V2 has
been found to be generally distributed throughout the epiphysis in very young fetal rat cartilage, and disappears as the cartilage develops [6,28]. The moderate amount of V2 seen in the resting and proliferative zones suggests that the fetal bovine cartilage used in this study was very young.

3.9. Field emission scanning electron microscopy results — spatial inhomogeneity

The limited resolution of the LEO 1430 VP SEM, compared to that of a field emission SEM (FESEM), gave rise to the question of whether there was spatial inhomogeneity of collagen XI in the hypertrophic region. While collecting data in the hypertrophic region of the sample labeled for collagen XI there were some data points with a very high dot count and others with a rather low dot count. Using the FESEM, data was collected to resolve the uncertainty. In Fig. 4, a high-resolution image of the hypertrophic region in the sample labeled for collagen XI, intense labeling, seen as bright dots, occurs around the periphery of the cells and diminishes as the distance from the cells increases. Depending on where the lower resolution images were taken, either a high or low density of dots could be observed, leading to the conclusion that there is spatial inhomogeneity. The frames encountered with high dot counts are still valid data, but they were analyzed separately from the frames with lower dot counts.

Quantification of the frames with high dot density showed that the labeling is highest within a distance of approximately two microns from the cell wall. Measurements beyond two microns revealed significantly diminished amounts of labeling. The distribution of collagen XI fibrils in a pericellular arrangement as well as collagen XI being localized to thin fibrils has been shown in previous work [5,19,20,24–26]. This present work then suggests that the fibrils directly around cells are both abundant in collagen XI and also of a finer nature.

4. Conclusions

The immuno-SEM technique is a useful tool to investigate the localization of specific proteins within the cartilaginous matrix and also within mineralized tissue. The technique provides ease of visualization of the location of specific collagens within cartilage as well as mineralized tissue. Due to the clear and definitive labeling that can be seen when using backscattered electron imaging, the technique of immuno-SEM can be used to quantify amounts of different collagens present in cartilage and mineralized tissue. The present study examined the use of the immuno-SEM technique to label various collagens within cartilaginous and mineralized tissue. Many consistencies were shown in the locations of collagen I and II, thus showing that the technique of immuno-SEM can be used with confidence. Collagen XI was shown in the lower hypertrophic region and also in a pericellular arrangement, within about two microns from cell walls, throughout the cartilaginous tissue. V1b was expressed in the articular surface, mineralized region, resting zone, and the distal edge of the diaphysis. The V2 was shown to have the greatest presence in the articular surface with some labeling seen in the distal edge of the diaphysis and in mineralized tissue. The results from this study suggest that collagen XI, V1b, and V2 play a critical role in the ossification process, and warrant further research as to their specific function within the ossification process.

References