PRODUCTION OF A MONOCLONAL ANTIBODY TO A METALLOPROTEASE ASSOCIATED WITH MATRIX VESICLES IN CHICKEN EPiphySEAL CARTILAGE

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Abstract: A monoclonal antibody to a protease associated with chicken matrix vesicles was obtained. It was confirmed that the protease was localized in or on matrix vesicles of epiphyseal cartilage by immunohistchemical electron microscopy. Possible roles of this protease in the initial calcification were discussed.

Key Words: Cartilage, Metalloprotease, Matrix vesicle, Monoclonal antibody

INTRODUCTION

Katsura et al(1) first reported that protease activity was detected in matrix vesicles. Fujiwara et al(2) reported the isolation of bovine metalloprotease associated with matrix vesicles, and Katura et al(3) reported more details of this enzyme of chicken epiphyseal cartilage. In this study, we produced a monoclonal antibody to metalloprotease associated with matrix vesicles, and confirmed the specificity for the protease.

MATERIALS AND METHODS

The assay procedures for protease activity, preparation of antigen and solubilization of protease were according to the previous report(3). The procedures for producing the making of monoclonal antibody were according to the method of Kohler and Milstein(4). The myeloma cell used in this experiment was NS-1, and 50% polyethylene glycol 4000 was used for fusion. The growth-positive hybridoma wells were screened to determine the immunospecificity for the metalloprotease of matrix vesicle by

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the trans blot and dot blot method.

Trans blot was performed as previously reported\(^5\). Samples and standards were electrophoresed with 7.5% polyacrylamide slab gel containing 0.1 per cent sodium dodecylsulphate\(^6\). After electrophoresis, proteins resolved in the polyacrylamide slab gel were transferred onto a nitrocellulose membrane (Bio Lad) by means of an electrophoretic transblotting apparatus according to the method of Towbin et al\(^7\). Dot blot was performed as follows. Thirty ng matrix vesicle fractions or solubilized protease in PBS were blotted on nitrocellulose membrane. Trans-blotted and dot-blotted nitrocellulose sheet was washed with TBS and blocked with 3% skim milk-TBS for 40 min in order to prevent a non-specific reaction. Immunobinding was performed by incubation of the nitrocellulose membrane for 1 h at room temperature with an antibody solution or the supernatant of the culture medium, and incubated with a second antibody, horse-radish peroxidase-conjugated goat anti-mouse immunoglobulin antibody (CAPPEL), and the membrane was stained with peroxidase substrate solution (4-chloro-1-naphthol)\(^8\).

For affinity chromatography, cyanogen bromide-activated Sepharose 4B gel (2 g; Pharmacia Fine Chemicals) was used. Twenty mg antibody was conjugated to this affinity column. The matrix vesicle fraction was applied to this column (70 X 12 mm) filled with the antibody-conjugated Sepharose 4B gel, and washed with 0.2 M Tris-HCl, pH 7.0. The protein bound to this antibody was eluted with the same buffer containing 1.5 M NaCl\(^5\).

To determine the subclass type of the antibody, a mouse monoclonal antibody isotyping kit (Amersham International plc) was used. The procedure is almost as same as that for the dot blot.

Immunohistochemistry was performed as previously reported\(^9,10\). Chicken cartilage was fixed with 4% paraformaldehyde, embedded in Lowicryl, and cut into 0.1 um thicknesses. The sections were incubated with antibody followed by reaction with protein-A
conjugated with gold particles. They were observed with a transmission electron microscope.

RESULTS AND DISCUSSION

SDS-PAGE of the crude matrix vesicle fraction used as immunogen is shown Fig. 1 (lane 2). This fraction was injected into mouse peritoneally, and hybridomas of spleen cells and NS-1 myeloma cells were obtained. Hybridomas were screened at first by reaction with crude matrix vesicle fraction, and then screened by reaction with the solubilized protease. The positive clones were screened finally by trans blot. Finally only one clone,

![Fig. 1: SDS-PAGE and immunoblot of matrix vesicle protein. Lane 1: molecular weight standards; Lane 2: crude matrix vesicle fraction; Lane 3: solubilized protease of matrix vesicles; lane 4: immunoblotting of solubilized protease of matrix vesicles to monoclonal antibody; lane 5: affinity purified protease of matrix vesicles. Molecular-weight standards were phosphorylase b(94K), bovine serum albumin(67K), ovalbumin(43K), carbonic anhydrase (30K), trypsin inhibitor(20K), and alpha-lactalbumin(14.4K). Lanes 1, 2, 3, and 5 were stained with coomasie brilliant blue.](image-url)
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term the 7B2 clone, was isolated. The subclass types of monoclonal antibody were IgGl and k-type.

In order to characterize the immunospecificity of this antibody, it was tested by transblotting to solubilized matrix vesicle protease. The results are shown in Fig. 1 (lane 4). A single band which shows a molecular weight of 33 KDa was stained clearly. Furthermore, the bound fraction which was purified by affinity chromatography conjugated with antibody displayed a single band on SDS-PAGE (Fig. 1, lane 5). The fraction which unbound to the affinity gel had no protease activity.

To determine if the antibody reacted with matrix vesicles in tissue, an immunohistochemical study was done. The results are shown in Fig. 2. Protein A gold particles are associated with matrix vesicles.

Nevertheless it is well known that the matrix vesicles play an important role in initial calcification, and there is insufficient information on the components of matrix vesicles. Previously, the presence of alkaline phosphatase, ATPase, and pyrophosphatase was reported in matrix vesicles. On the other hand, several proteases in cartilage were reported by some laboratories, but few workers demonstrated these proteases in

Fig. 2:
Immunohistochemical features of matrix vesicles shown by transmission electron microscopy. Protein A gold particles can be seen with matrix vesicles.
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association with matrix vesicles\(^1,11,12\). In this study, we
demonstrate that the 33KDa protease associates with matrix
vesicles immunohistochemically.

In calcifying fronts, noncollagenous proteins including
proteoglycans which might function as inhibitors of crystal
growth, must be degraded. It is reasonable that the matrix
vesicle protease functions when the matrix vesicles are ruptured.
Hydroxyapatite crystallites appear in interfibrillar spaces
between collagen fibrils and then they intrude into
intrafibrillar spaces of deployed fibril according to the
nanospace theory proposed by Katsura\(^{13}\)}. Matrix vesicle protease
should degrade such matrix proteins in interfibrillar spaces and
hoop-like binding materials which are tight scrolls of collagen
filaments\(^{14}\).

In this study it was shown that this protease is associated
with matrix vesicles, but we cannot determined whether it exists
in or on the matrix vesicles. However this protease may exist in
matrix vesicles because it was solubilized by a low concentration
of sodium deoxycholate\(^3\).

After the purification with affinity gel conjugated with
antibody, the protease activity was lost considerably. This is
due to the used eluting buffer. Several buffers were tested to
elute the purified protease, but all buffers tried inhibited the
protease activity in some extent. We suppose that the
instability is one of the characters of this protease. The
activity of the protease functioned in calcifying front must be
lost quickly to regulate the rate of calcification.

Since the crude matrix vesicle fraction was used as an
immunogen in this study, other antibodies to various molecular
weight proteins of matrix vesicles as shown in Fig. 1, lane 2
were obtained, and these antibodies had been cloned in our
laboratory\(^{15}\)}. Though several proteins and enzymes have been
shown\(^{11}\), the components of the matrix vesicles has not been well
characterized until now. We believe that studies with
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monoclonal antibodies to matrix vesicles will clearly demonstrate the matrix vesicle components and finally details of the mechanism of the initiation of calcification.

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