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Quantitative detection of anionic sites in rat femoral cartilage using cationic colloidal gold at low pH levels

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Summary. Quantitative analyses of anionic sites in the rat cartilage were performed by using post-embedding LR-Gold method with cationic colloidal gold (CCG) at different pH levels. Alteration of the pH levels affected the CCG staining patterns. The CCG particles were more observed at low pH levels, 1.0 and 2.5, than those at high pH 7.4. So, we quantitatively examined the superficial, intermediate and deep layers at pH 1.0. Morphometric analyses showed that CCG particles were increased from the superficial layer to the deep layer in both pericellular matrix (PM) and interterritorial matrix (IM). Moreover, they were more observed in PM than in IM. After chondroitinase ABC digestion, the CCG labeling was reduced in all matrices of the superficial and intermediate layers, though many CCG particles were still observed in PM of the deep layer. After hyaluronidase digestion, the CCG labeling was markedly reduced in the PM of the deep layer. These findings suggest that anionic sites in the cartilage matrix are detected by using the CCG only at low pH levels. In addition, the PM in the deep layer may consist of different components from those of the other two layers.

Key words: Hyaline cartilage, Catinonic colloidal gold, pH level, Proteoglycan, Anionic site

Introduction

The articular cartilage is a highly hydrated connective tissue, which consists of a relatively small number of chondrocytes distributed throughout abundant extracellular matrix. The principal constituents of the cartilage matrix are type II collagen fibrils and proteoglycans (PGs). The PGs are complex macromolecules containing protein cores, to which glycosaminoglycans (GAGs) are covalently attached. They are also highly negative-charged by virtue of carboxyl and sulphate groups (Hardingham and Fosang, 1992). So, several dyes, such as alcian blue (Schofield et al., 1975; Maitland and Arsenault, 1989), ruthenium red (Thyberg, 1977), ruthenium hexammine trichloride (Hunziker et al., 1983), cuprolinic blue (Orford and Gardner, 1985; Van Kuppevelt et al., 1987; Scott, 1990) and polyethyleneimine (Schurer et al., 1977, 1978; Sauren et al., 1991; Toriumi, 1996; Ueda et al., 1997), were used to demonstrate anionic sites at an ultrastructural level. Such stainings are based on the electrostatic attraction between polyanionic GAGs and positively-charged dyes. Although some previous reports have used the pre-embedding techniques for detecting anionic sites with cationic probes, such routine treatment with osmium tetroxide may induce ultrastructural changes of anionic sites (Yoshimura et al., 1991; Ueda et al., 1997; Leng et al., 1998)

Recently, Goode et al. (1991, 1992, 1993) have reported glomerular anionic sites on post-embedded ultrathin sections with cationic colloidal gold (CCG). Since then, it has often been used for investigating the distribution of the PGs in other tissues (Saga and Takahashi, 1993; Torihara et al., 1995), but there has been no report on the cartilage matrix. In the present study, we have examined the pH effect on CCG labeling patterns in rat femoral cartilages and have also compared them among three layers, such as superficial, intermediate and deep layers, at pH 1.0.

Materials and methods

Animals and tissue preparations

Five male Wistar rats, aged 4 weeks, were deeply anesthetized by sodium pentobarbital, as reported before (Ueda et al., 1997). Their femoral heads were cut into small pieces by razor blades and immersed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (SCB), pH 7.4, at 4 °C for 60 min. They were dehydrated in a series of ethanol at -25 °C and embedded in LR-Gold (Polysciences, Warrington, PA, USA), and polymerized by ultraviolet beam at -25 °C for 24 h.

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Toluidine blue staining for light microscopy

Semithin sections at 1 μm thickness were stained with 0.5% toluidine blue, and observed with a light microscope (Olympus BH-2, Tokyo, Japan). Three different layers; superficial, intermediate and deep, could be detected and routinely trimmed.

Cationic colloidal gold labeling

Ultrathin sections were put on nickel grids and treated with 50 mM ammonium chloride in SCB for 30 min to block the negative charge of free aldehyde residues (Torihara et al., 1995). Then they were incubated at different pH levels with cationic colloidal gold of 5 nm size (British Biocell, Cardiff, UK) at 1:50 dilution for 60 min. The three buffer solutions used were 0.1M SCB (pH 7.4), potassium hydrogen phthalate-HCl buffer (pH 2.5), or potassium chloride-HCl buffer (pH 1.0). After rinsing in each buffer solution, they were dried at room temperature. Subsequently, they were fixed again with 0.25% glutaraldehyde in SCB for 10 min, and counter-stained only with uranyl acetate. They were observed with an electron microscope (Hitachi H-600, Tokyo, Japan).

Enzyme-digested preparations

Ultrathin sections were digested with two enzymes before the CCG labeling, as reported before (Ueda et al., 1997). Some were treated with 50 TRU/ml hyaluronidase "Amano" (Amano, Osaka, Japan), which was derived from *Streptomyces hyalurolyticus* and specifically-digested hyaluronan, at 60 °C for 1 h. Others were also digested with 1U/ml chondroitinase ABC (Sigma, St Louis, MO, USA) at 37 °C for 1 h. Subsequently, they were labeled with the CCG and prepared in the same way as described above.

Quantitative analysis

Quantification of CCG labeling was randomly performed, as reported before (Ueda et al., 1997) and statistically analysed with a t-test. The labeling density was calculated by the number of CCG particles per μm^2 at 10 different parts of each matrix in superficial, intermediate and deep layers.

Results

It is well-known that femoral cartilages consist of the superficial layer (SL), intermediate layer (IL), deep layer (DL) and calcified layer. In this study, we have focused on the upper three layers.

Effect of pH on CCG labeling

Figure 1 shows CCG labeling at different pH levels in the IL. Alteration of pH levels had a marked effect on the distribution of CCG particles in the cartilage matrix. Some gold particles were localized in the PM (Fig. 1a) and IM (Fig. 1b) at pH 7.4. On the contrary, more gold particles were observed in both PM and IM regions at pH 2.5 (Fig. 1c,d). At pH 1.0, most gold particles were observed to assemble into small aggregates (Fig. 1e,f). The CCG particles in chondrocytes were scarcely distributed at the same level of background (data not shown).

CCG labeling in SL at pH 1.0

Figure 2 shows CCG labeling in the SL at pH1.0. The number of CCG particles in all matrices were remarkably decreased in comparison with those of DL (Fig. 2a, Table 1). Especially, the CCG particles in the superficial lamina (Fig. 2b) were less observed than those of IM (Fig. 2c). The particle numbers of territorial matrix (TM) or interterritorial matrix (IM) were

Table 1. Distribution of CCG particles in the rat cartilage at pH 1.0. (particle number per 1μm^2 of cartilage matrix).

LAYER	PM	TM	IM	La
SL	210.1±67.7**	98.7±38.8**†	63.4±18.6*†	17.6±3.9††
IL	500.8±52.2**	233.7±41.4**††	223.8±39.5††	
DL	1007.6±193.8	721.9±123.3†	206.2±49.2††	

PM: pericellular matrix; TM: territorial matrix; IM: interterritorial matrix; La: superficial lamina; SL: superficial layer; IL: intermediate layer; DL: deep layer. *: p<0.01, **: p<0.001; comparing the number of each matrix (PM, TM and IM) in SL and IL with that of the corresponding region in the DL. †: p<0.01, ††: p<0.001; comparing the number in the TM or IM with that of the PM in each layer.

Table 2. Distribution of CCG particles in the rat cartilage at pH 1.0 after chondroitinase ABC digestion (particle number per 1μm^2 of cartilage matrix).

LAYER	PM	TM	IM
IL	80.1±26.3**	67.2±23.6**	32.9±7.1**
DL	719.4±230.6	305.6±127.0**	144.1±16.5

PM: pericellular matrix; TM: territorial matrix; IM: interterritorial matrix; IL: intermediate layer; DL: deep layer. *: p<0.01, **: p<0.001; comparing each number with that of the corresponding region in non-enzyme treated preparations.

Table 3. Distribution of CCG particles in the rat cartilage at pH 1.0 after hyaluronidase digestion (particle number per 1μm^2 of cartilage matrix).

LAYER	PM	TM	IM
IL	1272.9±211.7	1367.4±316.8	976.7±167.5
DL	834.5±164.8*	1842.9±21.3*	1669.9±78.8**

PM: pericellular matrix; TM: territorial matrix; IM: interterritorial matrix; IL: intermediate layer; DL: deep layer. *: p<0.01, **: p<0.001; comparing each number in the DL with that in corresponding matrices of the IL.

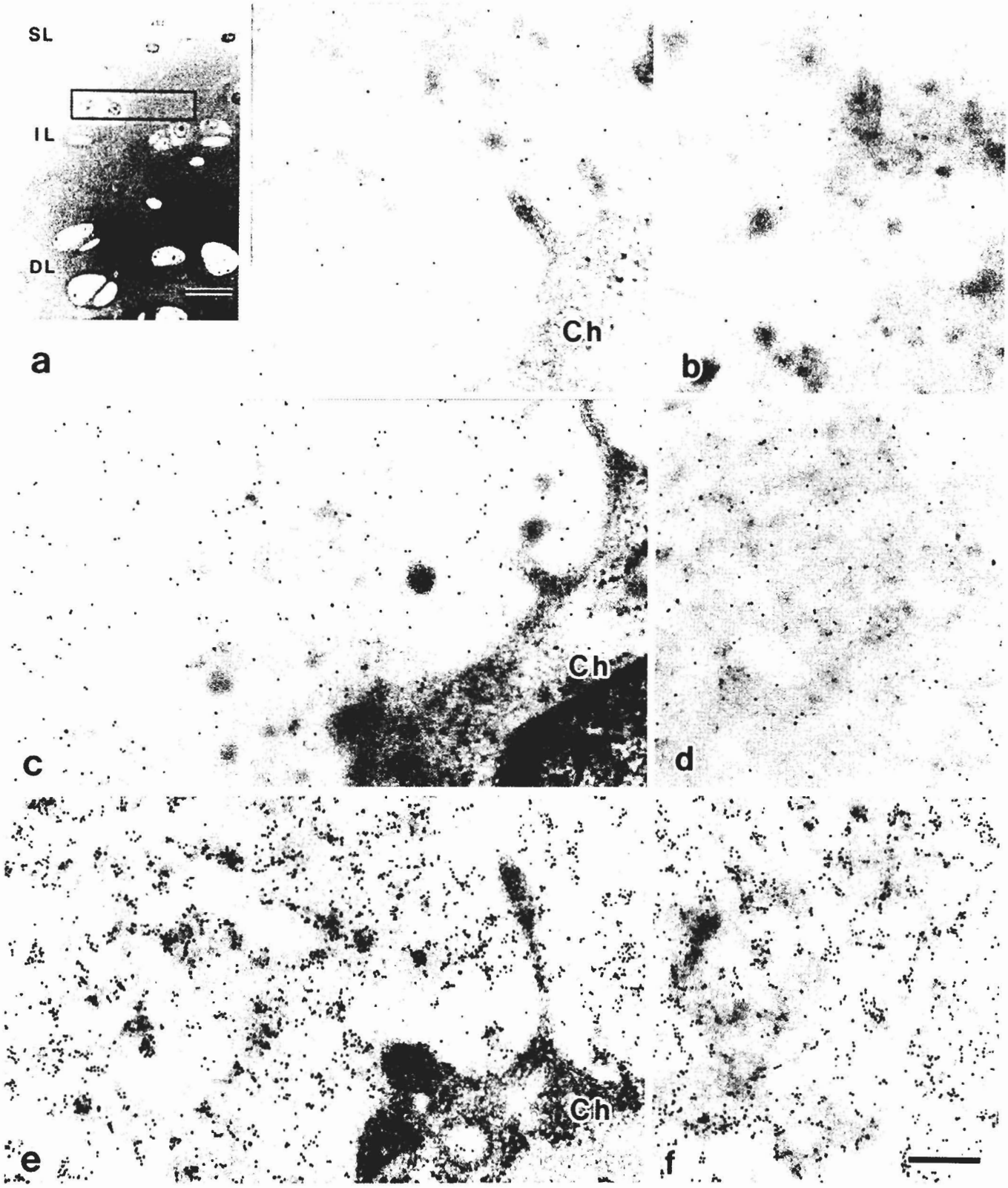

Fig. 1. CCG labeling at different pH levels in the intermediate layer. Some gold particles are localized in the pericellular **(a)** and interterritorial regions **(b)** at pH 7.4. Inset: toluidine blue staining. A square indicates the region examined by electron microscopy. Bar: 20 μm. DL: deep layer; IL: intermediate layer; SL: superficial layer. More gold particles are recognized in both regions at pH 2.5 **(c, d)** and pH 1.0 **(e, f)**. Ch: chondrocyte. Bar: 200 nm.

significantly reduced, as compared with those of PM, in the SL ($p<0.01$) or IL ($p<0.001$) (Table 1).

CCG labeling in DL at pH1.0

Figure 3 shows CCG labeling in the DL at pH1.0. The PM was distinguished from the TM, because more gold particles were observed in the PM than those in the TM (Fig. 3a). The number of gold particles was less in the IM than that in the PM (Fig. 3b). The statistical analysis clarified that the labeling density in the DL was significantly classified into three levels (Table 1, bottom).

Chondroitinase ABC digestion

Figure 4 shows different CCG labeling patterns in both IL and DL at pH1.0 after chondroitinase ABC digestion. In the IL, gold particles were decreased in both PM (Fig. 4a) and IM (Fig. 4b). The statistical analysis indicated that they were significantly decreased in all matrices of the IL ($p<0.001$; Table 2, top). On the contrary, most CCG particles still remained in PM of the DL even after the enzyme digestion (Fig. 4c), but they were remarkably decreased in IM (Fig. 4d). The statistical analysis indicated that CCG labeling density in all matrices was significantly reduced in the IL (Table 2, top).

Hyaluronidase digestion

Figure 5 shows various CCG labeling patterns in the IL and DL at pH 1.0 after hyaluronidase digestion. The labeling densities were increased, when compared with

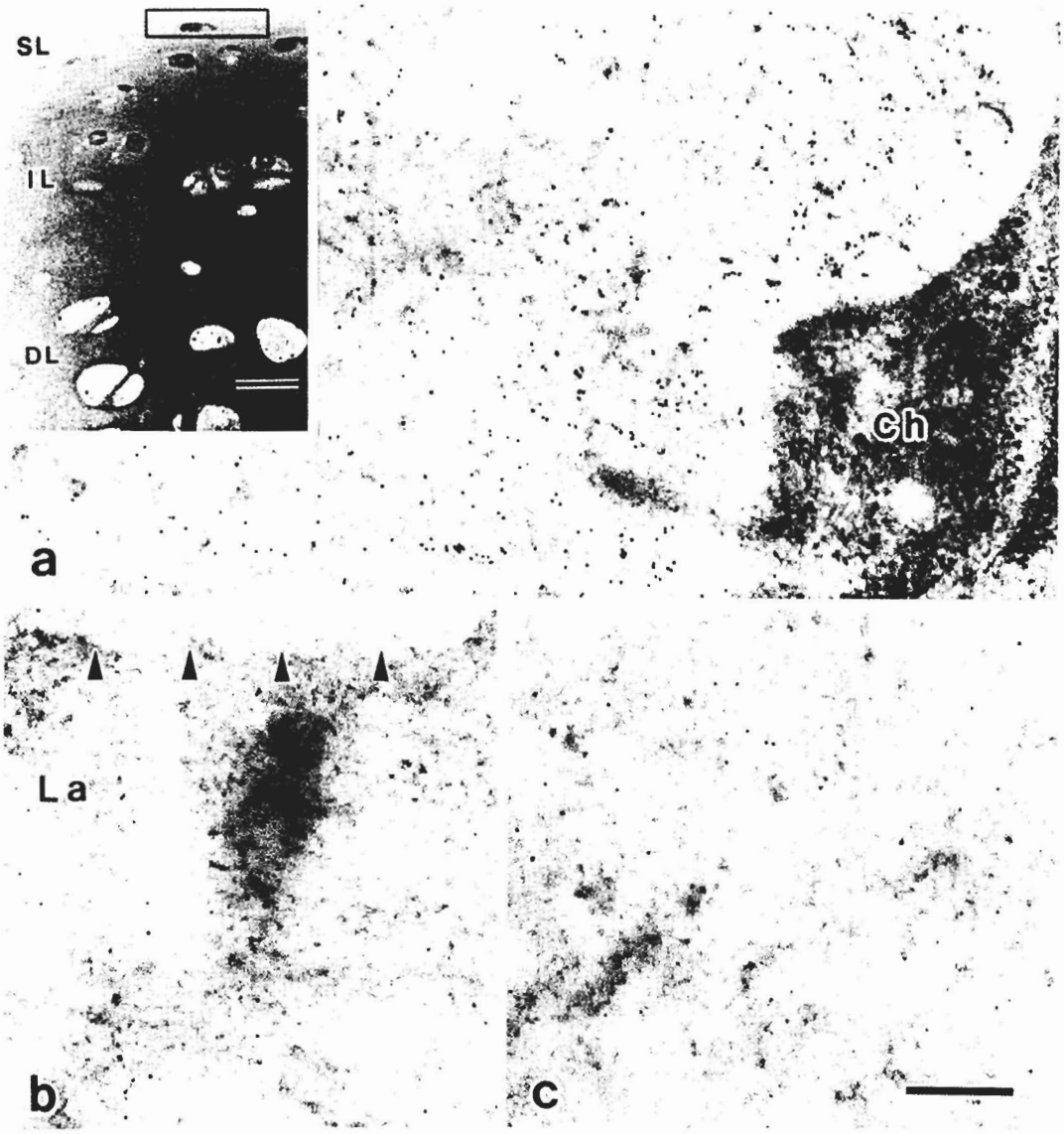

Fig. 2. CCG labeling in the superficial layer at pH 1.0. More gold particles are localized in both pericellular and territorial matrices **(a)** than those in the superficial lamina **(b)** and the interterritorial matrix **(c)**. Arrowheads indicate the surface of the femoral head cartilage. Inset: toluidine blue staining. A square indicates the region examined by electron microscopy. Bar: 20 μm.
DL: deep layer;
IL: intermediate layer;
SL: superficial layer;
La: superficial lamina;
Ch: chondrocyte.
Bar: 200 nm.

those without the enzyme digestion (Figs. 1e,f, 3a, b). In the IL, CCG particles were lined up or aggregated in the PM (Fig. 5a) and IM (Fig. 5b). In the DL, they were remarkably decreased in the PM (Fig. 5c), though they were still lined up or aggregated in the IM (Fig. 5d). The statistical analysis indicated that the gold particles of PM in the DL were significantly decreased, in comparison with those of PM in the IL (Table 3). However, they were significantly increased in both TM and IM of the DL.

Discussion

It has been known that carboxyl, phosphate or sulphate groups are negatively-charged components in animal tissues (Thiéry and Ovtracht, 1979). In addition, detection of such anionic sites depends on pH conditions. A sulphate group is strongly acidic and remains ionized below pH 1.0, though both carboxyl and phosphate groups are not ionized under such a condition (Spicer et al., 1967; Thiéry and Ovtracht, 1979; Kashio et al., 1992; Yang et al., 1996). Our findings indicated that alteration of pH levels affected the CCG labeling patterns, which were dramatically decreased at higher pH levels. There is a question why many fewer CCG particles were observed at pH 7.4 or pH 2.5, as shown in Figure 1. In our previous study (Ueda et al., 1997), the cartilage matrix was stained with a cationic dye, polyethyleneimine (PEI: MW 2,000), and PEI-positive products were detected at pH 7.4. However, the staining pattern at pH 7.4 was different from that at low pH levels. There might be two possible ideas in the discrepancy. One is that CCG particles are much larger than PEI molecule, so binding capacity of the CCG may

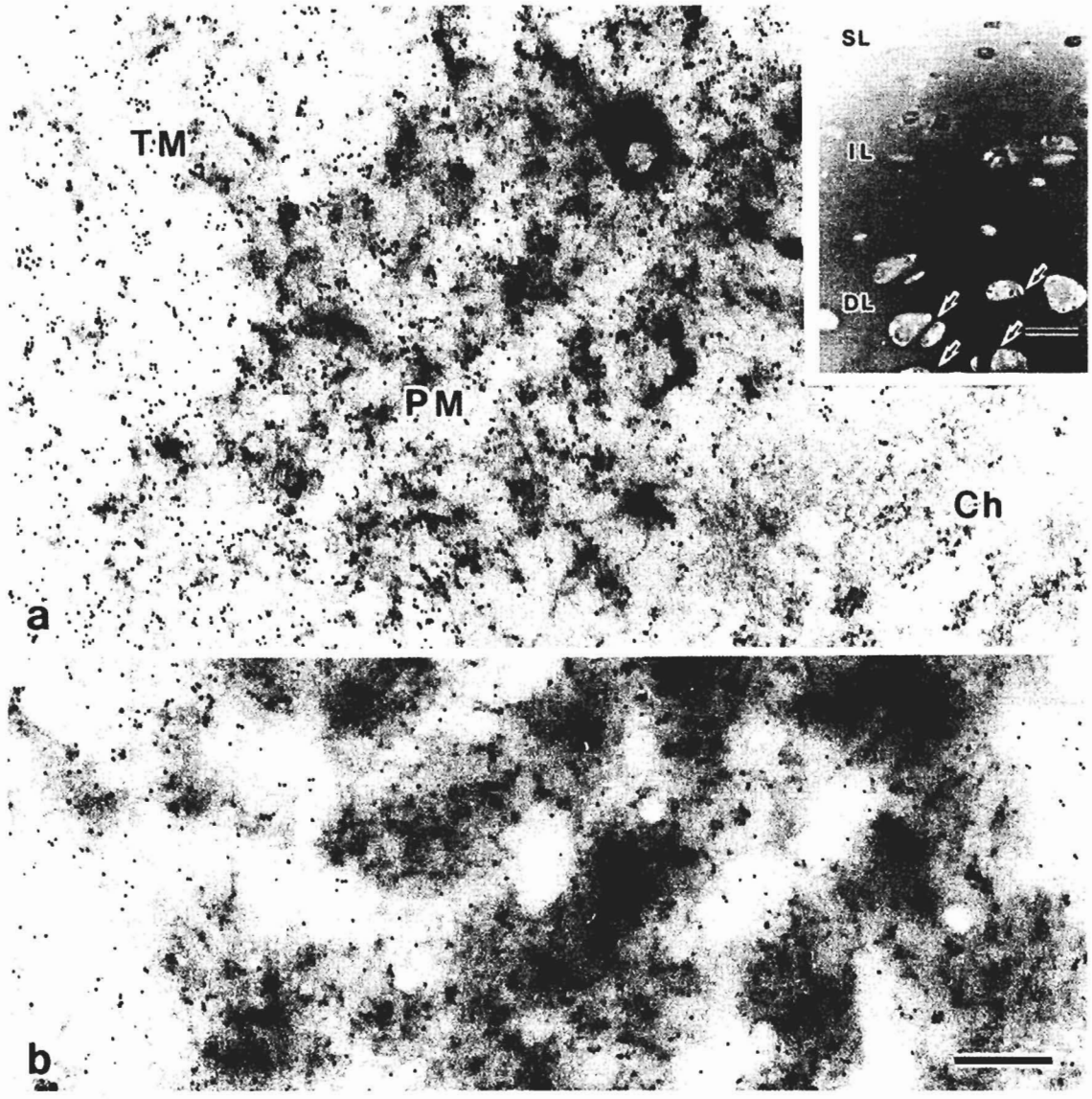

Fig. 3. CCG labeling in the deep layer at pH 1.0. **a.** Gold particles are localized in the pericellular matrix (PM) more than those in the territorial matrix (TM). Inset: toluidine blue staining. Arrows indicate that pericellular regions are stained with toluidine blue. Bar: 20 μm. **b.** Interterritorial matrix. Bar: 200nm.

be relatively weaker than that of PEI. Therefore, during the staining process, the CCG could not detect weakly-charged anionic sites. However, this idea cannot explain the reason why the CCG could not detect sulphate groups at pH 7.4. It was reported that, on the chemical basis of negative charge expression, the glycocalyx charge in kidney glomeruli was easy to detect below pH 3.0, but difficult at higher pH levels (Goode et al., 1991). The second possible idea is that negative charges of sulphate groups in cartilages may be decreased at higher pH. It was suggested that effects of low pH levels might alter the structural configuration, and thus render some negative-charge sites visible by CCG (Goode et al., 1991). During joint loading, mechanical compression of the cartilages can lead to some dynamic changes of components including interstitial ion concentrations or negative-charged structures (Garcia et al., 1996). Therefore, it is necessary to clarify a correlation between the mechanical cartilage compression and the negative-charge expression of sulphate groups in vivo.

The cartilages contain lots of sulphated PGs for keeping the solvents to resist the mechanical joint loading. A major PG in hyaline cartilages is an aggrecan, which has approximately 100 chondroitin sulphate and 30 keratan sulphate chains (Mörgelin et al., 1995). Our findings suggested that sulphate groups in the DL were

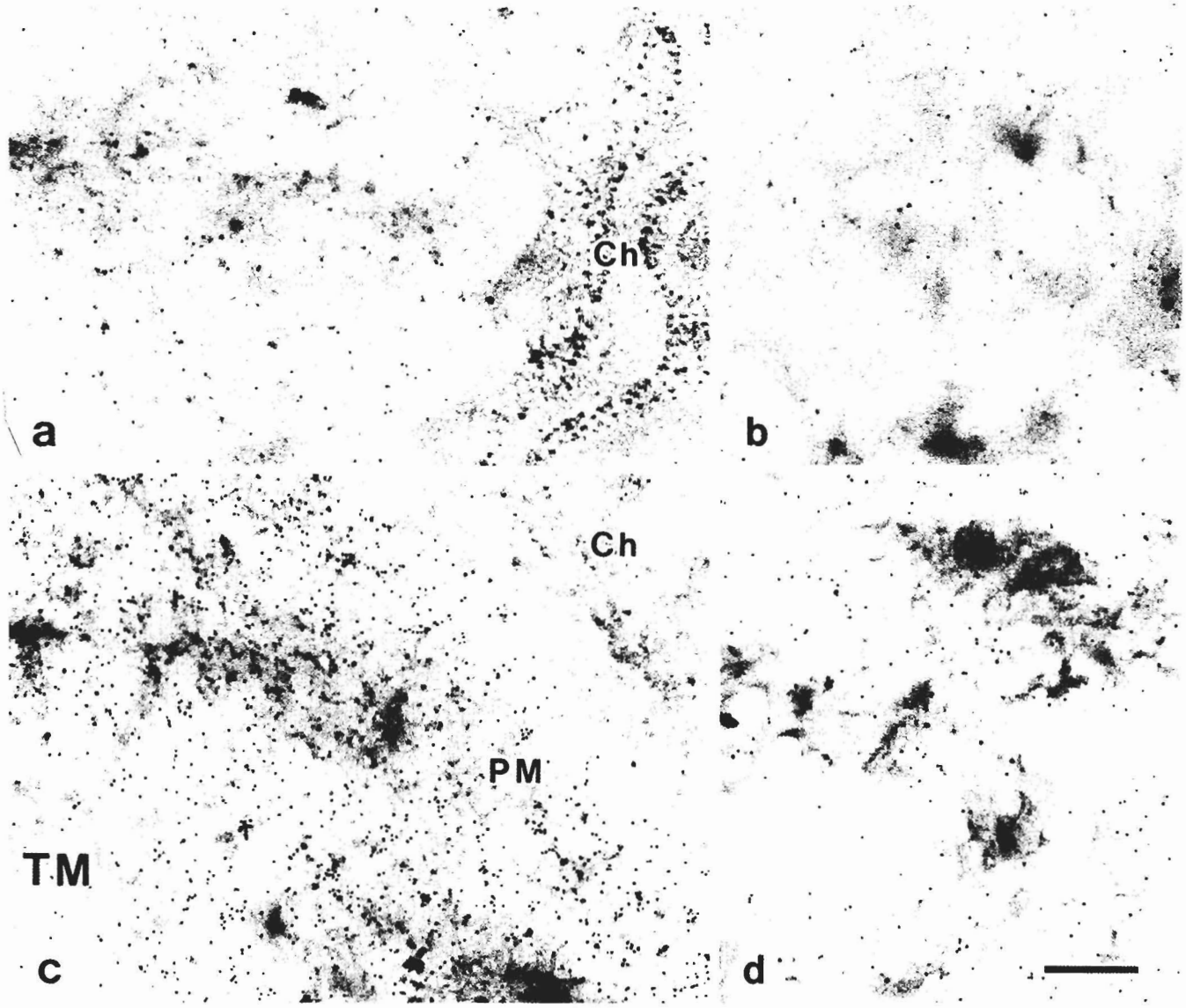

Fig. 4. Different CCG labeling patterns at pH 1.0 between the intermediate **(a,b)** and deep **(c,d)** layers after chondroitinase ABC digestion. In the intermediate layer, gold particles are considerably decreased in both pericellular (a) and interterritorial (b) matrix. In the deep layer, most gold particles are remained in the pericellular matrix (c) after the enzyme digestion. However, they are decreased in the interterritorial matrix as well as that in the same region without enzyme treatment (d). Ch: chondrocytes; PM: pericellular matrix; TM: territorial matrix. Bar: 200 nm.

localized more than those in the SL and IL. Especially, it was noted that PM in the DL was heavily negatively-charged. Preparations after digestion with chondroitinase ABC showed different CCG labeling patterns between the IL and the DL. Most of the CCG particles were eliminated in the IL, but many CCG particles still remained in the PM of the DL. So, the PM of the DL probably contains chondroitinase-resistant sulphates in addition to chondroitin sulphates. Moreover, the CCG labeling of the PM was dramatically decreased after the hyaluronidase digestion. It is reported that chondrocytes have surface receptors for hyaluronan in the same class as CD44 (Knudson, 1993), indicating that hyaluronan molecules with sulphated PGs encircle the chondrocytes in the DL. Hyaluronan-aggrecan complexes, which were considered to be a model for the PM and/or TM of cartilages, were proposed to extend from the cell coat in a brush-like configuration (Lee et al., 1993). Accordingly, such a decrease of CCG particles may be related to sulphated PGs detachment from the degraded hyaluronan.

Two different models of chondrocyte differentiation have been proposed for articular cartilages (Chen et al., 1995). One is the histological category, divided into

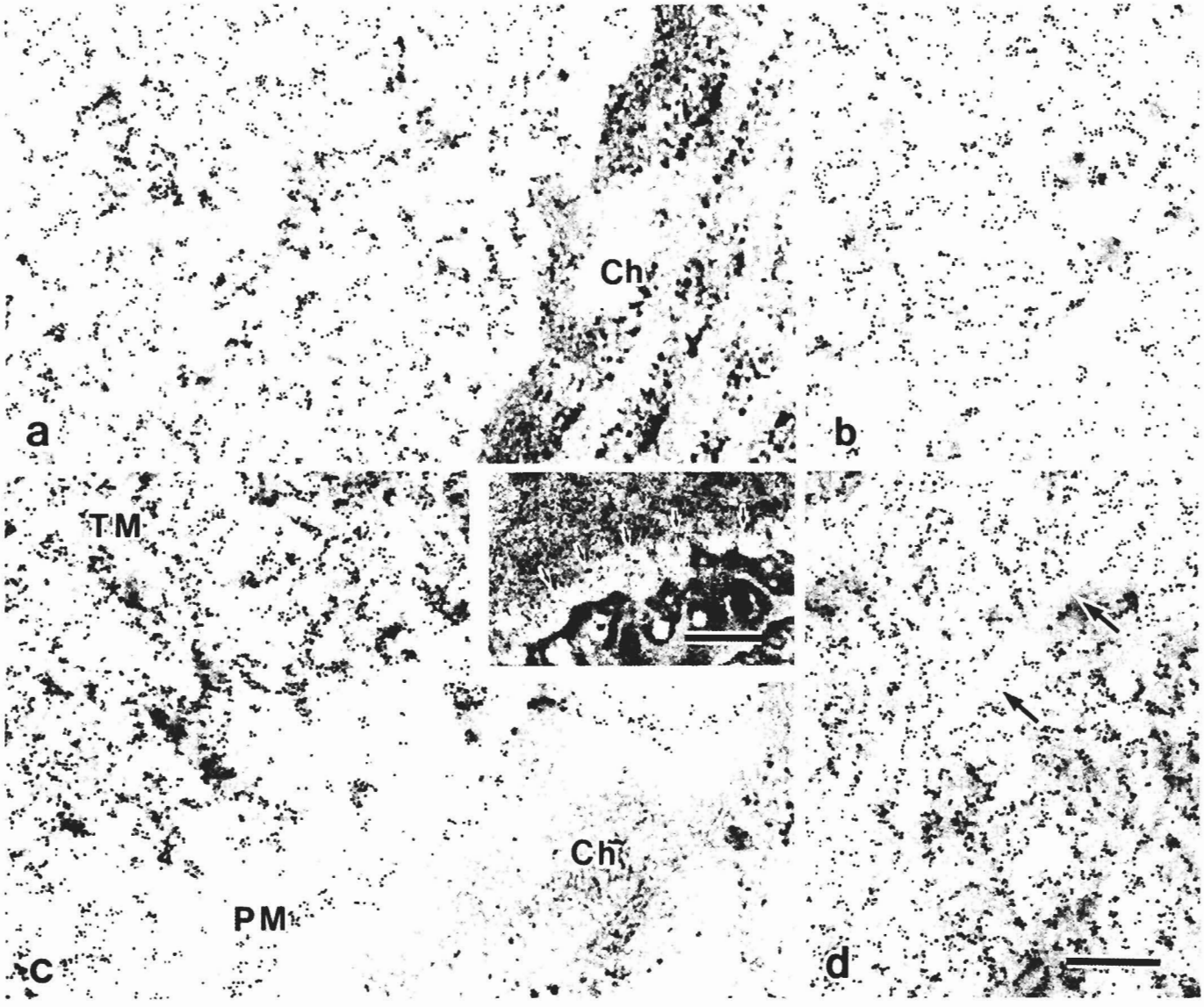

Fig. 5. Different CCG labeling patterns at pH 1.0 between the intermediate layer **(a, b)** and the deep layer **(c, d)** after hyaluronidase digestion. In the intermediate layer, gold particles are lined up or aggregated in the pericellular (a) or interterritorial (b) matrix. c. In the deep layer, gold particles are decreased in the pericellular matrix (PM) after the enzyme digestion. Inset: low magnified chondrocyte and the matrix. Small arrows indicate the area with less CCG labeling. d. Many gold particles are seen in the interterritorial matrix. Arrows indicate a thick collagen fiber. Ch: chondrocytes; PM: pericellular matrix; TM: territorial matrix. Bar: 200 nm. Inset, Bar: 1 μm.

three stages; proliferation, maturation, and hypertrophy (Kim and Conrad, 1977), which correspond to the SL, IL, and DL, respectively. The second model indicates that chondrocyte differentiation is divided into two stages (Castagnola et al., 1988). Chondrocytes are considered to be either in the proliferative state or in the hypertrophic state. In terms of cartilagenous anionic sites, the second model is supported because the CCG labeling patterns were different between the DL and the SL or IL. The hypertrophic chondrocytes produce type X collagen (Capasso et al., 1984), which is not produced by proliferative chondrocytes. So, some chondroitinase-resistant PGs might be produced in the hypertrophic chondrocytes.

In summary, CCG labeling in the rat cartilage is strongly detected at pH 1.0, but not at higher pH levels. In addition, the CCG labeling patterns in the DL are different from those in the upper two layers, and PM in the DL has lots of hyaluronans and chondroitinase-resistant sulphates. Further studies are needed to elucidate the function of such anionic sites in the cartilage matrix.

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