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Proteolytic enzymes in skeletal development: histochemical methods adapted to the study of matrix lysis during the transformation of a "cartilage model" into bone

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1. ABSTRACT

The replacement of a "cartilage model" by definitive bone is characterized by a series of localized excavations of the cartilage which are eventually followed by bone deposition. Each excavation requires lysis of cartilage components (defined here as the breakdown of a peptide bond) and their eventual resorption (defined here as microscopical visible cartilage loss). More precisely we have proposed that the lysis is affected by proteases capable of breaking down the main proteoglycan "aggrecan" and the main fibril element, "type II collagen". Four approaches combining biochemical, immunologic and microscopic techniques have been adapted to test this hypothesis. Each is applied to the rat tibial head's "cartilage model" where proteases have been shown to be major contributors to secondary ossification center formation. The approaches have been found both effective and distinct as cartilage resorbing enzymes have not only been identified but also detected *in situ* before and after activation. Achieved overall is an understanding of when, where and how specified proteases contribute to tissue component lyses. While the focus resides on the *in situ* proteolysis of cartilage, three of the approaches could be translated without change to other tissues, whereas one may require tissue specific adjustments before use.

2. INTRODUCTION

Most bones appear in the embryo in the form of a "cartilage model" that is initially a miniature of the definitive adult bone (1). The transformation of a "model" into genuine bone – necessarily requires the resorption of cartilage. Thus in development but also throughout childhood, the epiphyses and especially their growth plate components are active sites of skeletal change and these sites rely upon a normal progression from cartilage to bone to mediate bone shape and growth.

Though it had been suspected for some time that proteases likely contributed to the destruction of the "cartilage model" (reviewed in 2; 3; 4, 5, 6) it remained difficult to prove direct involvement. First, the modes of tissue destruction are complex and even today, it is impossible to reproduce the cartilage excavations of interest to us *in vitro*. Second, biochemical procedures in place for enzyme extraction from tissues are ill-suited to detect the trace amounts of enzyme present in the complex remodelling sites under study. Finally, even when substantial amounts of tissue are available, to quote a recent review " conventional methods of proteomics at present involve denaturation of all proteins in a mixture by two-dimensional electrophroretic methods and serial liquid chromatography steps before mass spectroscopy, but considerable information is lost as a result of degeneration or digestion (7)". For these reasons, and others described below, it was decided that four approaches should be applied - all of them adapted to histology.

The approaches are as follows. The first deals with the use of routine protease immunohistochemistry (Part 3). The second approach deals with neoepitopes indicative of cleavage site specific targets in order to identify cleaved substrates that may reveal enzyme activity in tissues (Part 4). The third approach makes use of a technique referred to as "histozymography" that is designed to detect specific functional proteases (Part 5). Part 6 is devoted to a complex immunohistochemical procedure named "protease neoepitope approach", in which biochemical and immunological techniques have been adapted to microscopy, in order to reach several goals, particularly the identification of enzyme producer cells and the detection of enzyme targets.

Finally, besides the approaches found instrumental in our own studies, others have applied an altogether different one based on the loss of protease gene function in mice (8-16). Interested readers are referred to these articles for a review of the features observed in animals lacking functional MMP-9 and/ or MMP-13 enzyme in particular (11; 15; 16) and, to evaluate the approach for its potential as a method.

2.1. Introduction to the test "model".

In a typical long bone like the rat tibia, the replacement of the bone's "cartilage model" begins before birth in the diaphysis as an erosion of the surface and is followed by excavation of the central marrow space, where new bony structures appear to build up the primary ossification center (17, 18, 19). The process begins by the aggregation of mononuclear TRAP positive cells around the "model". These cells are located at the level of the periosteum. The diaphysis center consists of intact calcified cartilage and will eventually become the future marrow space. The transformation of the "model" is attributed to the TRAP positive cells which differentiate progressively into multinucleated cells that invade the calcified cartilage region and transform it into a marrow space (14; 18; 19). Thus described is a transformation of cartilage that relies upon the complete removal of mainly calcified cartilage matrix (20) and this is achieved primarily by proteases elaborated by the osteoclast (21 and 22).

Intriguingly, after birth and during the first twenty days in a young rat's life, there is a slow but complete removal of the epiphysis center (for literature review, refer to 2). This removal in contrast to the one described above, is less well known and it encompasses a series of attacks that are launched against a hyaline cartilage "model" that is not calcified. The resorption of a "pure" cartilage (that is, cartilage that has escaped calcification) is one of the topics in the current review.

To understand the particulars of the resorptions taking place in the epiphysis, it is indicated to begin with the emergence of cartilage canals carrying blood vessels and osteoprogenitor cells (figure 1). This represents the first of four stages described (in the rat) as follows (2).

2.1.1. Stage I

Canal Excavation (figure 1B). Canals first appear as erosions at the surface of the "cartilage model's" epiphysis and then grow towards its center. In the past, this resorption has been explained by the involvement of undefined "factors" (23) presumed to arise from mononuclear cells termed "perivascular" (24).

2.1.2. Stage II

Marrow space formation (figure 1C). The space that will eventually be occupied by marrow in the "model" epiphysis arises from several canal blind ends approaching one another and fusing.

2.1.3. Stage III

First signs of ossification (figure 1D). Hypertrophic chondrocytes emerge close to the proximal wall of the marrow space. The nearby matrix calcifies. And this is soon followed by discrete bone deposition.

2.1.4. Stage IV

Primary and secondary growth plates are outlined (figure 1E). Finally, the marrow space further enlarges thereby defining the perimeter of the two growth plates.

The resorptions which collectively bring about changes to the model occur in two modes within distinct areas referred to as "remodeling sites". One mode is associated with endochondral bone formation and designated hereafter as "ossification-dependent" cartilage resorption. Here the loss of cartilage is mixed as the completely removed parts include uncalcified and calcified

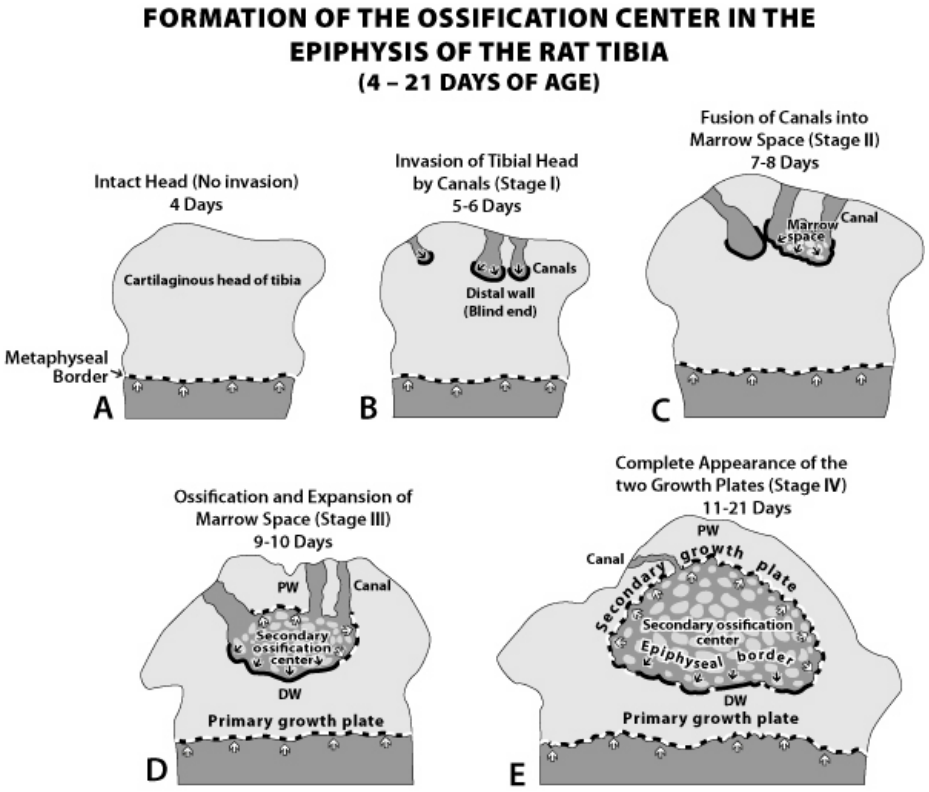

Figure 1. Stages in the development of the secondary ossification center in the proximal tibial epiphysis of 4-21 day old rats (modified from 2). Before the center development begins, the epiphysis is solidly cartilaginous A.. Each one of the four panels (B-E) represents one of the successive stages in center development. The sites undergoing "ossification-dependent" cartilage resorption are indicated by the simple broken line under which are shown small ascending white arrows. (The broken line signifies true interruptions between adjacent remodeling sites). Alternatively, the sites undergoing "independent" cartilage resorption are continuous as indicated by the black line above which are shown small black descending arrows. (In fact each black line corresponds to a "pre-resorptive layer" which is composed of a modified cartilage that differs from normal cartilage in three ways: chondrocytes are degenerating, proteoglycan particles are rare or absent and collagen fibrils are damaged (2)).

cartilage. Both are resorbed in the same locales in conjunction with bone formation (figure 2A, 25; 26). The other mode is not involved in any bone formation. In this mode all loss pertains to the "pure" cartilage form that has escaped calcification. The latter has recently been named "independent" cartilage resorption. In figure 1, indicative of our test "model", each panel depicts firstly, the rat tibial epiphysis at a specified age, secondly, the location at each age where active remodeling sites are found (arrows, figure 1) and, thirdly, the mode of cartilage resorption that drives the resorption in that locale.

Since the cartilaginous epiphysis is mainly comprised of the proteoglycan aggrecan and fibrillar type II collagen, we presumed its resorptions were due to proteases being in place to destroy these two elements (2; 3; 4; 5; 6). To test this hypothesis we asked which of the four major classes of enzymes - cysteine, serine, aspartic or metallo-proteases - were involved? Evidence for the involvement of a cysteine protease, cathepsin B, as well as members of the metallo-protease class of enzymes, are subsequently uncovered during the course of our inquiries – interestingly, so too are the mononuclear cells involved in their production. This review provides an historical account of these findings.

3. ROUTINE PROTEASE IMMUNOHISTOCHEMISTRY

3.1. Theoretical basis

The success of any immunohistochemical study of an enzyme depends on the use of monospecific antibodies that will only react with that enzyme. The general approach is to rely upon antibodies that have been prepared against a highly purified enzyme or part of an enzyme, and to inject it into a different species (mainly rabbits in the studies examined here) for production of an antiserum (27). In principle, antibodies can be made to respond to various "antigenic sites" present on the enzyme. The sites can be divided into two structural categories (28; 29). The first or "segmental site" is continuous; as antibodies bind to a sequence of adjacent amino acid residues that may be as short as n=4. The antigenic sites belonging to the other category consist of amino acid residues that are far apart in the primary protein sequence yet are brought together by how the protein folds in three dimensions. The former represents sequential epitopes

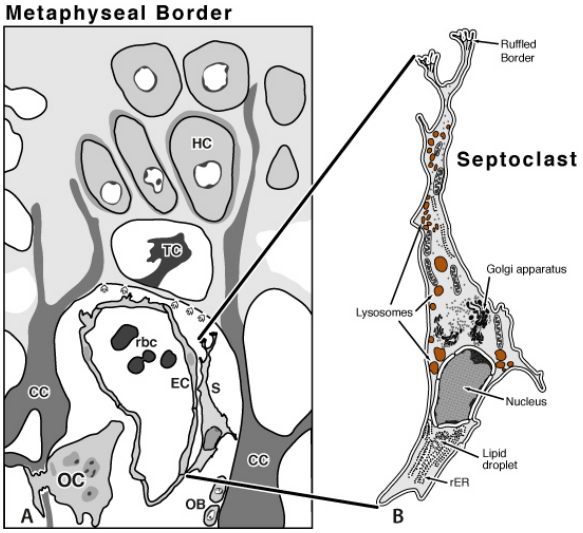

Figure 2. Structure of the metaphyseal border. The metaphyseal border is a narrow region located between the cartilaginous epiphysis and the diaphysis (as depicted in figure 1A), where an "ossification-dependent" cartilage resorption process erodes the epiphyseal cartilage boundary (indicated by small, white arrows). The metaphyseal border is usually described as the zone of interaction between invading capillaries and the cartilage zone rich in hypertrophic chondrocytes. The dark gray signifies calcified cartilage (cc), the light gray, represents the matrix of the cartilaginous epiphysis that is *not* calcified. **A–** refers to the framed part - this is a diagrammatic representation of the metaphyseal border. The upper half of the figure is occupied by the hypertrophic chondrocyte (HC) - including-cartilage, with degeneration shown in the lowermost one, named terminal chondrocyte (TC). The lower half of the figure is the region of invading capillaries. In the center, a capillary where EC denotes an endothelial cell, the lumen includes red blood cells (rbc), while on each side, a calcified, longitudinal cartilage septum (cc) appears as a dark-looking band running vertically. At left, an osteoclast (OC) cuts through a longitudinal septum. On the other side of the capillary, two osteoblasts (OB) may be distinguished. At right, between a transverse septum (identified by small arrowheads) and capillary, a cell described under the name "septoclast" has a ruffled apex that erodes nearby cartilage. An enlargement of the cell at right B. shows the elongated shape of the septoclast as well as the abundance of lysosomes (indicated by brown shading). The "metaphyseal border" represents a cartilage remodeling site where the resorption progresses as follows (25; 26). The calcified longitudinal septae, extend from the epiphysis into the metaphysis and are resorbed by osteoclasts although about 50% of their cartilage persists to serve as a substrate for bone deposition (figure 2A) (26). The non-calcified transverse septae that lie directly at the interface are also resorbed (arrowheads, figure 2A), but the cell involved in this resorption was not known. The collective events were those indicative of the "ossification-dependent" cartilage resorption mode.

(28); the latter are conformational or discontiguous epitopes (29). For a classical review describing antibodies to enzymes, with particular reference to the preparation and characterization of anti-lysosomal enzyme antibodies, the reader is referred to the review of Poole (27).

Lysosomal enzymes are found in most cells. These enzymes include members of diverse protease classes, respectively named: cysteine (cathepsin B, H, L, S and others), aspartic (cathepsin D) and, in specified cells, serine (cathepsin G, elastase, chymases and tryptases) (for review, refer to 30 and to MEROPS version 7.20 (31)). Cathepsin B is synthesized within the rER, and glycosylated in the Golgi by attachment of mannose to asparagine residues. The mannose residues are phosphorylated and via binding of them to the mannose-6-phosphate receptor the cathepsin B proenzyme is targeted to the lysosome (for review, 31; 32). On acidification, autoprocessing occurs whereby the propeptide is cleaved yielding the active protease (33). Until this point the enzyme is inactive thereby preventing autolysis. Besides actions in the lysosome, cathepsin B is implicated in extracellular events [bone resorption, (21, 22), cancer (34; 35), acute pancreatitis (36) and arthritis (37)].

Since cathepsin B degrades the non-helical extensions of collagens (38; 39) and aggrecan core protein (40) as well as link protein *in vitro* (41), we decided to test whether or not cathepsin B was involved in the resorption of cartilage during long bone development (3).

3.2. Procedure details

The antiserum used in the testing was prepared by Rowan, Mach and Mort against rat procathepsin B expressed in yeast (*Saccharomyces cerevisiae*) (33). Immunochemical characterization of the antiserum reveals that it binds to native cathepsin B proenzyme or active enzyme, and also to their corresponding denatured forms (33).

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To prepare the tissue for immunohistochemical examination animals are exsanguinated under anaesthesia by perfusion with lactated Ringer's solution into the left ventricle while the right atrium is opened (5). The Ringer's solution is then replaced with fixative solution (periodate-lysine-paraformaldehyde prepared as described by McLean and Nakane, 42). The tibiae are harvested and immersed in the same fixative overnight and then decalcified for 12 days in 10% EDTA (w/v in 0.1M Tris-HCL, pH 7.4) before embedding in O.C.T. compound (Somagen, Edmonton, AB). Cryostat sections are prepared at a thickness of 8 µm, mounted on gelatin coated slides and stored at -20^{0}C until further use (2). To detect the protease in the tissue sections, light microscopic immunochemistry followed a method described earlier (4) by which antibody reactivity is localized in the sections using an indirect immunoperoxidase technique performed at room temperature following the manufacturers' directions (Vectastain Elite ABC kit; Vector Laboratories Inc., Burlingame, CA).

3.3. Salient results

With the help of the described antiserum a cell named "septoclast" was identified at the metaphyseal border (figures 2B and 3B-E) (3). The antiserum bound to this cell's lysosomes and produced a cellular staining that

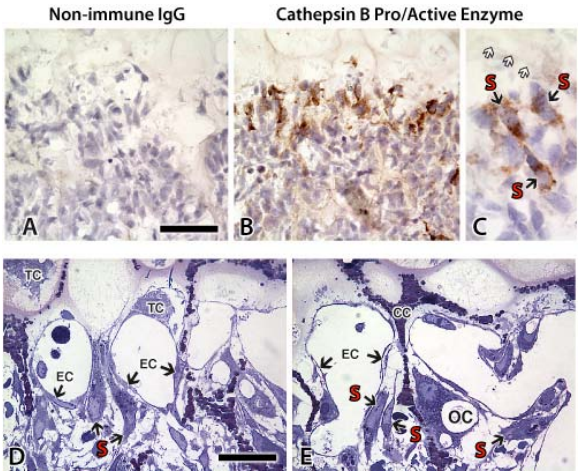

Figure 3. Septoclasts at the metaphyseal border of stage I epiphysis stained either with anti-cathepsin B antiserum (B and C) or with toluidine blue (D and E). The first row includes three panels, the first one A. has been exposed to non-immune IgG for control. The next panel B. displays the binding of the antiserum whose reaction is revealed by peroxidase as a brown reaction. The brown reaction is indicative of cathepsin B (as pro or active enzyme). The final panel at high power C. identifies three septoclasts (S) containing dot-like brown reactions which have been localized to lysosomes by electron microscopy (3). The white arrows identify a transverse septum that is unstained with the antibodies and is barely visible in the section. The second row's panels (D and E) also display septoclasts (S). This tissue has been exposed first to mixed aldehydes then treated with OsO4 before embedding in Epoxy resin. In D., at center and to the left, endothelial cells (EC) define the boundaries of two independent but adjacent vessels. Identified between the vessels are two septoclasts (S). The identified cells (arrows) have a single nucleus and a cytoplasm rich in lysosomes. The lysosomes, visualized here as blue dots, are varied in size and shape. In panel E. the distinctiveness of the septoclast is further emphasized by comparing its morphology to that of a neighboring osteoclast (OC). cc in E. denotes calcified cartilage. Bar in A = 50 µm (applies to B). Bar in D = 20 µm (applies to C and E).

was remarkable and drew attention to its presence (figure 3B and C). As for the nature of the cell the figure 2B diagram depicts the lysosomal reactivity indicative of cathepsin B. The cell possesses a single, central nucleus, and an apical cell membrane that terminates in a small ruffled border. The ruffled border suggests that the septoclast may be related to the osteoclast lineage. However, when tested against common markers of osteoclasts or macrophages, the cathepsin B rich septoclasts have been unreactive, and it has been concluded that septoclasts are not related to either one. Still today, it is not known what the cathepsin B enzyme is lysing. However, the evidence is conclusive that a distinctive cathepsin-B-rich cell plays a role in cartilage resorption, even though the details of this involvement remain to be clarified.

3.4. Potential of the method

To interpret the findings it is necessary to recall that proteases are synthesized by producer cells as inactive proenzymes which must be activated for attack on the target. Many antisera while proven monospecific to the enzyme of interest may not be specific to only one of its forms as was the case for the antiserum used here (33). Hence under "routine" immunostaining conditions it is not usually possible to assign the reactions produced in tissue section to either the inactive proenzyme or the activated, mature enzyme form. For lysosomal enzymes like cathepsin B, whose targets are subjected to complete breakdown within the lysosome it was likely that any antibody label was due to cathepsin B rather than procathepsin B as the latter is rapidly converted to mature enzyme within the acidic environment of the lysosome. Thus the data suggests that the reaction revealed by peroxidase in figure 3B and C likely represents the activated cathepsin B enzyme, but the limitations in place make it impossible to prove.

4. SUBSTRATE NEOEPITOPE IMMUNO-HISTOCHEMISTRY

Immunologists were the first to coin the term "neoepitope" (for historical review see, 43). Like any epitope, a neoepitope represents the region of the antigen that produces an immunological response. However, while an epitope is a normal part of the antigen, a neoepitope is a novel part that has been acquired. Thus the term neoepitope was first applied in relation to the activation of the complement system, where individual components undergo structural changes leading to the disappearance of epitopes and the acquisition of mainly conformation dependent neoepitopes in the activation products (43; 44). Even earlier however, it had been recognized in the case of some enzymes like porcine pepsin that major differences existed in conformation between the enzyme and proenzyme forms (27). Since conformational changes did occur in some enzymes following activation, it was possible in these cases to monitor the transformation with antibodies that could discriminate the change.

While the concept of neoepitope first evolved in relation to enzymes and was mainly conformation based, it subsequently became clear that the term "neoepitope" could be extended to include enzyme substrates where in contrast, as will be made clear below, antibodies are produced to target specific sequential epitopes present on the enzyme's substrate but only after it is cleaved.

To test if "link" protein may exhibit "neoepitopes" after peptide bond cleavage, in 1992 John Mort and Peter Roughley (Shriners Hospital for Children, Montreal) in collaboration with Clare Hughes and Bruce Caterson (then at University of North Carolina, Chapel Hill), prepared monoclonal antibodies that were specific to the new N-terminus present on link protein cleavage products derived from the digestion of link protein by an enzyme belonging to the matrix metalloproteinases (MMPs) subfamily (45). This study represented the first to demonstrate with the help of "anti-neoepitope" antibodies,

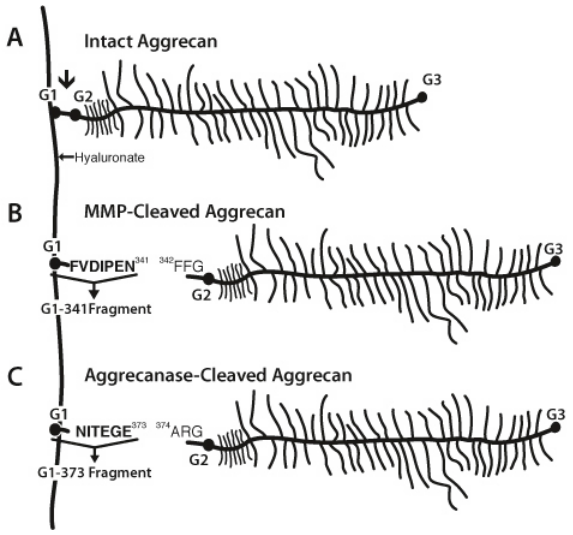

Figure 4. Three aggrecan molecules anchored on a filament of hyaluronate (vertical line at left; modified from 2). A.. Intact aggrecan molecule composed of an aggrecan core protein attached to the hyaluronate filament. Along the core protein, three globular domains are respectively labeled G1 (bound to the hyaluronate), G2 (separated from G1 by the first "interglobular region" - arrow) and G3 (ending the core protein and separated from G2 by the second "interglobular region, along which are attached many glycosaminoglycan chains, represented by short lines). B. Aggrecan cleaved by a so far unidentified member of the matrix metalloproteinase (MMP) subfamily. The cleavage takes place in the first interglobular region at the N^{341}–F^{342} bond. . C. Aggrecan cleaved by a member of the aggrecanase subfamily The hyaluronate-attached fragment ends in the amino acid sequence NITEGE. Antibodies against a peptide identical to the neoepitope are used to detect the cut and, at the same time, the enzyme subfamily that has made the cut.

that specific peptide bond cleavage events could be detected within cartilage extracellular matrix proteins by immunological methods (for review see, 46; 47).

4.1. Theoretical basis of the method

The attack of a substrate by an enzyme is generally carried out under well defined conditions - a protease cleaves a protein at a specific bond. The cut yields two protein fragments and the ends on either side of the cut represent neoepitopes. When antibodies are prepared against such neoepitopes, the application of the antibodies to the tissue, followed by immunostaining, will recognize the sites where neoepitopes are retained in the tissue. In so doing, the presence of the enzyme capable of producing the cut is revealed. Hence, as substantiated below, the cut end is a neoepitope. Further still it allows the detection of the enzyme that makes the cut. Finally, the antibodies that make the detection possible are produced to specifically respond to antigenic determinants formed by sequential amino acid residues located adjacent to the bond that is hydrolyzed.

In 1998 and in 2001, as was mentioned earlier, we had postulated that the described resorptions of the epiphyseal cartilage are carried out by proteases directed to the two major cartilage components: aggrecan and type II collagen (2; 4). This hypothesis implied that the sites of cartilage resorption contain enzymes capable of breaking down these two substances. Hence in 1998, attention was first given to finding such enzymes in resorption sites (4).

4.2. Procedure details

The first enzymes to be investigated were those cleaving aggrecan. Briefly, the structure of the aggrecan molecule will be reviewed (48; 49). The molecule essentially consists of a long core protein, attached by its N-terminus to a hyaluronate filament (figure 4A). Along the core protein, three globular domains are distinguished (labeled G1, G2 and G3 in figure 4A) and between them are two interglobular regions. The first is rather short and extends from G1 to G2. The second, which is longer, extends from G2 to G3. Numerous glycosaminoglycan side chains are attached along the length of the longer region.

Two groups of aggrecan-attacking enzymes have been investigated (reviewed by 47; 49). One group consists of matrix metallo-proteases (MMPs) that have been shown *in vitro* to cleave the short interglobular region at the N^{341}-F^{342} bond (figure 4B). The cut yields two fragments, one of which is brief – referred to as G1-341 fragment – and remains attached to hyaluronate, while the other fragment is lost from the cartilage. We postulated that should the same cleavage take place during the resorption of the "cartilage model", it would be possible to detect the G1-341 fragment at remodeling sites

The second group of aggrecan-attacking enzymes (also belonging to the metallo-protease class) is composed of proteases initially named "aggrecanases", of which at least four members have been identified (reviewed by 47). These enzymes have been shown *in vitro* to cleave the aggrecan core protein at a bond located 32 amino acids beyond the MMP cut, that is, at the E^{373}–A^{374} bond (figure 4C).

The N-terminal cuts, indicative of each enzyme subfamily, have been modeled by producing immunizing peptides with two glycines as spacers and one cysteine added for coupling to ovalbumin. (The coupling has been carried out using the method of Hughes *et al.* (45)), resulting in the immunizing peptides, "CGGFVDIPEN" to prepare anti-FVDIPEN antibodies for the detection of MMP cuts and, CGGNITEGE to prepare anti-NITEGE antibodies for the detection of aggrecanase cuts). The immunizing peptides have been purified by reverse phase chromatography using acetonitrile gradient in 0.1% trifluoroacetic acid. The immunization of rabbits has been done as described earlier (4).

4.3. Salient results

The results reveal that every cartilage resorption site, involved in the removal of uncalcified cartilage, is immunostained by either the anti-FVDIPEN antiserum or

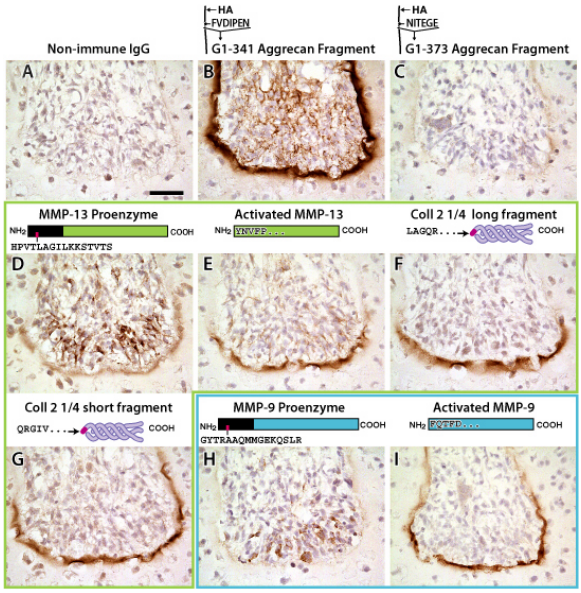

Figure 5. Light microscopic immunostaining of the stage I canal blind end region after exposure to various antibodies, whose immunostaining is indicated by a brown reaction. The canal blind end is chiefly composed of the pre-resorptive cartilage layer. In every panel, the canal blind end forms a half circle that is oriented toward the picture top, as emphasized by strong staining in several panels. The canal content mostly consists of cells and capillaries. The first row includes three panels, the first one of which A. has been exposed to non-immune IgGs for control. The next panel B. displays intense immunostaining by anti-FVDIPEN antibodies. The final panel C. displays the binding of anti-NITEGE antibodies, which are directed to the G1-373 fragment. The second row's first panel (D) records immunostaining by anti-MMP-13-proenzyme antibodies. The second panel in the second row (E) shows immunostaining by anti-activated MMP-13 antibodies. Panel F. shows immunostaining by anti-LAGQR antibodies, directed to collagen 2-¼-long fragments. The third row's first panel (G) shows immunostaining by anti-QRGIV antibodies that are directed to the collagen 2-¼-short fragments. Note the immunostaining in panel E. is distributed in exactly the same way as the two types of fragments (F, G) produced when this enzyme cleaves collagen 2. The second panel of this row (H) records immunostaining by antibodies directed to the MMP-9 proenzyme. The canal blind end is not stained. The third row last panel (I) depicts immunostaining by anti-activated MMP-9 antibodies, that is, a fairly strong reaction of the canal blind end indicative of the presence of activated MMP-9 in the pre-resorptive layer. Bar in A = 50 µm (applies to B – I).

the anti-NITEGE antiserum or both and, therefore, contains the cuts made either by an MMP, an aggrecanase or both. Hence every cartilage resorption sites respectively include either a MMP (so far unidentified), an aggrecanase (identified as aggrecanase-1, (50)) or both enzymes.

As an example of light microscopic immunostaining by anti-FVDIPEN antibodies, figure 5 (first row) depicts the region around the canal blind end at stage I. Since these antibodies are directed to the cuts produced by the unidentified MMPs, it is concluded that the dark reactivity in figure 5B, which is definite at the canal blind and along the nearby canal walls, represents the sites where MMPs cleave the local aggrecan. In contrast, as indicated by the absence of immunostain observed in figure 5C, no cuts are produced at this site by an aggrecanase. So, only the MMPs actions against aggrecan core protein have been demonstrated at this cartilage remodeling site. The results on aggrecan cleavage by MMPs at the electron microscopic level are illustrated in figure 6.

In summary, either the unidentified MMP or both the MMP and aggrecanase-1 are present in each cartilage resorption site during remodeling (2; 4; 50). Thus a neoepitope made it possible to identify the enzyme group involved in the lysis of aggrecan core protein at the specified remodeling sites.

4.4. Potential of the method

The neoepitope approach has been used in cartilage with success not only to examine proteolysis of link protein (45) and aggrecan (4; 47, 51), as mentioned above, but also to dissect the cleavage of type II collagen occurring either as intrahelical domain cleavage (52) or as cleavage within the nonhelical telopeptide domain (53).

The selection of an amino acid sequence as a potential immunogen for the detection of an antigen target requires several conditions. If we wish to localize a cut made by a protease in a substrate target, the sequence of amino acids on one side of the cut could be initially selected as the immunogen for the detection of the cut. First, the target must be retained in the tissue and throughout tissue-processing for histology, a question that may be answered by reviewing the target properties in the literature. The second requirement is that the target sequence not be homologous as an end terminus to that of other tissue protein(s) present, a decision made after subjecting this sequence to data analysis (National Center for Biotechnology Information. (NCBI) by comparing it to short nearly exact matches generated by a BLAST protein search). If these steps lead to rejection of the selected immunogen, the sequence on the other side of the cut could instead be considered as the target. Incidentally, it is prudent to use at least two rabbits for each immunogen. Finally, once antisera have been obtained, they should be submitted to affinity chromatography to purify and concentrate them. The more potent responder would then be used.

Over the years our Electron Microscopy Unit has been involved in making more than 5 anti-neoepitope substrate antibodies. All antibodies produced the expected outcome and were as successful as the search for aggrecan-cleaving enzymes in tissue. However, whilst this approach provides important information with regard to the identification of the group(s) involved in protein cleavage in the tissue, it cannot be used to specifically identify the

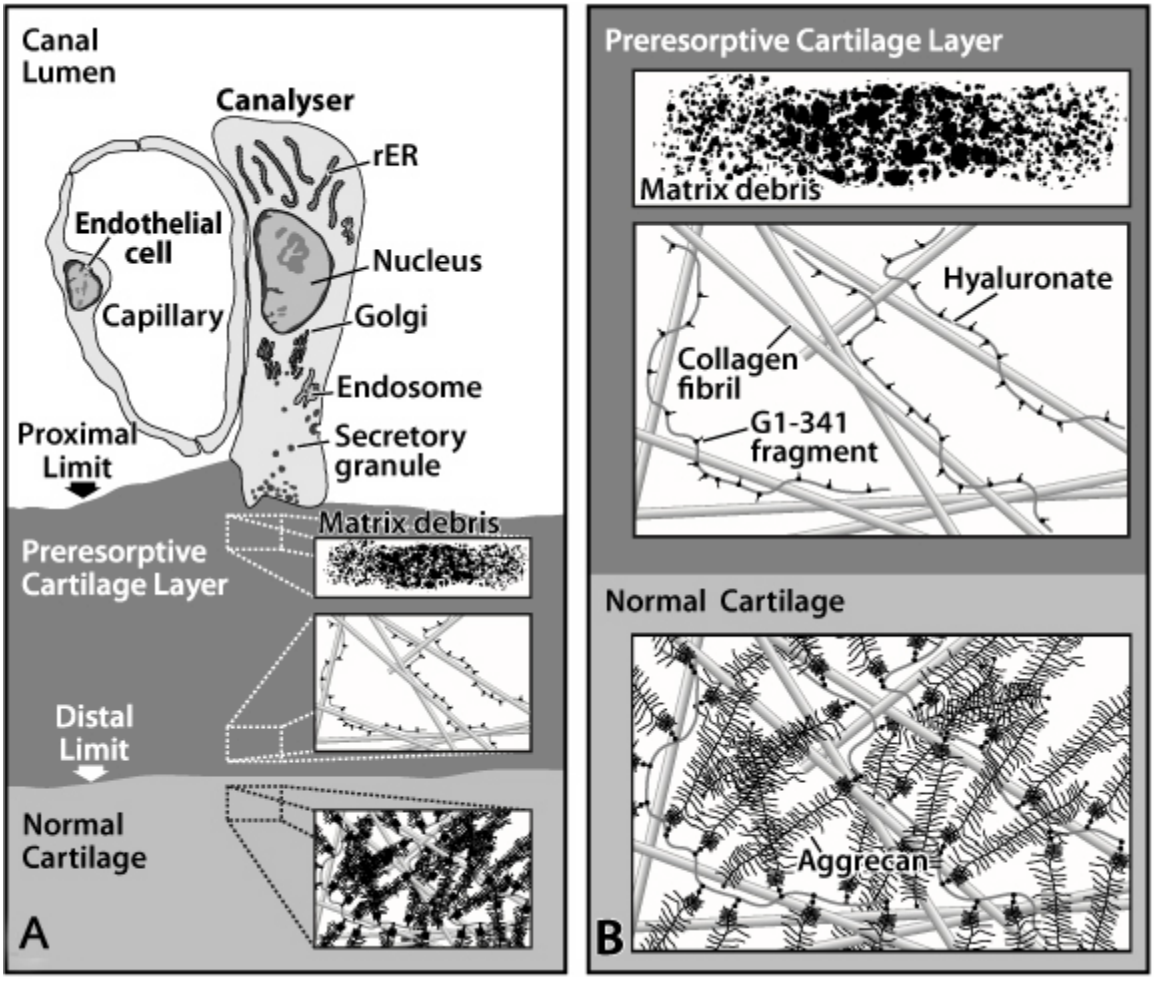

Figure 6. Structure of the pre-resorptive layer shown at low A. and high magnification B. A.. Normal cartilage is depicted in solid gray, the pre-resorptive layer is depicted in a dark tone, whereas the canal lumen with its light background occupies the upper half of panel A.. Only shown are two of the structures present in the lumen: a capillary in which an endothelial cell is pointed out and a canalyser cell showing the cytoplasmic components. B. Diagrammatic representation of the fine structure of the regions. (The relative positions of the enlarged regions are shown as inserts in A). B. Normal cartilage consists of gray rods representing collagen fibrils, a fine black filament represents the hyaluronate filaments and finally, bottle brush-like structures is indicative of the aggrecan molecule. The middle rectangle of panel B. depicts the pre-resorptive layer. The fine black filaments representing hyaluronate are easily recognizable, but the aggrecan molecules are no longer present except for a very small piece of them referred to as G1-341 fragment. Finally, the top rectangle depicts the layer's debris. In conclusion, the diagram summarizes the fact that MMPs cleavage of aggrecan core protein has been revealed as a key step in cartilage resorption (2).

protease that produces the actual cut. Other approaches were needed to address this important issue.

5. HISTOZYMOGRAPHY

5.1. Theoretical basis of the method

Histozymography has been used for the detection of enzymes in frozen sections that have not been subjected to histological fixation. Hence the enzymes are in a state that is somewhat similar to their living condition. This technique has been inspired by previous methods in which unfixed frozen sections are applied onto slides bearing a target substrate that can be digested by the enzyme suspected of being present in the tissue, as initially done by Adams and Tuqan (54) and Daoust (55).

While Adams and Tuqan (54) relied upon the gelatin present in X- ray film as the test substrate, the main tool used here for histozymography is a slide bearing photographic emulsion that has been blackened by exposure to light and then subjected to photographic development and fixation. Upon microscopic examination, this emulsion is seen to be composed of minute black silver grains embedded in gelatin.

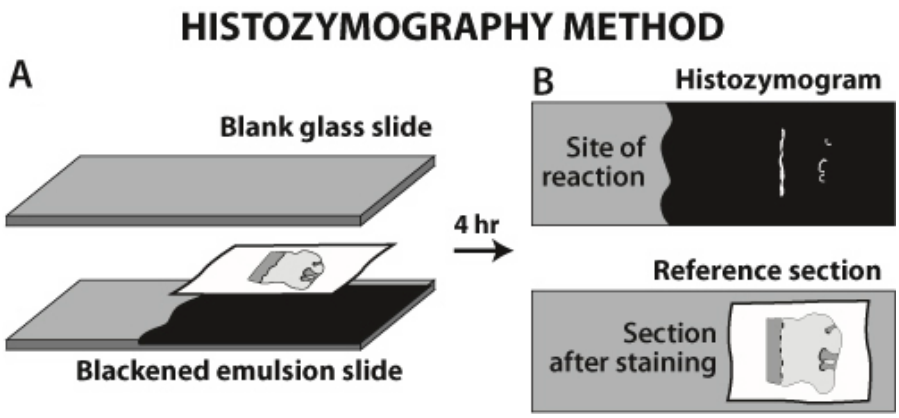

Figure 7. A three-step description of the gelatin histozymography method. Before the procedure, "blackened emulsion slides" are prepared by dipping slides into photographic emulsion, such as the Kodak NTB-2 emulsion, exposing them to light for five minutes and finally developing, fixing and drying them. A.. The first step in histozymography. The first step is the cutting of a frozen section and its deposition on transparent Scotch tape 800. In the second step, the tape mounted section with section surface facing down, is deposited on a blackened emulsion slide, as shown at lower left. A blank glass slide such as seen as upper left is placed on top. And finally, the entire slide section sandwich complex is transferred to a humid chamber at 21 ^{0}C. Section and emulsion are thus kept in contact for some time. (Much of our work was done using a four hour exposure).B. . The third step is the separation of section and emulsion after the exposure has been terminated. Thus the tape with the attached section is detached from the emulsion and stained using a routine histology stain such as toluidine blue. The section is then mounted on a glass slide in the same orientation as the emulsion image - referred to as histozymogram. Comparison of section and emulsion makes it possible to compare each light spot in the emulsion (indicative of gelatin digestion) with the section site that has produced it and, therefore, contains an enzyme capable of digesting the emulsion gelatin. An example is presented in figure 8.

The histozymographic procedure is carried out by placing an unfixed frozen tissue section on the blackened emulsion (figure 7A). After a suitable time in a damp atmosphere (usually for four hours in our experience), the section is removed and the emulsion is examined. Thus, after exposure of a longitudinal section of newborn rat tibia to a light-fogged emulsion layer, light spots appear in the emulsion of the histozymogram (figure 7B) due to loss of the gelatin, implying loss of the included black silver grains. The interpretation is that the tissue areas (figure 7B, observed in the reference section) facing the light spots contains an enzyme capable of digesting gelatin, presumably a protease. The three areas facing a light spot in figure 7B correspond respectively to the junction between diaphysis and epiphysis (indicative of the site of attack coming from below) and the blind ends of two emerging canals (indicative of the attack originating from the superior surface of the epiphysis). The former junction includes a thin tissue layer, referred to as "metaphyseal border", that is active in resorbing the epiphyseal cartilage in the mode described previously as "ossification-dependent" cartilage resorption. The latter is active in the mode described as "independent" cartilage resorption.

5.2. Details of the procedure (5)

To prepare the fogged emulsion bearing slide, a histological glass slide is dipped into Kodak NTB2 emulsion, exposed to the room light for 5 minutes, and subjected to standard photographic development and fixation. The blackened emulsion is stable at room temperature.

Anesthetized rats 4 – 21 days old are exsanguinated by perfusion with lactated Ringer's solution into the left ventricle (5, 6). The tibial epiphyses are removed, divided in half along their length by a single cut with a razor blade and placed cut surface down on a cryomold containing 5% polyvinyl alcohol in water for freezing on dry ice. They may then be stored in a laboratory freezer. For the actual histozymography, fresh frozen sections are prepared by the method of van Noorden and colleagues. (56). The sections are cut at a 20 µm thickness on a motor driven Brights cryostat using a tungsten carbide coated knife at a -20^{0}C cabinet temperature.

A piece of transparent 800-Scotch tape is adhered to the block surface and the microtome knife is advanced at a low constant speed to cut below the tape and produce a high quality section attached to the tape. After the section has been cut, the free ends of the section-carrying tape are attached by shiny 600-Scotch tape onto a glass slide.

The section-transparent-tape complex is applied onto the blackened emulsion at room temperature (figure 7A) and kept in place, usually for four hours as stated above. The section-transparent-tape complex is then removed from the emulsion and stained by a routine histological method. The emulsion shows light spots wherever it is in contact with a tissue site containing gelatin-digesting protease. Comparison between emulsion and section makes possible the assignment of the light emulsion spots to tissue sites, as shown in figure 7B.

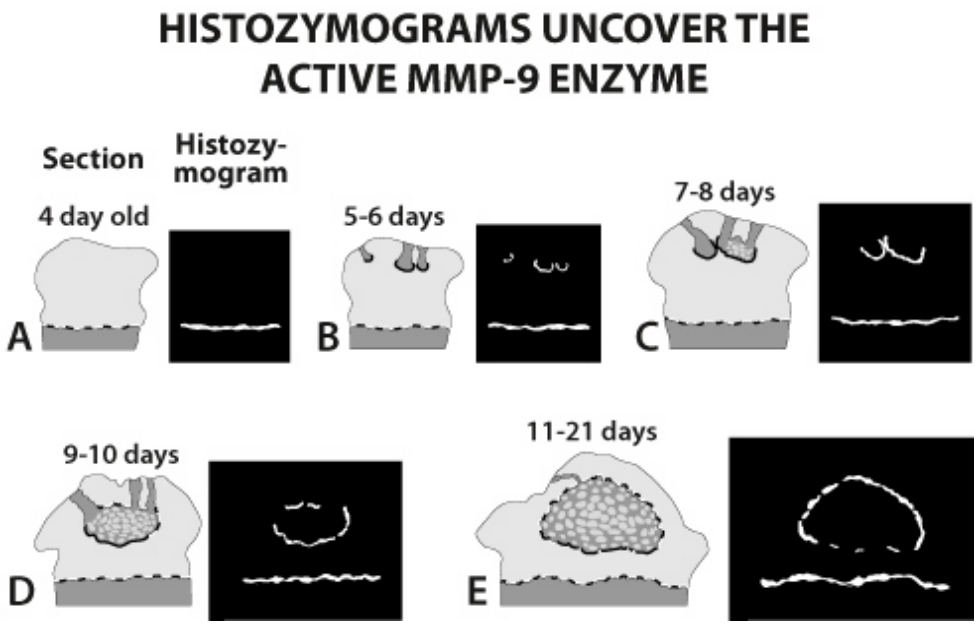

Figure 8. Histozymograms of rat tibal epiphysis at the four stages in secondary ossification center formation. We have demonstrated that reactive histozymograms are produced by the presence in the adjacent tissue of the enzyme known as gelatinase B or MMP-9 (5, 6). A.. Before center formation begins, a clearly distinct reactive histozymogram corresponds to the junction between epiphysis and diaphysis referred to as "metaphyseal border" (described in figures 1 and 2). The metaphyseal border of corresponding histozymograms can be recognized in every other panel of this figure. B.. Stage I, when canals arise, there are positive, faint pale spots evident on the histozymogram corresponding to the blind ends of the small canals. C. . Stage II, when canals fuse to form the marrow space, there is a gelatin loss from the emulsion at sites corresponding to the distal marrow space wall at right and a large canal at left. D.. Stage III, when the initial signs of ossification appear, there is a reaction on the histozymogram produced along almost all the marrow space wall. E.. Stage IV, when the enlarged marrow space is associated with distinct primary and secondary growth plates, reactions are produced on the histozymogram by the proximal and lateral walls of the marrow space.

To identify the effective protease, we repeated the procedure in the presence of suspected protease inhibitors and antibodies (5, 6). Significantly, only antibodies able to neutralize the protease MMP-9 blocked all reactions. The conclusion is that MMP-9, also called gelatinase B, is the gelatin-digesting protease.

5.3. Actual and potential results of the method

The articles just mentioned (5; 6) report that all cartilage resorption sites in the tibial head of young rats contain a protease identified as MMP-9 or gelatinase B (figure 8A-E). Thus it was concluded that MMP-9 was involved in both modes of cartilage resorption.

This procedure is similar to that developed by Daoust (55) who applied fresh frozen sections to slides coated with suitably stained target-containing gelatin to detect enzymes such as ribonuclease and others. However, due to his untimely death, the potential of the method was essentially realized by others (for review see, 57). Today, various options are open to the investigator, the use of gelatin alone (58), a photographic emulsion as was used here or more recently films made of quenched fluorescent substrates, which upon cleavage produce a fluorescent signal (59; 60). Clearly, the nature of the problem best determines which test is selected. Regardless of the decision taken, however, the ability to make definitive conclusions rests upon protease specific inhibitors or preferably monospecific antibodies that also possess the ability to inactivate or neutralize protease cleavage (5; 6). Without the latter aids, the identity of the gelatin degrading protease present in the tissue remains unknown.

6. PROTEASE NEOEPITOPE IMMUNO-HISTOCHEMISTRY

6.1. Theoretical basis of the procedure

In the course of this investigation, it has become clear that proteolytic enzymes, particularly MMPs, play a major role in the early stages of skeletal development by insuring "ossification – dependent" and "independent" resorptions.

Before considering the specifics of protease involvement further, a few introductory remarks about MMPs in general. The enzymes of this group function optimally at neutral pH, rely upon a zinc ion for catalysis and cleave multiple substrates *in vitro* including structural proteins; thus explaining the derivation of the acronym MMPs (matrix metalloproteinases). This acronym is incorporated into the name of each member and precedes all of the 24 numbered human MMP gene products (8; 61; 62).

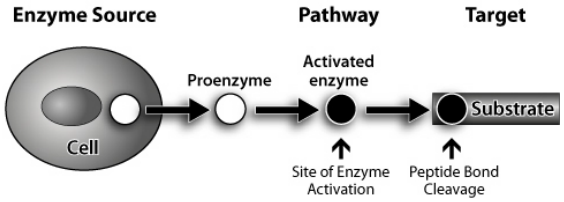

Figure 9. Diagram outlining enzyme's life-history. At left, an enzyme producer cell is elaborating the "proenzyme" - a functionally inactive form of the enzyme – which is then secreted by the cell to outside tissue. Eventually the secreted "proenzyme" loses its propeptide domain and thus becomes capable of functional activity as an "activated enzyme". Finally the "activated enzyme" binds to its substrate to carry out its function such as, shown here, peptide bond cleavage. The goal of the "protease neoepitope approach" was to reveal this life-history directly in the tissue.

MMPs are elaborated by cells in the proenzyme form. The proenzyme, which lacks functional activity, is composed of at least three domains: (1). At the N-terminus is the enzyme propeptide domain that imparts latency, a term indicating that the presence of this domain prevents the proenzyme from having functional activity. (2). Succeeding the propeptide domain is the catalytic domain which is capable of functional activity, but can only exert it after the propeptide has been proteolytically removed. (3). Finally, the hemopexin-like domain (or fibronectin-like domain, in MMPs 2 and 9 enzymes, also referred to as gelatinases A and B, respectively), which is crucial for enzyme attachment to target.

Although enzyme studies have progressed markedly in recent years, such problems as the identification of enzyme producer cells or the detection of enzyme tissue targets under living conditions have often been neglected or examined by methods that are not fully adequate (figure 9). Furthermore, when an enzyme is secreted no adequate method exists to trace its migration nor to identify where and how it takes on the "activated enzyme" form capable of attacking the target. The study of MMPs enzymes has required solving these problems and to solve them suitable techniques have been required.

The "protease neoepitope approach" is based on the hypothesis that (i). antibodies can be used to localize activated MMP enzyme and the target to which it binds in the tissue while other (ii) antibodies can be used for microscopic localization of the enzyme as the pre-activated form or proenzyme.

6.2. Procedure details

The method has been applied to two MMP enzymes, the MMP-9 enzyme as proof of principle, and the MMP-13 enzyme, also known as collagenase-3 to directly search for its involvement (unpublished data; Lee E.R, L. Lamplugh, B. Kluczyk, J.S. Mort & C.P. Leblond).

The strategy for each enzyme is as follows. A set of anti-peptide antibodies have been prepared using two immunizing peptides, one modeled from a 16 mer sequence in the rat propeptide domain to obtain antibodies capable of binding to this domain and thus recognizing the inactive proenzyme. The other is modeled from the first, five amino acids beginning the rat catalytic domain to obtain antibodies capable of binding to that domain and thus recognizing the activated enzyme. Specificities have been proven in both cases with the help of two procedures, that is, examination of peptides in direct and competitive ELISA assays or by analyzing partially activated recombinant human MMP-9 or MMP-13 enzymes by SDS-PAGE and Western blotting techniques. The various tests have shown that it is possible to distinguish between the proenzyme and the activated enzyme forms in tissue and thus discriminate the changes that occur in enzyme domain structure with activation. In both cases the specific targets are antigenic determinants comprised by a sequence of adjacent amino acid residues. However, only the antibodies designed to detect the activated enzyme are anti-neoepitope antibodies.

6.3. Actual results

The results reveal that every cartilage resorption site is immunostained by the anti-MMP-9 neoepitope antibodies. These sites therefore contain activated MMP-9, confirming what had been uncovered by histozymography. As an example of the light microscopic immunostaining by the anti-activated MMP-9 enzyme antibodies figure 5I depicts the region around the canal base at stage I. In contrast and as an example of light microscopic immunostaining, the anti-MMP-9 proenzyme antibodies were shown to immunostain mononuclear cells within the canal blind end and identifies these cells as the MMP-9 enzyme producer (figure 5H). Controls using a non-immune IgG (figure 5A) show no immunostaining in either cells or matrix thereby confirming the specificity of the reactions observed with both antibodies.

Since it has been found that these mononuclear cells also make MMP-13 and cathepsin B, the cells have been designated "canalyser" cells. Canalysers possess a fully differentiated cytoplasm that is rich in rER and Golgi components indicative of a secretory cell (figure 6A). Like the septoclast mentioned earlier, canalysers display no common markers indicative of osteoclasts, macrophages or endothelial cells. Yet while sharing this feature and the presence of cathepsin B, the structure of the septoclasts and canalyser differs enough to suggest that these two cells may either be distinct or distantly related (compare, figures 2B and 6A).

Hence, the "protease neoepitope approach" can provide definitive information about enzyme targeting and synthesis *in situ*.

6.4. Potential of the method

Collagenases share the ability *in vitro* and at neutral pH to cleave the triple helical domain of type 2 collagen at a specific Gly/Leu bond to generate

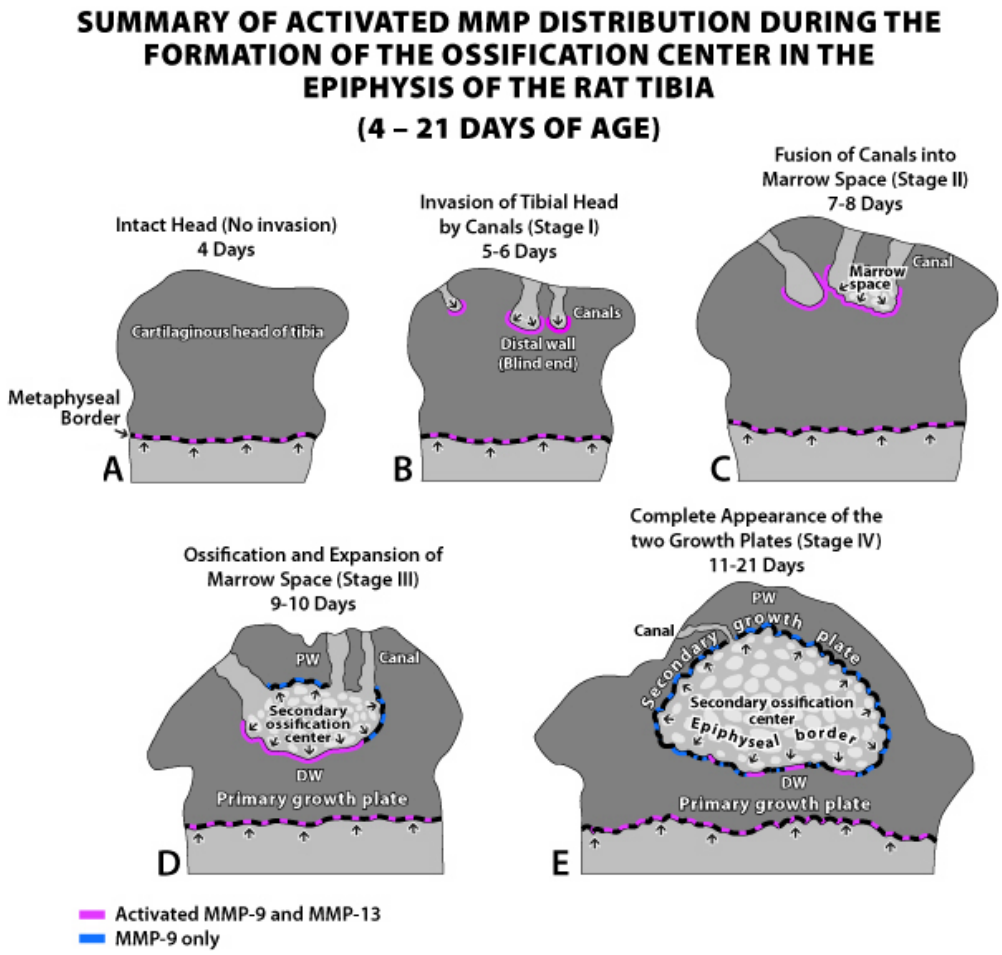

Figure 10. Localization of the activated MMP-9 and MMP-13 enzyme forms. The five panels represent the same series of the four stages taking place during secondary ossification center development that have been illustrated in figure 1. Here, however, the distribution of the two MMPs is indicated by color. First the purple color represents the sites including both activated MMP-9 and MMP-13; these sites are first the metaphyseal border up to stage IV (E); and second, the canal blind end (B, C) and the distal marrow space wall (C, D). Next the blue color represents the sites including the activated MMP-9 alone; these sites are part of the distal wall of the marrow space at stage III (D) and the whole marrow space wall at stage IV (E). In considering the formation of the center alone, the results definitively indicate that MMP-9 and MMP-13 enzymes together function during "independent" cartilage resorptions whereas only the MMP-9 enzyme participates in the "ossification-dependent" cartilage resorptions observed along the walls of the center observed in animals aged 9 – 21 days old.

characteristic ¼ and ¾ fragments (63; 64; 65; 66). Three human collagenases exist; MMP-1 (also called collagenase-1 or interstitial collagenase), MMP-8 (collagenase-2 or neutrophil collagenase) and MMP-13 (collagenase-3). In addition, two other MMP proteases also exhibit the potential to cleave the collagen 2 intrahelical domain *in vitro*. These are the membrane type MT-MMP-1 (MMP-14) (67) and gelatinase A (MMP-2) (68).

Testing the "protease neoepitope approach" with MMP-13 has also yielded substantial results (unpublished data; Lee E.R, L. Lamplugh, B. Kluczyk, J.S. Mort & C.P. Leblond). At specified cartilage remodeling sites anti-MMP-13 propeptide domain antibodies identify two proenzyme sites, collagen fibrils and canalyser cells. The latter is considered the MMP-13 producer cell. Anti-MMP-13 anti-neoepitope enzyme antibodies detected activated MMP-13 sites along collagen fibrils - considered the target. Finally, anti-collagen 2-1/4 fragment antibodies demonstrate fragments indicative of collagenase activity. The identical localization of MMP-13 and collagen fragments in collagen resorption sites demonstrates that MMP-13 is the collagenase responsible for the collagen cutting that is occurring in these sites. Thus the "protease neoepitope approach" has revealed the identity of the collagenase responsible for the demise of fibrillar collagen as well as its source and *in situ* pathway.

As an example of the light microscopic immunostaining by the anti-MMP-13 antibodies figure 5 depicts the region around the canal blind end at stage 1. Since the anti-MMP-13 proenzyme antibodies stain the

canalyser in the canal lumen, these are the producer cells (figure 5D). In the same panel the anti-MMP-13 proenzyme antibodies also immunostain the canal blind end (figure 5D). Moreover, the anti-MMP-13 neoepitope antibodies that detect the activated enzyme also immunostain the canal blind end (figure 5E) a result indicating that the activated enzyme is present there. Finally, antibodies against ¼ collagen fragments also immunostain the canal blind end (figures 5F and G). Indeed the association of the MMP-13 and collagen fragments is close enough as confirmed by electron microscopy, to prove that the fragments are produced by MMP-13 cleavage of cartilage collagen type 2, as mentioned above.

In conclusion, the "protease neoepitope approach" is effective at discriminating between the pre and post-activation forms of MMP-9 and MMP-13 enzymes *in vitro* and *in situ*. The same approach also works to detect cleavages within intracellular enzymes (calpain-1 (69); caspase-3, (70); both cysteine proteases) as well as those present on circulating complement components (C1s, serine protease, (71)). Interestingly, beyond the proteases named, the same approach has also been successful in detecting proteolyzed forms of a transferase (protein kinase C, (72)). Hence, enzymes other than protease enzymes appear to lend themselves to analysis by the approach.

To determine if an enzyme is suitable for analysis by the "protease (enzyme) neoepitope approach", basic information about the enzyme is required. Most importantly, knowledge of its amino acid composition is essential for the preparation of the required antibodies. Such information is now accessible for all MMPs and collectively, for enzymes as a group (MEROPS version 7.20; (31)). Key to the success of the "protease neoepitope approach" is the starting purity of the peptides imitating the enzyme domains. When coupled to ovalbumin as HPLC purified peptides and injected in rabbits according to a schedule, a good immune response can be generally anticipated (4; 73). However, the importance of rigorously characterizing the resulting IgG's can not be over-emphasized. As for every immunological based assay, non-immune controls must be exercised at each step. These collectively described steps and cross checks embody the "protease neoepitope approach".

7. CONCLUSIONS

This review provides the reader with an historical account of how difficult problems have been solved by implementing approaches adapted to histology. Four approaches have been described - all of them are shown effective - as each of them has contributed in one or more ways to uncovering the involved; 1). protease(s), 2). producer cell, 3). target and/or 4). mechanism behind lyses in the pre-resorptive layer of the test "model" under study. Intriguingly, while much is known about the cells involved in the resorption of calcified cartilage or bone, that is the osteoclast, relatively little was known about the protease producer cell that resorbs the uncalcified elements of the test "model". Advances are therefore made by relating cause and effect directly in the tissue as only under these conditions do all of the needed elements co-exist. Finally, as summarized in figure 10 where the activated MMP-9 and MMP-13 enzymes are directly portrayed in space and time, *in situ* analyses clarify how a tissue remodels in three dimensions, and in the tissue under study, shows precisely when and where these proteases function to transform a "cartilage model" into a secondary ossification center..

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