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Complement Cls, a classical enzyme with novel functions at the endochondral ossification center: immunohistochemical staining of activated Cls with a neoantigen-specific antibody

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Abstract. The secondary ossification center of 14- to 16-day-old hamster tibiae was examined immunohistochemically with active and inactive Cls-specific antibodies, RK5 and RK4, respectively. At the ossification center, chondrocytes differentiate from proliferating and hypertrophic to degenerating stages, and their site is occupied by the bone marrow. Cls was strongly immunostained in hypertrophic chondrocytes. In order to discover whether Cls is activated at a particular site, the cartilage was immunostained with RK5 and RK4. RK5 mainly reacted with degrading matrix around invading vessels. In contrast, RK4 strongly stained hypertrophic chondrocytes. Immunoelectron microscopy revealed Cls on degrading fragments of chondrocytes and fibers of cartilage matrix. Decorin, one of the major matrix proteoglycans, was dose and time dependently degraded by Cls. Type II collagen and type I gelatin were also degraded. Articular cartilage from patients with rheumatoid arthritis was positively immunostained (11/12 cases) with an anti-Cls monoclonal antibody (mAb) PG11, whereas normal articular cartilage (5/5 cases) was negative, suggesting Cls participation in the etiology of rheumatoid arthritis.

Key words: Complement Cls – Neoantigen – Cartilage – Hypertrophic chondrocyte – Immunohistochemistry – Rheumatoid arthritis – Decorin – Syrian hamster – Human

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Abbreviations: mAb, Monoclonal antibody; *PLP*, periodate-lysine-paraformaldehyde; *SDS-PAGE*, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; *HE*, hematoxylin and eosin; *DFP*, diisopropyl fluorophosphate; *NMP-9*, matrix metalloproteinase 9

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Introduction

In the classical complement cascade, Cls activated by Clr cleaves C2 and C4 which have been known as sole substrates of Cls. Recently, however, we have demonstrated that Cls degrades type II collagen (Yamaguchi et al. 1990), a major constituent of cartilage matrix and gelatin, and activates zymogen of matrix metalloproteinase 9 (MMP-9) (Sakiyama et al. 1994). MMP-9 is known to be synthesized by chondrocytes in vivo (Toyoguchi et al. 1996) and in vitro (Nakagawa et al., 1997), and colocalized with Cls at the primary ossification center of human femur (Sakiyama et al. 1994). Therefore, Cls is thought to have a role in cartilage degradation.

Cartilage consists of chondrocytes and extracellular matrix, which contains several types of collagen and proteoglycans. During development and growth of long bones, chondrocytes pass through resting, proliferating, maturing, hypertrophic and degenerating stages. This naturally occurring cell death aids in tissue remodeling. Degenerated chondrocytes disappear, leaving enlarged lacunae and surrounding matrix which is eventually degraded by monocytes (Hunziker et al. 1984) and chondroclasts (Lewinson and Silberman 1992), and the site is replaced by bone marrow (Cancedda et al. 1995; Caplan and Pechak 1987). Programmed cell death of chondrocytes is accompanied by an expression of an s-myc protein (Asai et al. 1994) and tissue transglutaminase (Aeshimann et al. 1993; Fesus et al. 1991). In most tissues, dead cells are usually cleaned away by phagocytic cells. In cartilage, an avascular tissue, however, degenerating cells disappear before phagocytic cell invasion. Apoptotic signaling is known to induce various kinds of proteolytic enzymes (Nicholson et al. 1995; Seamus and Green 1995). Degrading enzymes may play a major role in the cleaning mechanisms of cartilage. We have shown that Cls synthesis increases in accordance with chondrocyte differentiation both in vivo (Toyoguchi et al. 1996)

Table 1. Antibodies specific to peptides (*arrow*, cleavage site on activation)

Antibody	Residues[a]	Sequences[a]
RK3	560–583	ERRNIVIQLRGAKLPVTSLE €€€€€€€€€€€€€€€€€€€€€€€€€€€€€€€€↓
RK4	410–440	VCGVPTEPFRIQQRIFGGFPAKIQSFPWQVF
RK5	424–431	IFGGFPAK

[a] Predicted from cDNA sequences of hamster Cls (Kinoshita et al. 1989)

and in vitro (Nakagawa et al., 1997). Cls synthesized by chondrocytes as well as that delivered from the blood stream may participate in chondrocyte and matrix degradation. Cls should be activated in situ to achieve the function. On activation, hamster Cls is cleaved between 423Arg and 424Ile (Kinoshita et al. 1989), generating a new NH_2-terminus of the L chain. In order to demonstrate the activation in the local tissues, we have developed antibodies specific to inactive and active Cls: RK5 recognizes the new epitope of the L chain of the active Cls, and RK4 recognizes an uncleaved peptide (Sakiyama et al. 1996). In the present studies, Cls activation in the endochondral ossification center was examined using these antibodies. We also examined degradation of a proteoglycan, decorin, which binds to and stabilizes collagens in cartilage matrix (Scott 1988; Hedbom and Heinegard 1989; Svensson et al. 1995).

Materials and methods

Antibodies and immunohistochemistry

Peptide-specific antibodies against hamster Cls are shown in Table 1. RK3 recognizes the active center and inhibits Cls esterase activity (Toyoguchi et al. 1995). RK3 reacts with both active and inactive Cls. RK4 and RK5 specifically bind to the inactive and active form of Cls, respectively (Sakiyama et al. 1996). The antibodies were purified by affinity chromatography on immobilized peptides. A monoclonal anti-hamster Cls antibody, PG11 (Sakiyama et al. 1991), and an anti-active-form-specific human Cls monoclonal antibody (mAb), M241 (Matsumoto and Nagaki 1986), were previously prepared and characterized.

Tibial epiphyseal cartilage was obtained from 14- to 16-day-old Syrian hamsters. Tissues were fixed with periodate-lysine-paraformaldehyde (PLP) at 4° C overnight, decalcified with 5% EDTA at 4° C in 0.15 M NaCl solution, soaked sequentially in 10%, 15%, and 20% sucrose and then 20% sucrose plus 1% glycerol and frozen in OCT compound (Miles Scientific, Naperville, Ill., USA). Sections were treated with sheep testis hyaluronidase (50 U/ml; EC 3.2.1.35; Seikagaku Kogyo, Tokyo, Japan), incubated with each antibody (20 μg/ml) at 4° C overnight followed by an incubation with biotinylated-anti-rabbit or anti-mouse IgG $F(ab)_2$ (Boehringer Mannheim Biochemica, Mannheim). The samples were then incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Boehringer Mannheim Biochemica), and counter-stained with hematoxylin. For immunoelectron-microscopic observations, the samples treated with anti-Cls antibodies and biotinylated anti-mouse or anti-rabbit antibodies were incubated with gold particle (10 nm)-conjugated strepatavidin (Amersham Japan, Tokyo). They were postfixed with 2% glutaraldehyde, 2% OsO_4 in PBS, followed by dehydration and embedded in Epon. Ultrathin sectinos were stained with uranyl acetate and lead citrate and then examined with the electron microscope JEM-1200EX (Nihondenshi, Tokyo, Japan).

Digestion of matrix components by human Cls

Digestion of decorin was first tested by incubating decorin (8 μg; gift of Dr. M. Pierschbacher, Telios) with Cls in enzyme-to-substrate ratios ranging from 1:50 to 1:5 at 37° C for 24 h in PBS. After termination of the reaction with 3 mM diisopropyl fluorophosphate (DFP), the samples were incubated with chondroitinase ABC (0.02 U/ml, Seikagaku Kogyo) at 23° C overnight. The reaction mixtures were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and stained with Coomassie brilliant blue. Time-dependent decorin digestion by Cls was also carried out in an enzyme-to-substrate ratio of 1:25 for 2–24 h, and the digestion products were analyzed as described above. Five micrograms of mouse type II collagen (gift of Dr. M. Suzuki) and type I gelatin (Sigma, St. Louis, Mo., USA) were incubated with Cls (1 μg) in 10 mM TRIS-HCl (pH 7.4) containing 0.15 M NaCl overnight at 37° C. The digestion products were analyzed on SDS-PAGE.

Immunostaining of Cls in human articular cartilage

Human knee articular cartilage in normal and rheumatoid arthritis was obtained from patients undergoing total replacement surgery with informed consent. The specimens were fixed with 4% paraformaldehyde and immunostained with PG11 (20 μg/ml), which cross-reacted with human Cls (Sakiyama et al. 1991).

Results

Immunohistochemistry and immunoelectron microscopy of Cls in hypertrophic chondrocytes

A longitudinal section of a 14-day-old hamster proximal tibia is shown in Fig. 1A. Bone marrow is being formed at the secondary ossification site. Chondrocytes differentiate from flattened proliferating cells at the periphery of the cartilage to enlarged hypertrophic cells toward the center. At the light-microscopic level, the cytoplasm of hypertrophic chondrocytes appears vacuolated (Fig. 1B, arrows). Such vacuolation proceeds in accordance with hypertrophy and results in dilatation of lacunae (Fig. 1B, arrowheads). Finally, the chondrocytes become undetectable. At this stage, no phagocytes are seen in the lacunae (Fig. 1C, arrowheads). The death of hypertrophic chondrocytes is programmed to make room for the hematopoietic marrow. Morphologically, however, this type of programmed chondrocyte cell death appears to be different from that of other cells such as lymphocytes. As seen in Fig. 1B, the hydrated hypertrophic chondrocytes do not show nuclear condensation and fragmentation and plasma membrane convolution that are known as common features of apoptosis (Allen 1987; Kerr et al. 1987).

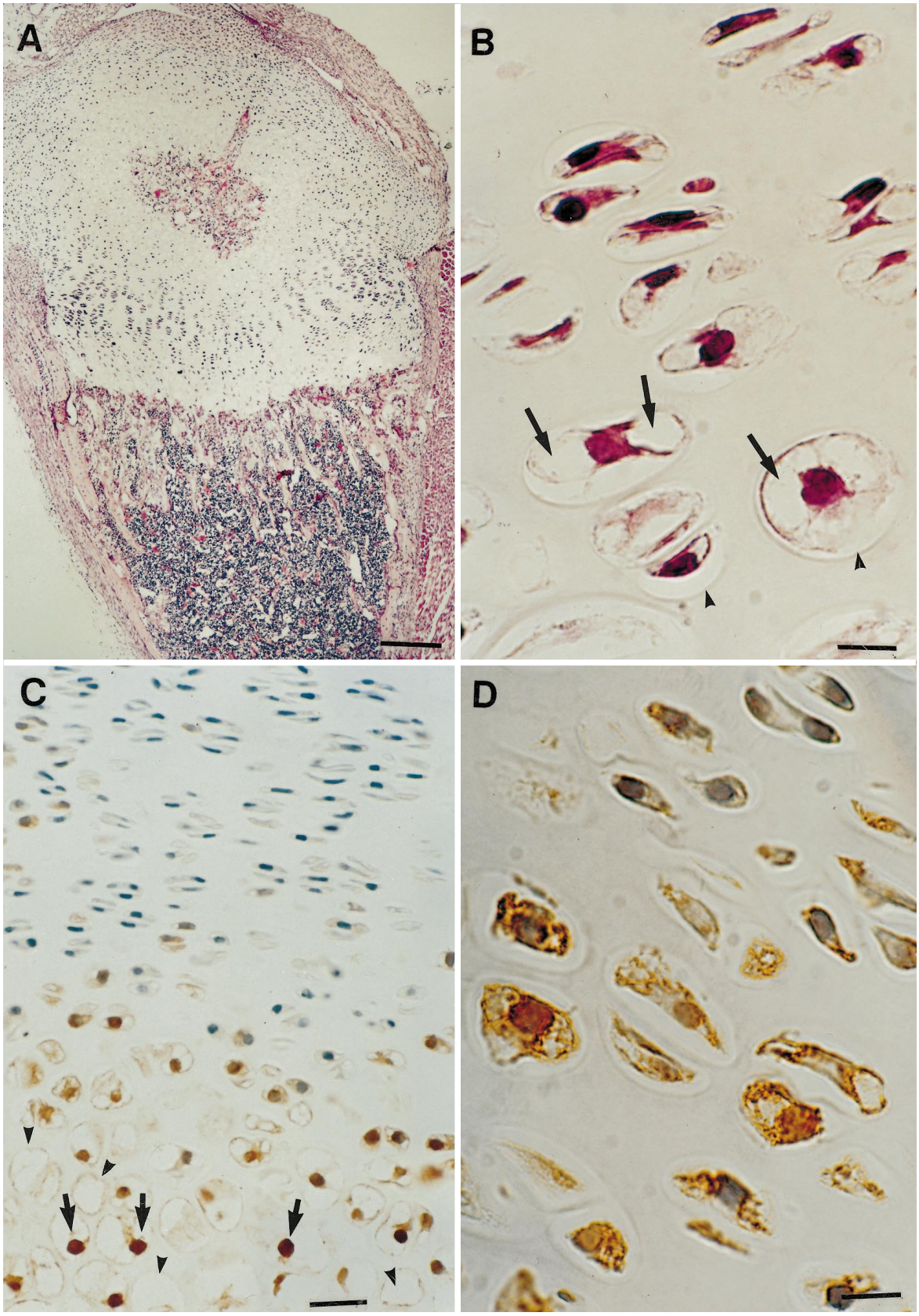

Fig. 1A–D. Morphology and immunostaining of Cls at the secondary ossification site of hamster tibia. Proximal tibia of 14-day-old hamster fixed with PLP and frozen-sectioned. **A** Staining with HE. *Bar:* 270 µm; ×37. **B** Higher mangification of **A**. *Bar:* 10 µm; ×925. **C** Section stained with anti-hamster Cls mAb PG11 (20 µg/ml), post-stained with hematoxylin. Proliferating chondrocytes negative in the staining with PG11, but degenerating hypertrophic chondrocytes strongly positive (nuclei and cytoplasm). *Bar:* 54 µm; ×185. **D** Higher magnification of hypertrophic chondrocytes. Degrading edges of cytoplasmic holes were stained predominantly. *Bar:* 10 µm; ×925

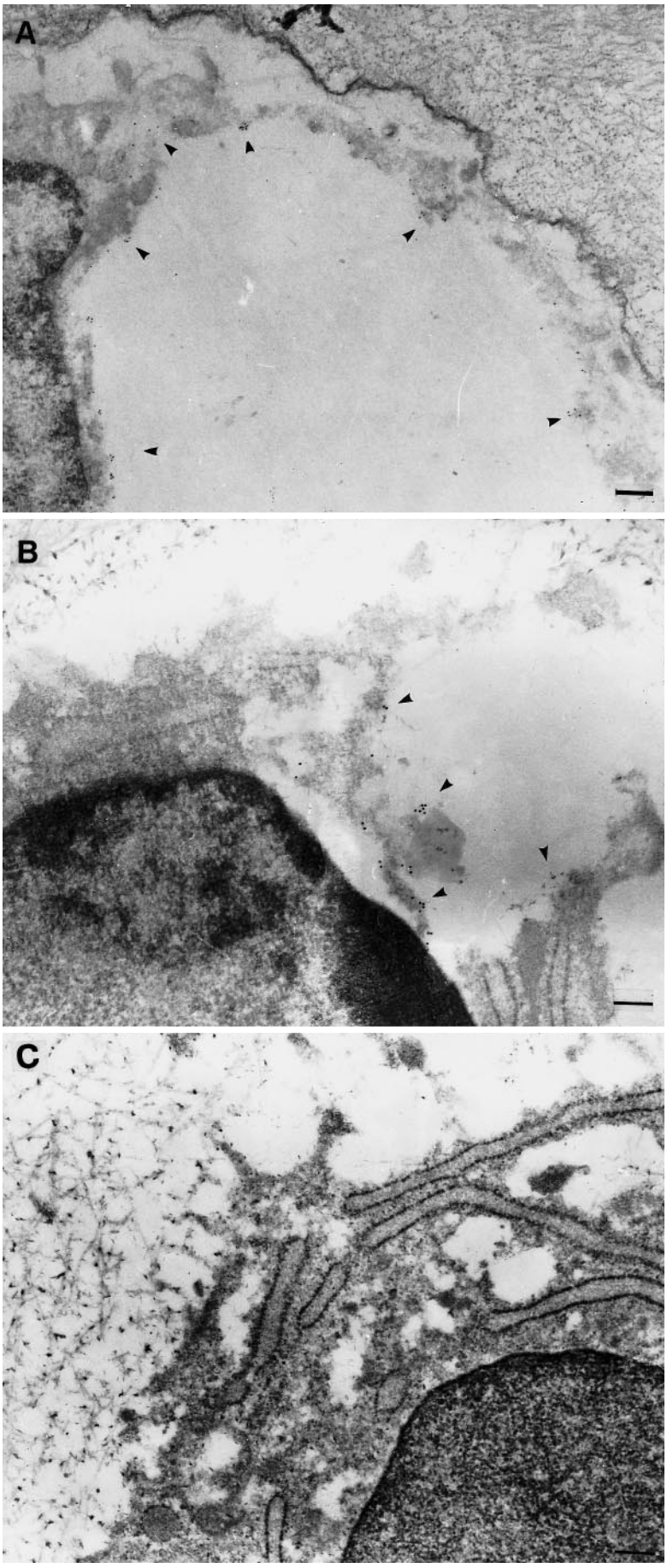

Fig. 2A–C. Immunoelectron microscopy of hypertrophic chondrocytes. Epiphyseal cartilage of 14-day-old hamster tibia fixed with PLP and immunostained with PG11 (**A**, **B**) or M241 (**C**), followed by biotin-anti-mouse IgG and streptavidin-gold as described in the text. Gold particles (*arrowheads*) in **A** and **B** are seen on degrading cellular fragments. **A** *Bar:* 267 nm, ×30000; **B, C** *bar:* 200 nm, ×40000

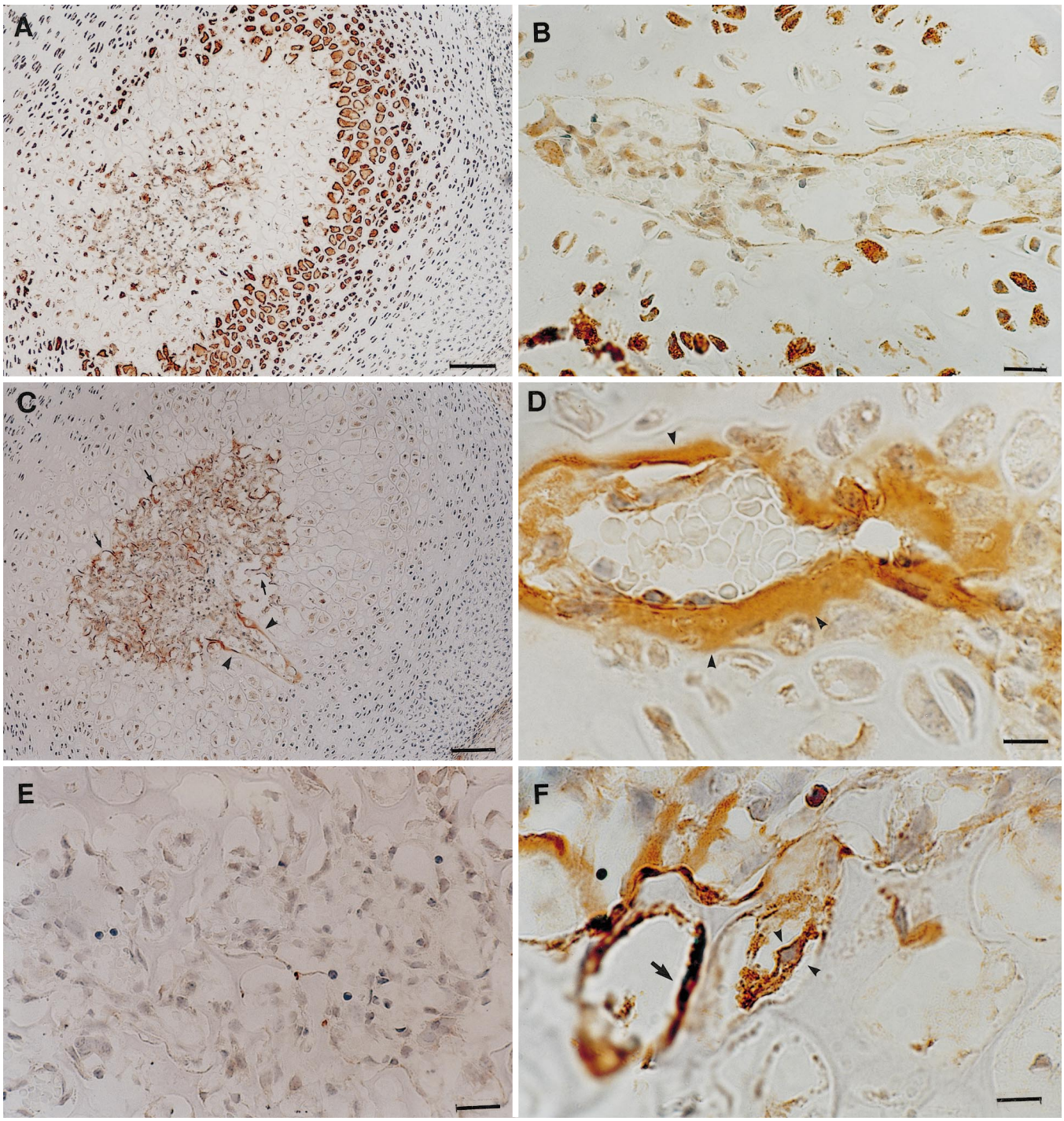

Fig. 3A–F. Differential immunostaining of active and inactive Cls. Epiphyseal cartilage fixed and frozen-sectioned as described in the text. The samples were incubated with either RK4 (20 μg/ml; **A, B**), RK5 (20 μg/ml; **C**, **D**, **F**) or normal rabbit IgG (20 μg/ml; **E**) and post-stained with biotin-anti-rabbit IgG $F(ab)_2$ and HRP-streptavidin. Inactive and active Cls were found mainly in hypertrophic chondrocytes (**A**, **B**) and extracellular matrix around invading vessels (**C**, **D**, **F**). **A**, **C** *Bar:* 108 μm, ×65; **B** *bar:* 27 μm, ×260; **D, F** *bar:* 10 μm, ×700; **E** *bar:* 54 μm, ×130

Hamster tibial secondary ossification center was immunostained with anti-hamster-Cls mAb PG11 (Fig. 1C, D). In the hypertrophic zone, edges of lacunae and cytoplasmic holes of hydrated chondrocytes and some, but not all, nuclei were strongly immunostained (Fig. 1C, D), whereas proliferating chondrocytes were negative (Fig. 1C). The strongly stained cells in Fig. 1C (arrows) may correspond to the dark condensed cells reported by Farnum and Wilsman (1987). M241, a mAb specific to human activated Cls, did not react even with degenerating hypertrophic chondrocytes (data not shown). Immunoelectron micrographs revealed that Cls

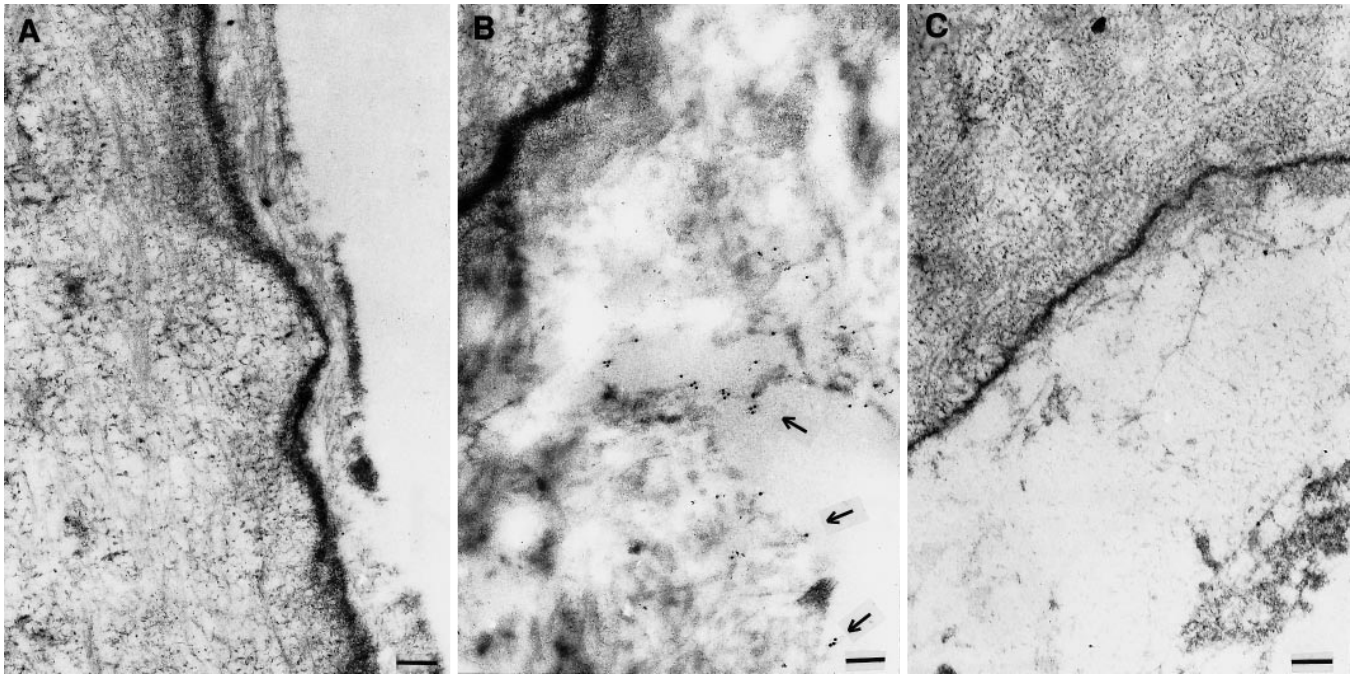

Fig. 4A–C. Immunoelectron micrograph of matrix around invading marrow. The sections were incubated with RK4 (**A**), RK5 (**B**) or normal rabbit IgG (**C**) and then biotin-anti-rabbit IgG $F(ab)_2$, followed by streptavidin-gold. Gold particles (*arrows*) were observed on amorphous mat-like structures at the degrading side of the matrix. *Bar:* 200 nm; ×40000

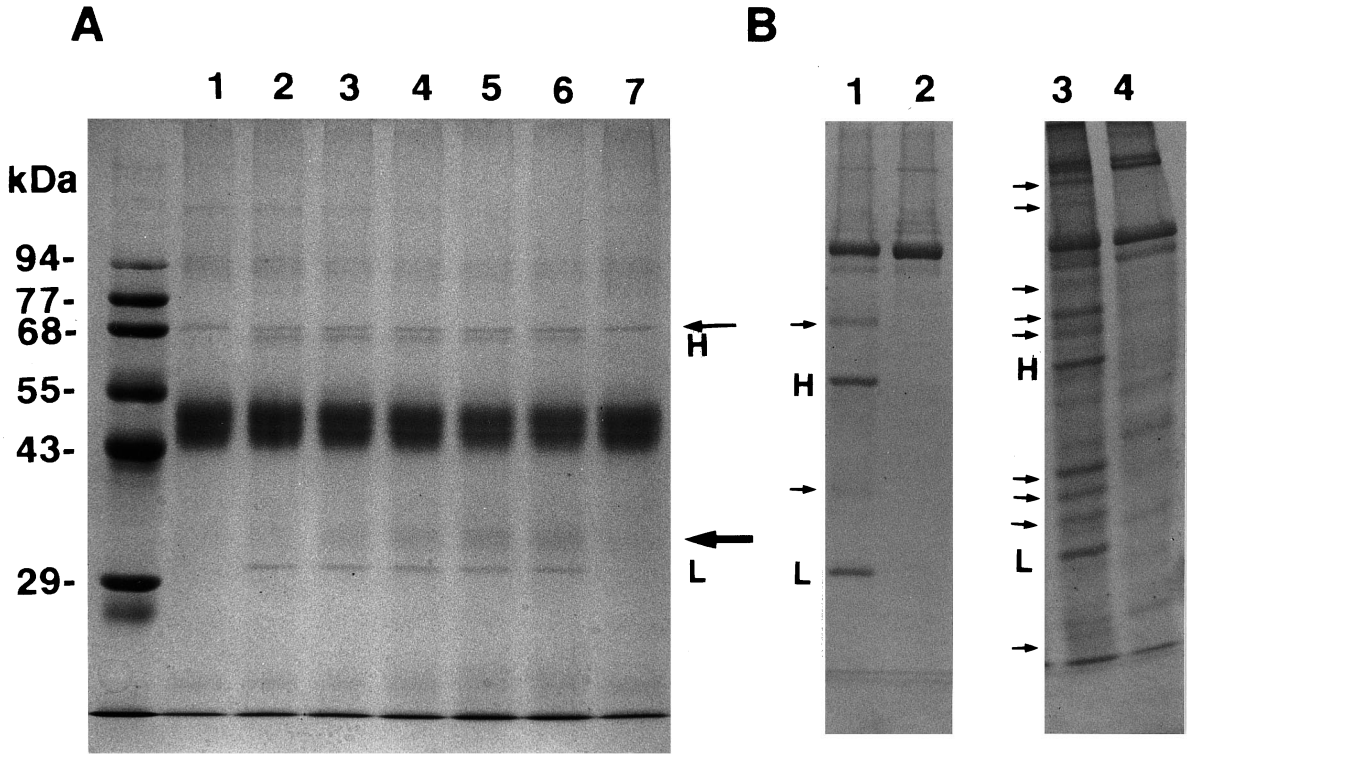

Fig. 5A, B. Digestion of matrix components by Cls. **A** Time-dependent degradation of decorin by Cls. Decorin (8 μg) was incubated with (*lanes 2–6*) or without (*lanes 1, 7*) Cls at a Cls-to-decorin ratio of 1:25 in PBS for 0 h (*lane 1*), 2 h (*lane 2*), 4 h (*lane 3*), 6 h (*lane 4*), 12 h (*lane 5*) and 24 h (*lanes 6, 7*) as described in the text. After the incubation, samples were analyzed on SDS-PAGE under reducing conditions and stained with Coomassie blue. *Large arrow*, Degradation product of decorin; *small arrow*, chondroitinase ABC; *H, L*, H and L chains of Cls, respectively. **B** Degradation of type II collagen (*lanes 1, 2*) and type I gelatin (*lanes 3, 4*) by Cls. Five micrograms of each protein was incubated with (1 μg; *lanes 1, 3*) or without (*lanes 2, 4*) Cls in TRIS-HCl buffer (pH 7.4) overnight at 37° C. The incubation mixtures were analyzed on SDS-PAGE as described in the text. *Small arrows*, Digestion products of the substrates; *H, L*, H and L chains of Cls

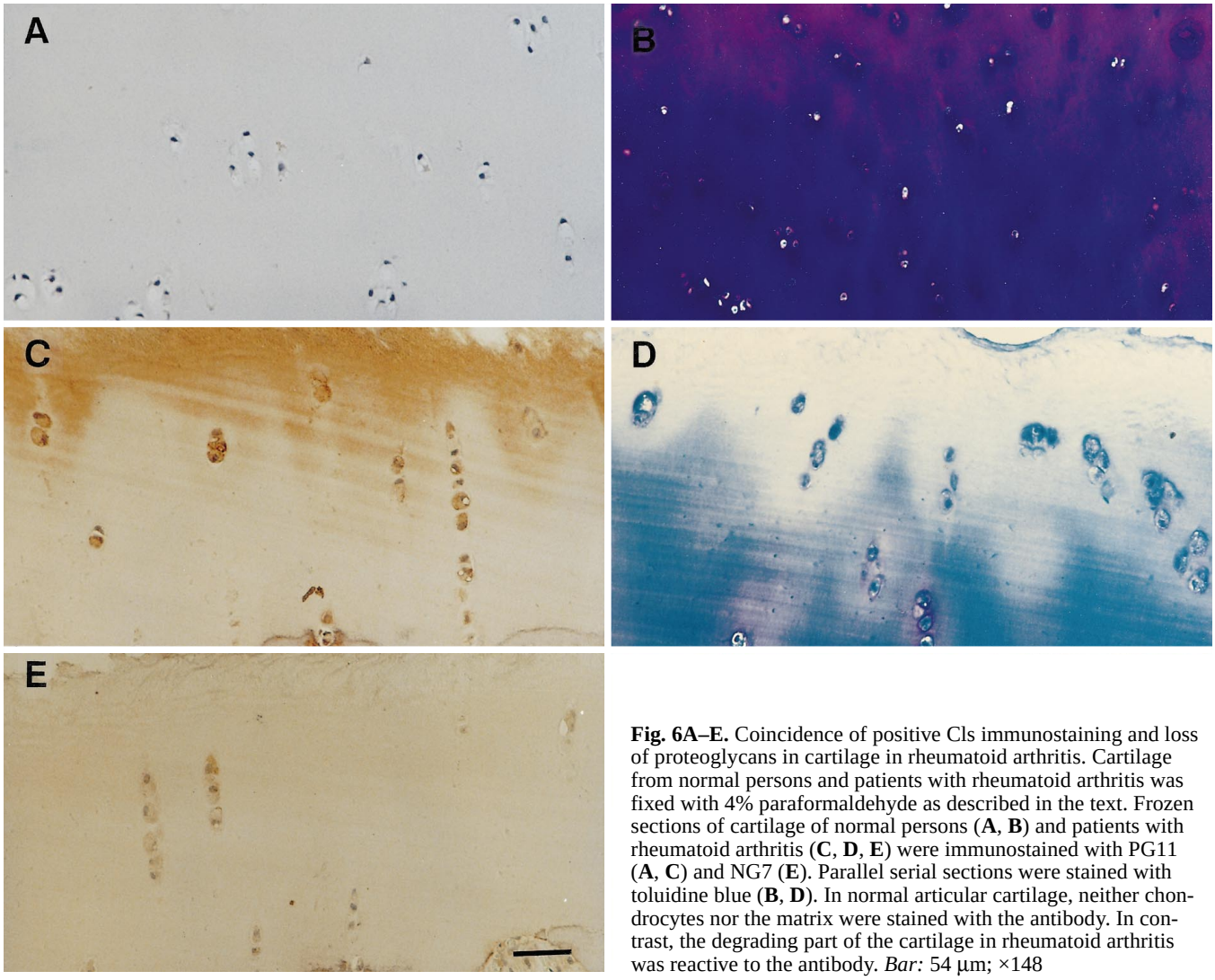

Fig. 6A–E. Coincidence of positive Cls immunostaining and loss of proteoglycans in cartilage in rheumatoid arthritis. Cartilage from normal persons and patients with rheumatoid arthritis was fixed with 4% paraformaldehyde as described in the text. Frozen sections of cartilage of normal persons (**A**, **B**) and patients with rheumatoid arthritis (**C**, **D**, **E**) were immunostained with PG11 (**A**, **C**) and NG7 (**E**). Parallel serial sections were stained with toluidine blue (**B**, **D**). In normal articular cartilage, neither chondrocytes nor the matrix were stained with the antibody. In contrast, the degrading part of the cartilage in rheumatoid arthritis was reactive to the antibody. *Bar:* 54 μm; ×148

was localized at degrading edges of cytoplasmic vacuoles and cellular fragments (Fig. 2A, B). No gold particles were visible when M241 was used (Fig. 2C).

Differential immunostaining of active and inactive Cls in situ

Since localization of Cls in the late hypertrophic zone suggested its involvement in cartilage degradation, we further examined whether Cls is activated at the degrading sites. When the specimen was treated with RK4, which reacts only with inactive Cls, hypertrophic chondrocytes (Fig. 3A, B) were positively stained. In contrast, RK5, the antibody specific to the neoepitope of activated Cls, stained Cls in the extracellular matrix around the invading blood vessels (Fig. 3C, D, arrowheads), the edges of the degrading matrix (Fig. 3C, F, arrows) and degenerative chondrocytes (Fig. 3F, arrowheads). These staining patterns displayed a striking contrast in the location of active and inactive Cls. Control rabbit IgG showed negligible staining (Fig. 3E).

Immunoelectron-microscopic study around the marrow revealed that activated Cls was localized on the fine filamentous mat-like structure at the degrading side of the demarcation line. No definite labeling was seen in the samples stained either with RK4 or non-immune Rig (Fig. 4A, C).

Digestion of matrix components by Cls

Since Cls was found to be activated at the ossification center, we examined whether it degraded matrix proteins. Decorin is known to stabilize collagen fibrils by binding to the molecules (Scott 1988; Hedbom and Heinegard 1989; Svensson et al. 1995). The susceptibility of decorin to Cls was also examined in the present study. When decorin was incubated with active Cls at enzyme-to-substrate ratios of 1:50, 1:40, 1:30, 1:25, 1:20, 1:15, 1:10 and 1:5, it was degraded in concentration-dependent manner (data not shown). Time-dependent degradation of decorin is shown in Fig. 5A. A major decorin degradation fragment of 33 kDa molecular mass was

generated after 2 h incubation with Cls (1:25) and increased with incubation time up to 24 h (Fig. 5A, large arrow). At the same time the bands of decorin became thin.

Cls split type II collagen into 2:1 fragments (Fig. 5B, lane 1, arrows), which were different from digestion fragments of collagenase (Harper 1980). Gelatin was degraded into several pieces by Cls (Fig. 5B, lane 3, arrows). The activities of Cls were inhibited by DFP and M241 (data not shown). These results confirmed our previous observations (Yamaguchi et al. 1990).

Cls immunostaining in human articular cartilage

Articular cartilage in rheumatoid arthritis (11/12 cases) was positively immunostained in both matrix and chondrocytes with PG11 (Fig. 6C), whereas normal cartilage (5/5 cases) was negative (Fig. 6A). The staining pattern of Cls in cartilage with rheumatoid arthritis coincided with the loss of proteoglycans, which was detected by toluidine-blue staining (Fig. 6D). Cartilage in rheumatoid arthritis showed poor reactivity to NG7 (Fig. 6E), a mAb against hamster Cls (Sakiyama et al. 1991), which is poorly cross-reactive to human Cls.

Discussion

In the present work we have shown by immunohistochemistry that Cls was activated at the secondary ossification center of hamster tibia. At the central part of the ossification center, degenerating late hypertrophic chondrocytes disappeared before bone marrow cell invasion. Since no phagocytic cells were seen in the lacunae at this stage, chondrocytes might be digested by their own degrading enzymes. It is possible that Cls is one of the degrading enzymes, because (1) Cls synthesis increases with hypertrophic changes of chondrocytes and (2) and active Cls-specific antibody RK5 immunostains the edges of degrading fragments of chondrocytes. Immune complex-independent activation of Cls is induced by exposure to various cellular components such as cell membranes (Storrs et al. 1983), intermediate filaments (Linder et al. 1979), DNA (Ziccardi 1982), cardiolipin (a mitochondrial inner membrane component) (Kovacsovics et al. 1987) and polyanionic substances (Rent et al. 1975). These cellular components may be available for Cls binding at the ossification center where chondrocyte degradation proceeds. Proteoglycans (belonging to the polyanionic molecules) are also abundant in the cartilage matrix. Cls around blood vessels may be permeated from vessels and/or secreted from endothelial cells, since endothelial cells are reported to synthesize Cls (Gulati et al. 1993) and to be immunostained by PG11 (Sakiyama et al. 1994). Cls reaching cartilage matrix can be activated rapidly, because Cls at this site was reactive to RK5, but not to RK4.

Our previous studies have shown that Cls has the ability to degrade collagens and activate proMMP-9 (Sakiyama et al. 1994). The present study further demonstrates that decorin is also a substrate of this enzyme, indicating that Cls has a broad range of substrates.

In hamster articular cartilage, Cls is not detected by either immunostaining or in situ hybridization (Toyoguchi et al. 1996). Consistent with these observations, normal human articular cartilage displayed no immunostaining with anti-Cls mAb PG11. However, since strong Cls immunostaining is seen in cartilage with rheumatoid arthritis, it is suggested that Cls may participate in pathological cartilage matrix degradation as well as in the physiological turnover observed in the ossification center.

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