Osteoarthritis and Cartilage (1996) 4, 63–76
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1063–4584/96/010063 + 14 $12.00/0

OSTEOARTHRITIS and CARTILAGE

Pulsed electromagnetic fields influence hyaline cartilage extracellular matrix composition without affecting molecular structure

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Summary

Pulsed electromagnetic fields (PEMF) influence the extracellular matrix metabolism of a diverse range of skeletal tissues. This study focuses upon the effect of PEMF on the composition and molecular structure of cartilage proteoglycans. Sixteen-day-old embryonic chick sterna were explanted to culture and exposed to a PEMF for 3 h/day for 48 h. PEMF treatment did not affect the DNA content of explants but stimulated elevation of glycosaminoglycan content in the explant and conserved the tissue's histological integrity. The glycosaminoglycans in sterna exposed to PEMF were indistinguishable from those in controls in their composition of chondroitin sulfate resulting from chondroitinase ABC digestion. Specific examination with [^{35}S]-sulfate labels showed that PEMF treatment significantly suppressed both the degradation of pre-existing glycosaminoglycans biosynthetically labeled *in ovo* and the synthesis of new [^{35}S]-sulfated glycosaminoglycans. The average size and aggregating ability of pre-existing and newly synthesized [^{35}S]-sulfated proteoglycans extracted with 4 M guanidinium chloride from PEMF-treated cartilage explants were identical to controls. The chain length and degree of sulfation of [^{35}S]-sulfated glycosaminoglycans also were identical in control and PEMF-treated cultures. PEMF treatment also reduced the amount of both unlabeled glycosaminoglycans and labeled pre-existing and newly synthesized [^{35}S]-sulfated glycosaminoglycans recovered from the nutrient media. [^{35}S]-Sulfated proteoglycans released to the media of both control and PEMF-treated cultures were mostly degradation products although their glycosaminoglycan chain size was unchanged. These results demonstrate that exposure of embryonic chick cartilage explants to PEMF for 3 h/day maintains a balanced proteoglycan composition by down-regulating its turnover without affecting either molecular structure or function.

Key words: Pulsed electromagnetic fields, Cartilage extracellular matrix, Proteoglycan, Glycosaminoglycan.

Introduction

CARTILAGE EXTRACELLULAR MATRIX establishes a highly concentrated gel of proteoglycan immobilized within a dense network of collagen fibrils [1]. Proteoglycans are high molecular weight molecules composed of a core protein to which a large number of negatively charged and extremely hydrophilic glycosaminoglycan chains are attached covalently. Under normal physiological conditions sulfated proteoglycans interact with hyaluronic acid through one end of the protein core to form large multimolecular aggregates [2–4]. The high concentration and extreme hydrophilicity of proteoglycan aggregates create swelling pressure within the extracellular matrix which is constrained by a relatively inextensible collagen network. The association between proteoglycans and collagens provides articular cartilage with the unique physical properties of reversible compressibility and tensile strength enabling it to both withstand mechanical stress and protect underlying bone [5]. Loss of proteoglycan associated with osteoarthritis results in a disruption of cartilage integrity and an inevitable loss of biomechanical function [6]. Maintenance and/or restoration of a functional extracellular matrix is of significant interest to the repair of damaged cartilage.

Pulsed electromagnetic fields (PEMF) promote fracture healing and are widely applied to the successful clinical treatment of delayed- or non-union fracture in patients [7–12]. Although the biological mechanism of action of electrically induced osteogenesis is unclear, PEMF is generally thought to stimulate endochondral ossification by initiating a series of events in cartilage. Considerable evidence has accumulated indicating that PEMF affects the cytodifferentiation and phenotypic expression of cartilage *in vitro* [13–15] and

Submitted 21 November 1994; accepted 30 August 1995.

This work was supported by grants from Electro-Biology, Inc., Upper Pond Road, Parsippany, NJ 07054-1079, U.S.A.

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in vivo [16–17]. Of particular interest is the ability of PEMF to modulate cartilage extracellular matrix composition. In most studies, proteoglycan content and/or synthesis is elevated in response to PEMF treatment [16, 18–21]. However, in other studies glycosaminoglycan synthesis is decreased [22–24] or unaffected [25]. Conflicting effects of PEMF on collagen metabolism [19, 22–24], alkaline phosphatase activity [23, 26], cell differentiation [15, 22–23, 25, 26], cyclic AMP synthesis [26–27] and calcium incorporation [13, 15–16, 28] have also been reported. Such a wide variation in results can be attributed to the diversity of both PEMF driving signals employed and range of applications. Similarly, many different culture models of cartilage have been studied, including embryonic chick sternum or limb bud, rat or rabbit costal chondral junction, rabbit, bovine or human articular cartilage or bovine growth plate etc. Nevertheless, such responses suggest that with the appropriate signal and targeted cell population, PEMF could modulate cartilage extracellular matrix by influencing chondrocyte behavior. In recent studies, PEMF has been shown to preserve cartilage extracellular matrix integrity [29–31] and stimulate cartilage extracellular matrix restoration by mature bovine joints [21] and human osteoarthritic cartilage explants [32]. The preliminary clinical application of PEMF stimulation to some articular cartilage diseases is encouraging [33]. There is no evidence that PEMF evokes the appearance of abnormal cell phenotypes within a treated population.

Previously we have investigated the influence of different PEMF forms and treatment regimens on cell proliferation and sulfated glycosaminoglycan synthesis and degradation by embryonic chick sternal cartilage explanted to culture [29–31]. The results demonstrate that PEMF is capable of modulating the extracellular matrix composition of developing cartilage explants by suppressing both degradation and synthesis of sulfated glycosaminoglycans and these effects are dose-related [30–31]. The present studies have further analyzed the optimal treatment regimen, 3 h/day, of one PEMF signal [31] on the turnover of both total and [^{35}S]-sulfated glycosaminoglycans and the structure of [^{35}S]-sulfated proteoglycans in embryonic chick sternal cartilage explanted to culture. Results confirmed previous findings and further demonstrated that the decreased synthesis and degradation of sulfated glycosaminoglycans are accompanied by preservation of this component within the matrix following PEMF treatment, and that the treatment does not affect the structure and function of either newly synthesized or pre-existing sulfated proteoglycans.

Materials and Methods

MATERIALS

Ham's F12 medium was from Sigma Chemical Co., Poole, Dorset, U.K. Fetal calf serum was from ICN Flow, Scotland, U.K. Carrier-free [^{35}S]sulfuric acid was from Amersham International plc, Amersham, U.K. Hyaluronic acid from human umbilical cord, chondroitin sulfate from whale or shark cartilage, and keratan sulfate from bovine cornea were from Sigma Chemical Co. Papain was from Hopkin & Williams, Essex, U.K. Chondroitin ABC lyase from *Proteus vulgaris* was from ICN Biochemicals, Division of Biochemical Inc., Cleveland, OH, U.S.A. Dimethylmethylene Blue was from Aldrich Chemical Co., Gillingham, Dorset, U.K. *Streptomyces griseus* pronase was from Boehringer Mannheim, Lewes, East Sussex, U.K. Dowex 1 (AG1-X2, 200-400 mesh) was from Bio-Rad Laboratories Ltd., Watford, Herts, U.K. Sepharose CL-2B and Sephacryl S-200 were from Pharmacia, Uppsala, Sweden. DNP-alanine was from Sigma Chemical Co. All other reagents were of analytical reagent grade.

ORGAN CULTURE

Sixteen-day-old White Leghorn embryonic chick sternal cartilage was used in all experiments. Sterna were dissected free of surrounding and adherent tissues in cold calcium- and magnesium-free Tyrode's solution, pH 7.4. Equal numbers of explants were randomly placed to control and experimental culture dishes (plastic tissue, Sterilin Ltd, Hounslow, U.K.) according to the experimental groups described below. Cultures were maintained in Ham's F12 nutrient medium supplemented with 10% fetal calf serum, 1 mM glutamine, 0.1 mg/ml ascorbate and 50 units/ml penicillin and streptomycin (ICN Flow, Scotland, U.K.) in a humidified atmosphere at 37°C under 5% CO_2 in air for 48 h [30, 34]. As generally agreed in organ culture of embryonic chick cartilage [35–36], sternal explants maintain the differentiated phenotype of chondrocytes and readily proliferate and increase in size and extracellular matrix content with time under the experimental conditions. Metabolic activity of the cultured tissue is high because of its embryonic nature. However, in terms of proteoglycan synthesis, the rate of incorporation of [^{35}S]-sulfate is constant with time over the indicated incubation period [30].

PEMF TREATMENT

The apparatus for treatment exposure of cultures to PEMF was provided by Electro-Biology, Inc., Parsippany, NJ, U.S.A. This apparatus consists of an electric control unit and a pair of horizontally placed circular Helmholtz magnetic coils (14 cm diameter, 13 cm intercoil distance) mounted on a plastic framework. The PEMF signal consists of 30 ms duration bursts of pulses repeated at 1.5 Hz [Fig. 1(a)]. During each pulse the magnetic field rises from 0 to 1 gauss in 230 μs and then returns to 0 gauss in 30 μs [Fig. 1(b)]. Culture dishes were placed between the coils and stacked as close as possible to the vertical and horizontal center. Experimental cultures were exposed to PEMF for 3 h/day for the duration of the culture period. This treatment was found to be the most effective from our previous work [31]. Control cultures were maintained within the coils and under identical environmental conditions but in the absence of PEMF.

CONTENT OF GLYCOSAMINOGLYCANS

Freshly isolated embryonic chick sternal explants were placed into 35 mm tissue culture dishes each containing three sterna and 2 ml of the above nutrient medium. The culture dishes were randomly designated either control or experimental, all in triplicate, and incubated for 48 h in the absence or presence of PEMF treatment. Explants and media were recovered separately at the end of the culture period. Glycosaminoglycans either retained by the explants or released into the media were analyzed by reaction with dimethylmethylene blue following papain digestion [37]. Chondroitin sulfate from shark and whale was used as the standard to calibrate the assay. Portions of papain digest were further treated with chondroitinase ABC to determine the proportion of chondroitin sulfate in the mixture [37–38].

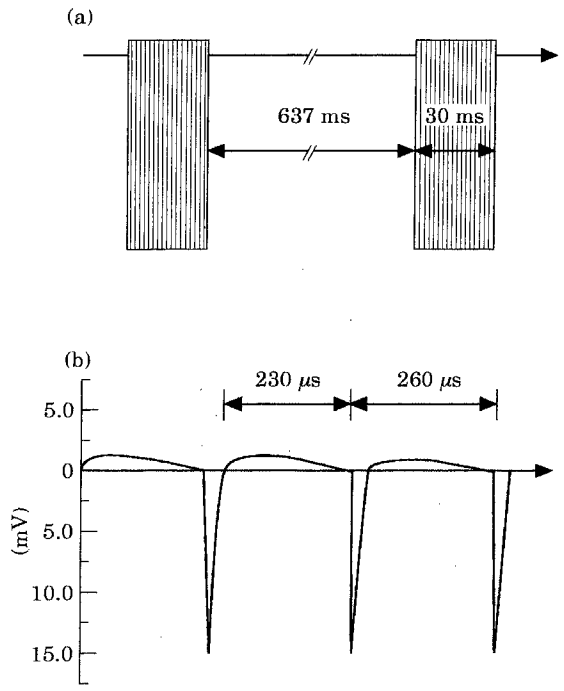

FIG. 1. Schematic representation of the pulsed electromagnetic field. PEMF signal employed in this study consists of 30 ms duration bursts of pulses repeated at 1.5 Hz (a). During each pulse the magnetic field rises from 0 to 1 gauss in 230 μs and then returns to 0 gauss in 30 μs (b). Sternal cartilage explanted to culture for 48 h were exposed to this signal for 3 h per day.

LABELING OF SULFATED GLYCOSAMINOGLYCANS

To determine the synthesis of sulfated proteoglycan *in vitro*, 5 μCi/ml carrier-free [^{35}S]sulfuric acid was added to control and experimental cultures for the final 24 h of the 2-day culture period. At the end of the culture period, explants and nutrient media were harvested separately and the incorporation of [^{35}S]-sulfate into sulfated proteoglycans analyzed. In separate experiments, 10 μCi carrier-free [^{35}S]-sulfuric acid in 100 μl distilled water was administered onto the chorioallantoic membrane through a small hole in the egg shell on the sixth day of embryonic development. Following administration of isotope, the hole in the shell was sealed with transparent adhesive tape and eggs were returned to the incubator to permit continued development [39–40]. Ten days later, the sterna were isolated from these biosynthetically radiolabeled embryos and analyzed for [^{35}S]-sulfated proteoglycan content either immediately or following explant culture for 2 days in the presence and absence of PEMF. Explants and nutrient media were again harvested and analyzed separately.

TURNOVER OF SULFATED GLYCOSAMINOGLYCANS

Quantitative analysis of sulfated glycosaminoglycan turnover was studied by determining the rates of *in vitro* incorporation of [^{35}S]-sulfate into glycosaminoglycans and *in vitro* breakdown of pre-existing [^{35}S]-sulfated glycosaminoglycans labeled *in ovo* as previously described [30, 39]. In both synthesis and degradation experiments, each sample comprised three sterna which were cultured in a 35 mm tissue culture dish containing 2 ml culture medium. All treatments were performed in at least triplicate. At the end of the culture period, [^{35}S]-sulfated glycosaminoglycans either remaining in the explants or released into

the media were determined by ion exchange chromatography on Dowex 1 (1 × 2-400) following *Streptomyces griseus* pronase digestion of the samples.

EXTRACTION OF SULFATED PROTEOGLYCANS

Separate experiments were performed to analyze the effect of PEMF on the structure and function of [^{35}S]-sulfated proteoglycans. Embryonic chick sternal cartilage was labeled for the newly synthesized and pre-existing sulfated proteoglycans and cultured with or without PEMF treatment in essentially the same way as the above described methods except that each culture was composed of 20 sterna and maintained in a 60 mm tissue culture dish containing 10 ml medium. The recovered explants were sliced into small pieces with a scalpel and extracted twice with 10 times their wet weight of 4 M guanidinium chloride in 0.05 M sodium acetate buffer, pH 5.8, at 4°C for 24 h under continuous agitation. Extracts were centrifuged at 1000 ***g*** for 15 min, supernatant retained and residues washed once with small volume of cold buffered 4 M guanidinium chloride. Extracts and residual washes were pooled and the residues were digested with papain in 0.05 M phosphate buffer, pH 6.5, containing 2 mM N-acetylcysteine and 2 mM EDTA at 65°C for 2 h [41]. Media, extracts and residues were dialyzed against 0.01 M sodium acetate buffer, pH 6.8 at 4°C to remove unbound [^{35}S]-sulfate and re-associate proteoglycans. Pepstatin A (1 μg/ml), 1,10-phenanthroline (1 mM), iodoacetic acid (1 mM), and phenylmethanesulphonyl fluoride (1 mM) were present as proteinase inhibitors in all extraction and dialysis buffers. Samples were analyzed for total [^{35}S]-sulfate incorporation into sulfated proteoglycans either retained within the explants or released into the media using an LKB 1214 Rackbeta Liquid Scintillation Counter and an aqueous ACSII counting scintillant (Amersham International, Amersham, U.K.).

GEL FILTRATION CHROMATOGRAPHY

Molecular size distribution of [^{35}S]-sulfated proteoglycans in explants and media was determined by gel chromatography on Sepharose CL-2B columns (100 cm × 0.66 cm analytical column, Whatman, Kent, U.K.) under dissociative and associative conditions [42–43]. Under dissociative conditions, 1 ml of dialyzed sample was applied to the column at room temperature and eluted with 0.5 M sodium acetate buffer containing 4 M guanidinium chloride, pH 7.0, at 3 ml/h. Under associative conditions, samples were mixed with hyaluronic acid (30 μg/ml) at 4°C for 4 h and then eluted with 0.5 M sodium acetate buffer, pH 7.0. The size distribution of glycosaminoglycan chains was determined by gel chromatography on Sephacryl S-200 (100 cm × 0.66 cm analytical column, Whatman) [44]. Peak fractions eluted from Sepharose CL-2B columns under associative conditions were pooled, digested with papain at 65°C overnight, applied to Sephacryl S-200 columns and then eluted at room temperature with 0.5 M sodium acetate buffer, pH 7.0, at 1.5 ml/h. Fraction of 0.5 ml was collected and aliquot analyzed for [^{35}S]-sulfate radioactivity. Proteoglycan aggregate and DNP-alanine were used as markers of the void (V_o) and total (V_t) volumes of the columns. The proteoglycan aggregate prepared from pig laryngeal cartilage was a gift from Michael T. Bayliss, Kennedy Institute of Rheumatology, London, U.K.

CELLULOSE ACETATE ELECTROPHORESIS OF GLYCOSAMINOGLYCANS

The degree of sulfation and charge density of the sulfated glycosaminoglycans were determined by cellulose acetate electrophoresis [45]. Peak fractions eluted from the Sephacryl S-200 column were pooled, dialyzed against distilled water at 4°C overnight and lyophilized. Lyophilized samples were re-dissolved in distilled water. Duplicate samples were spotted onto Cellogel strips (57 mm × 140 mm, Whatman, Chemetron, Italy) and subjected to electrophoresis in 0.1 M KH_2PO_4/0.1 M HCl, pH 2.0, with a potential gradient of 5 V/cm for 3 h. Hyaluronic acid, chondroitin sulfate and keratan sulfate were included as standards. Following electrophoresis, one strip was cut into 0.25 cm sections and analyzed for radioactivity. The other was stained with 0.02% alcian blue in 0.05 M $MgCl_2$/0.05 M sodium acetate buffer, pH 5.8, for 45 min at room temperature and then destained in the same buffer without alcian blue.

HISTOLOGICAL PROCEDURES

Sterna from 16-day-old chick embryos either freshly dissected or cultured for 48 h in the absence or presence of PEMF treatment were fixed for 24 h in buffered formaldehyde sublimate. Following standard histological procedures, tissues were embedded in paraffin wax, serially sectioned at 7 μm and stained with safranin O and Mayer's haematoxylin.

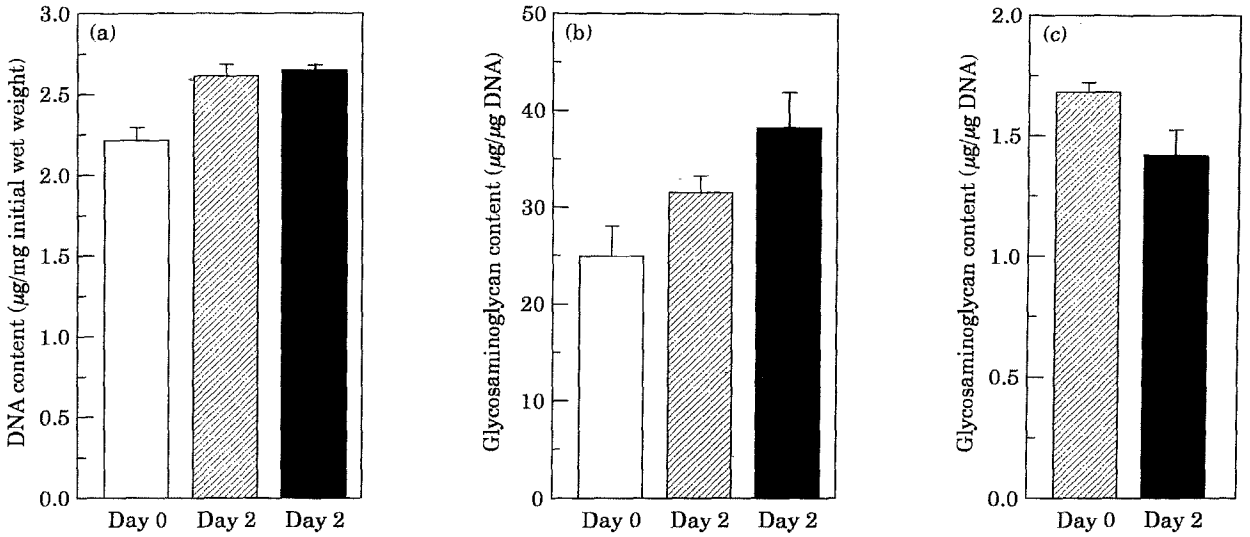

FIG. 2. DNA and glycosaminoglycan contents of explant cultures. Sternal cartilage was explanted to culture for 2 days. DNA content (a) and the amount of glycosaminoglycan in the recoveries from explants (b) and media (c) were determined at the beginning (day 0) and the end of the experiment (day 2) in either the absence (hatched columns) or presence of PEMF (solid columns). All data are expressed as means ± S.D. of at least three measurements.

OTHER ANALYSES

DNA content of sternal cartilage was measured by a fluorometric method of Royce and Lowther [46]. Briefly, aliquot from papain or pronase digests of cartilage explants prepared in all of the above described quantitative experiments was pre-treated with RNase from bovine pancreas [Sigma Chemical Co.] and then mixed with ethidium bromide. The content of DNA was estimated from the intensity of fluorescence emitted by ethidium bromide intercalated into DNA. Statistical analysis was performed using the Student's *t*-test.

Results

EFFECTS OF PEMF ON THE CONTENT OF GLYCOSAMINOGLYCANS

The amount of DNA and glycosaminoglycan in the tissue at the beginning (day 0) and the end of the experimental treatment (day 2) was determined and, together with the amount of glycosaminoglycan released from the explant into the medium, is summarized in Fig. 2. Control cultures showed a 17.5% increase in the amount of DNA content per initial wet weight after the culture period under the experimental conditions ($P < 0.01$). PEMF treatment of 3 h/day for 2 days did not cause any difference from controls in DNA content [Fig. 2(a)]. Thus, the observed results to be described are all expressed with reference to DNA content to avoid artificial effects of each sample's relative cellularity.

Glycosaminoglycan content retained by control explants [Fig. 2(b)] was increased by 25.7% after 2 days in culture ($P < 0.05$) and glycosaminoglycan content released from explants to media during the culture period [Fig. 2(c)] was 5.1% of the total. After the exposure of cultures to PEMF for the same period, glycosaminoglycan content retained by the explants was 21.4% higher than in controls ($P < 0.05$) whereas glycosaminoglycans released to the media were 30.6% lower compared to controls ($P < 0.02$). As a result, PEMF treatment promoted a net production of glycosaminoglycans in the matrix of the tissue examined.

The composition of glycosaminoglycans in papain digests of cartilage explants was determined by chondroitin ABC lyase digestion. Of glycosaminoglycans from day 0 explant 92.6 ± 0.8% (mean ± S.D.) were digested with the specific enzyme. This proportion of chondroitin sulfate in glycosaminoglycans of the tissue examined was the same as observed in embryonic chick cartilage [36, 40]. After explant culture for 2 days, the proportion of the enzyme-digested glycosaminoglycans in all cultures was the same as that of day 0 and there was no difference in this respect between control (92.3 ± 1.5%) and PEMF-treated (92.8 ± 1.9) cultures.

EFFECTS OF PEMF ON PROTEOGLYCAN SYNTHESIS

Incorporation rate of [^{35}S]-sulfate into sulfated glycosaminoglycans

Embryonic chick sternal cartilage was explanted to culture for 48 h in the absence or presence of

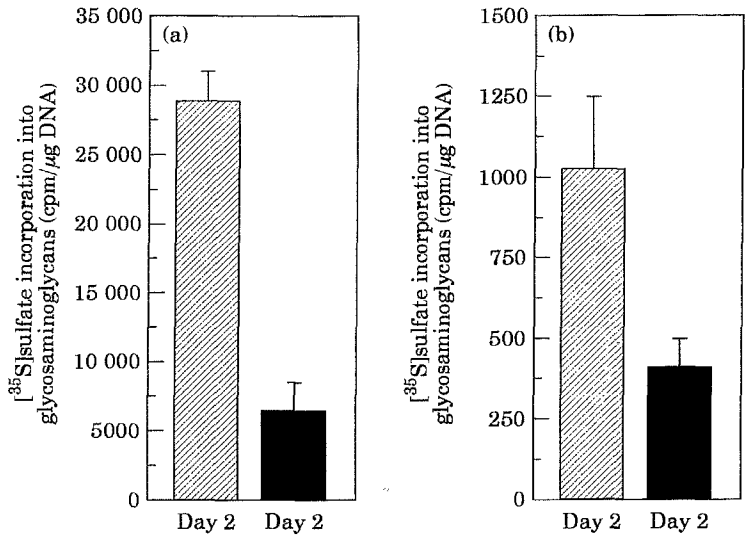

FIG. 3. Synthesis of [^{35}S]-sulfated glycosaminoglycans in explant cultures. Sternal cartilage was explanted to culture for 2 days in either the absence (hatched columns) or presence (solid columns) of PEMF and incubated with [^{35}S]sulfuric acid for the final 24 h. The rate of [^{35}S]-sulfate incorporation into glycosaminoglycans, indicative of glycosaminoglycan synthesis, was calculated as the recoveries of radioactivity from the explants (a) and the media (b). All data are the means ± S.D. of at least three measurements.

PEMF and [^{35}S]-sulfate added during the final 24 h. The incorporation rate of [^{35}S]-sulfate into glycosaminoglycans was determined as a measure of proteoglycan synthesis and is shown in Fig. 3. It was found that the level of newly synthesized sulfated glycosaminoglycans recovered from PEMF-treated explants was 76.6% lower than that from untreated controls ($P < 0.001$) [Fig. 3(a)]. It was also found that the PEMF treatment reduced the level of newly synthesized glycosaminoglycans recovered from the media [Fig. 3(b), $P < 0.02$]. The reduced release of newly synthesized glycosaminoglycans in PEMF-treated cultures appears not to be a consequence of the reduced synthetic rate of the molecules since the release rate of newly synthesized sulfated glycosaminoglycans from cultured cartilage tissue is regulated by a mechanism independent from their synthetic rate in cartilage organ culture [47]. These data demonstrate that PEMF treatment significantly suppresses the biosynthesis of sulfated proteoglycans and their release by embryonic chick sternal cartilage explanted to culture.

Size distribution and aggregation ability of newly synthesized proteoglycans

Newly synthesized [^{35}S]-sulfated proteoglycans were extracted from cultures with 4 M guanidinium chloride as described in Methods. This non-disruptive procedure resulted in an 83.1–88.3% extraction of labeled proteoglycans. The extracts were therefore regarded as an effective representation of total sulfated proteoglycan content of the cartilage explants [48–50] and were subjected to structural analysis. Sulfated proteoglycans extracted from the explants and recovered from the nutrient media were eluted on Sepharose CL-2B columns under dissociative and associative conditions. Newly synthesized proteoglycans extracted from the explants in control cultures were eluted as monomers under dissociative conditions and showed a broad size distribution [Fig. 4(a)] reflecting the polydispersed property of normal sulfated proteoglycans [51–52]. Under associative conditions, the majority of the sulfated proteoglycans aggregated with hyaluronic acid and eluted in the void volume. In the presence of PEMF, the size distribution and aggregation ability of newly synthesized proteoglycans were identical to controls although the amount was reduced owing to the suppressive effects of PEMF on sulfated proteoglycan synthesis [Fig. 4(b)]. The average molecular size of newly synthesized proteoglycans released into the media in the absence of PEMF was significantly reduced compared with that extracted from the explants. The majority of [^{35}S]-sulfated material recovered with the nutrient medium was unable to aggregate with hyaluronic acid [Fig. 4(c)], indicating that they might be degradation products. Proteoglycans released to the nutrient medium in the presence of PEMF showed a similar elution profile to the controls but the total amount was again reduced [Fig. 4(d)].

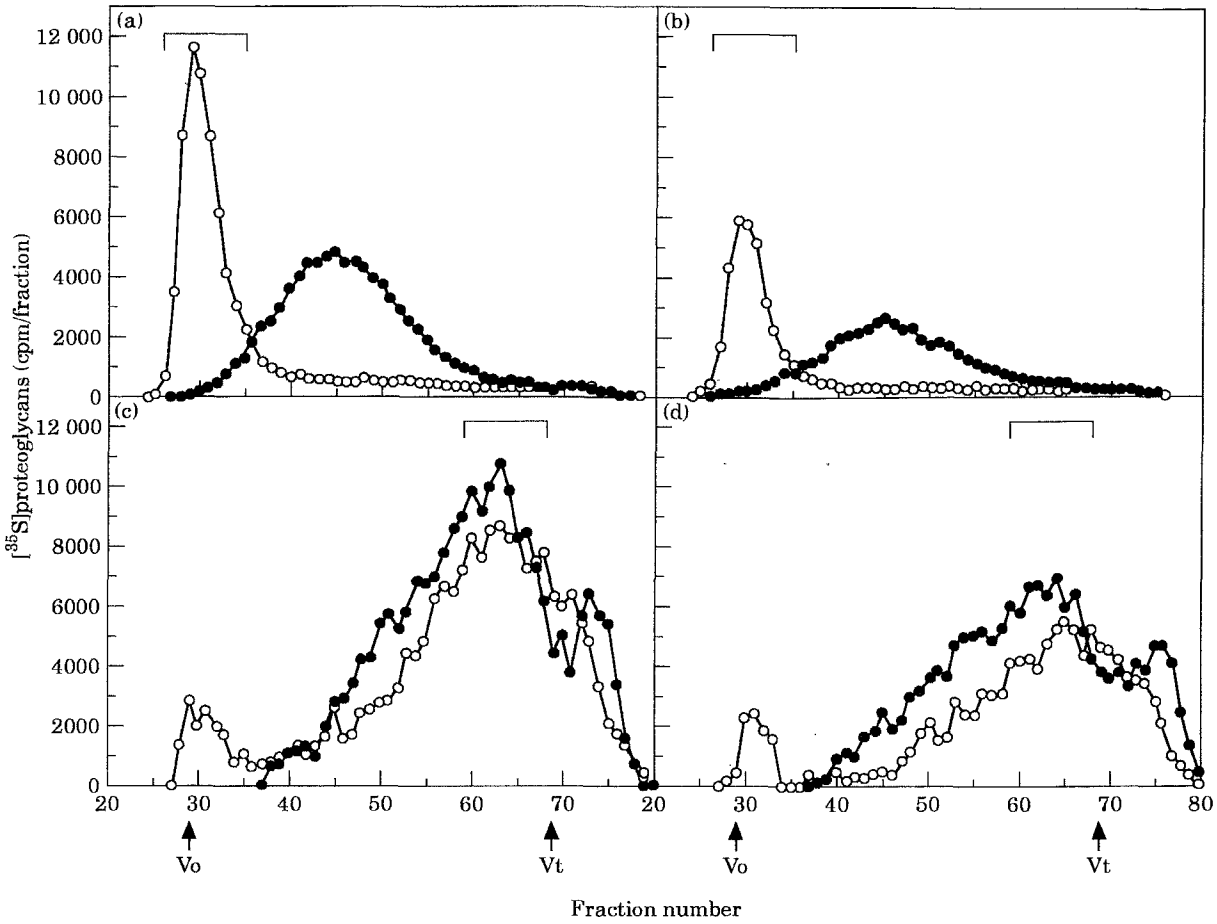

FIG. 4. Sepharose CL-2B chromatography of newly synthesized [^{35}S]-sulfated proteoglycans. Sternal cartilage explants were cultured in the absence [(a), (c)] or presence of PEMF [(b), (d)] for 48 h and incubated with [^{35}S]sulfuric acid for the final 24 h. Samples of sulfated proteoglycan from 4 M guanidinium chloride extracts [(a), (b)] and media [(c), (d)] were dialysed and applied to Sepharose CL-2B columns under associative (open circles) and dissociative (solid circles) conditions. Columns were eluted at room temperature at a rate of 3 m/h. Fractions were analyzed for radioactivity. V_o and V_t indicate the void volume and the total bed volume of the columns, respectively.

Size distribution of newly synthesized sulfated glycosaminoglycans

Peak fractions eluted from Sepharose CL-2B columns under associative conditions were pooled as indicated, digested with papain to generate glycosaminoglycan chains and subjected to Sephacryl S-200 chromatography. The elution profiles are shown in Fig. 5. The average molecular size of newly synthesized sulfated glycosaminoglycans recovered with the explants [Fig. 5(a)] or nutrient media [Fig. 5(b)] was the same in PEMF-treated and control cultures although the amount was reduced in the presence of PEMF. Newly synthesized [^{35}S]-sulfated glycosaminoglycans solubilized from cartilage explants also were subjected to Sephacryl S-200 chromatography and demonstrated no difference in the size distribution between control and PEMF treated samples (data not shown).

Degree of sulfation of newly synthesized sulfated glycosaminoglycans

Peak Sephacryl S-200 fractions of sulfated glycosaminoglycans extracted from explants were pooled as indicated, dialyzed, lyophilized and subjected to cellulose acetate electrophoresis in 0.1 M HCl. The migration distance of sulfated glycosaminoglycan chains was identical in either the absence or presence of PEMF (Fig. 6). The migration rate of individual polysaccharides depends exclusively on their degree of sulfation under the experimental conditions employed in this study [44]. Therefore, the sulfated glycosaminoglycans newly synthesized in the presence of PEMF were sulfated and charged to the same degree as untreated controls. The mobility of sulfated glycosaminoglycans from embryonic chick sternal cartilage was reduced compared with the chondroitin sulfate standard from shark or

whale cartilage due to over-sulfation of the latter [53–54].

EFFECTS OF PEMF ON PROTEOGLYCAN DEGRADATION

Degradation rate of pre-existing proteoglycans

[^{35}S]Sulfuric acid was administered onto the chorioallantoic membrane of the 6-day-old chick embryos and radiolabeled sterna harvested 10 days later. Sterna were either immediately analyzed for the level of the [^{35}S]-sulfated glycosaminoglycans or explanted to culture in the absence or presence of PEMF. The recoveries of [^{35}S]-sulfated glycosaminoglycans from explants and media were summed and the percentages of [^{35}S]-sulfated glycosaminoglycans remaining in the matrix after culture for 2 days are shown in Fig. 7. In control cultures, recovery of pre-existing [^{35}S]-sulfated glycosaminoglycans from the explants was 86.1%. Following treatment with PEMF over the same period 93.9% of the pre-existing [^{35}S]-sulfated glycosaminoglycans were recovered from the explants ($P < 0.05$). These results demonstrate that PEMF treatment

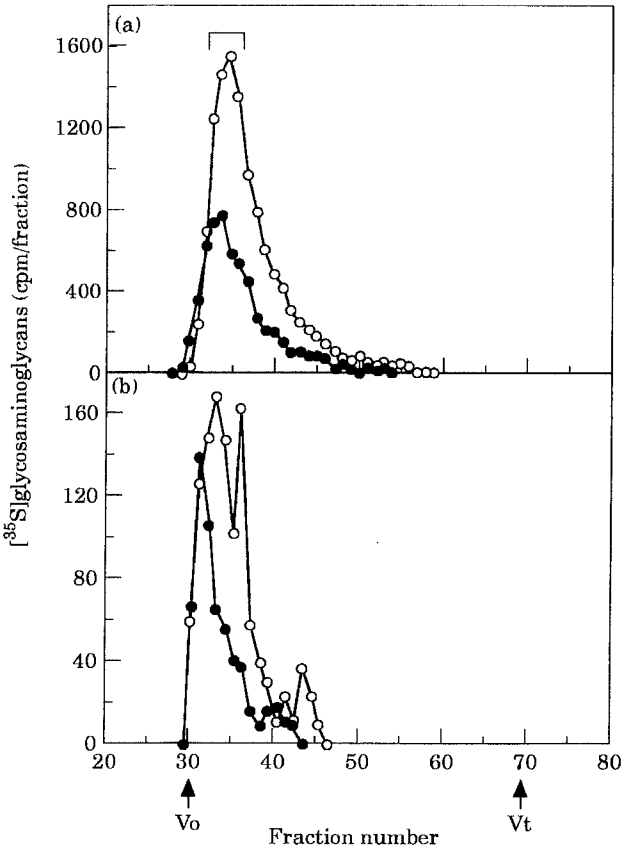

FIG. 5. Sephacryl S-200 chromatography of newly synthesized [^{35}S]-sulfated glycosaminoglycans. Pooled proteoglycan fractions were digested with papain and applied to Sephacryl S-200 columns. [^{35}S]-Sulfated glycosaminoglycans from control (open circles) and PEMF-treated (solid circles) cultures were eluted at room temperature with 0.5 M sodium acetate, pH 7.0, at a rate of 1.5 m/h. Fractions were analyzed for radioactivity. V_o and V_t indicate the void volume and the total bed volume of the columns, respectively. (a): extract, (b): medium.

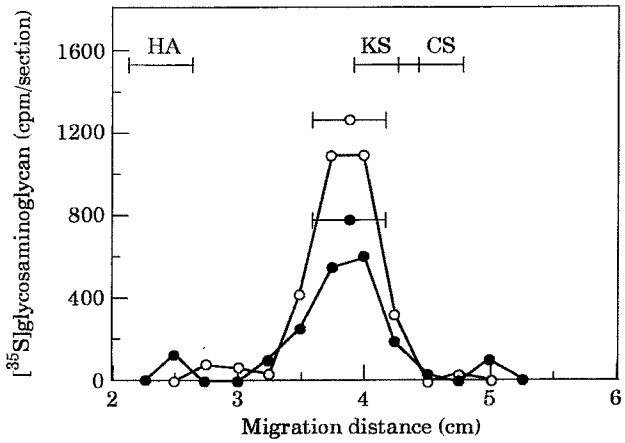

FIG. 6. Cellulose acetate electrophoresis of newly synthesized [^{35}S]-sulfated glycosaminoglycans. Pooled glycosaminoglycan fractions were dialyzed, lyophilized and subjected to electrophoresis. Samples from control (open circles) and PEMF-treated (solid circles) cultures were run on cellulose acetate strips in 0.1 M KH_2PO_4/0.1 M HCl, pH 2.0, with a potential gradient of 5 V/cm for 3 h. Hyaluronic acid, chondroitin sulfate and keratan sulfate were included as standards. Strips were either stained with alcian blue, denoted by the bars, or sectioned and analyzed for radioactivity.

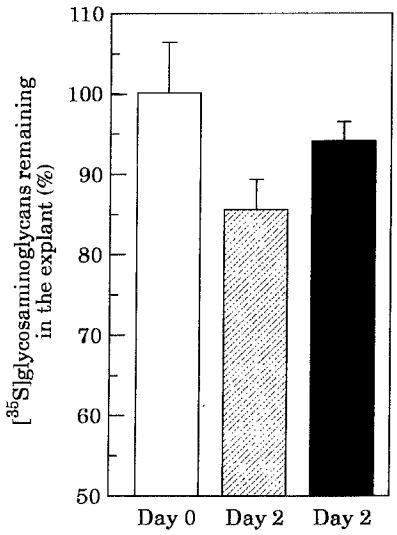

FIG. 7. Degradation of pre-existing [^{35}S]-sulfated glycosaminoglycans in explant cultures. Sterna were isolated from embryos previously administered with [^{35}S]sulfuric acid *in ovo*. The fate of the pre-existing [^{35}S]-sulfated glycosaminoglycans after 2 days culture in the absence (hatched column) or presence (solid column) of PEMF was determined and expressed as percentage of radioactivity retained by the explant. All data are the means ± S.D. of at least three measurements.

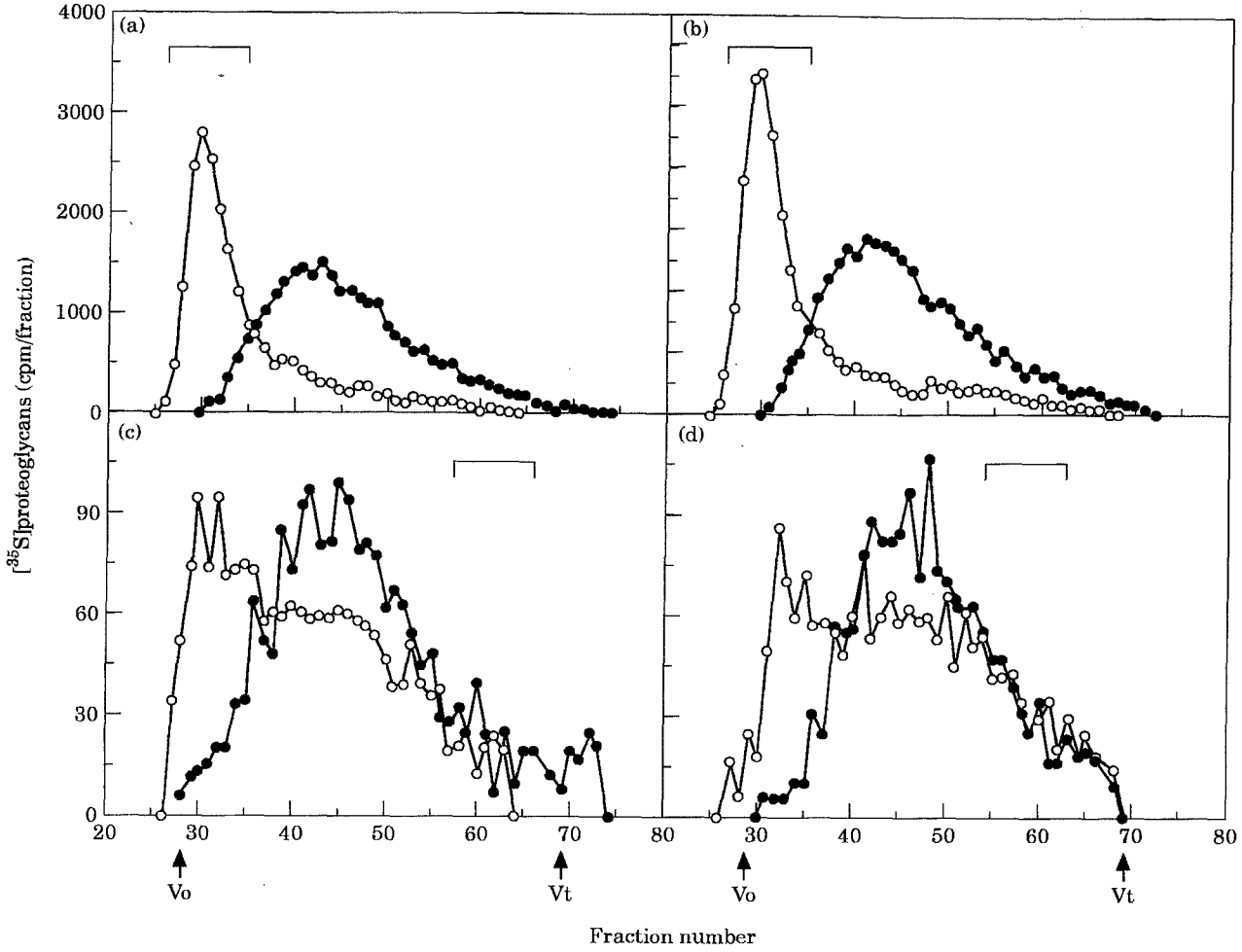

FIG. 8. Sepharose CL-2B chromatography of pre-existing [^{35}S]-sulfated proteoglycans. Sterna isolated from embryos previously administered with [^{35}S]sulfuric acid were explanted to culture in the absence [(a), (c)] or presence of PEMF [(b), (d)] for 48 h. Samples of sulfated proteoglycan from 4 M guanidinium chloride extracts [(a), (b)] and media [(c), (d)] were dialysed and applied to Sepharose CL-2B columns under associative (open circles) and dissociative (solid circles) conditions. Columns were eluted at room temperature at a rate of 3 m/h. Fractions were analyzed for radioactivity. V_o and V_t indicate the void volume and the total bed volume of the columns, respectively.

significantly suppresses the *in ovo*-labeled pre-existing sulfated proteoglycans of chick sternal cartilage from breakdown and release.

Size distribution, aggregation ability and degree of sulfation of pre-existing proteoglycans

Like the newly synthesized sulfated proteoglycans *in vitro*, about 90–92% of the *in ovo*-labeled pre-existing sulfated proteoglycans were extracted with 4 M guanidinium chloride from the tissue and they displayed very similar structural characterizations. Fig. 8 shows that the size distribution and aggregation ability of pre-existing sulfated proteoglycans in the presence of PEMF exhibited the same average molecular size and ability to re-aggregate as those in the absence of PEMF, and that the average molecular size and aggregation ability of pre-existing sulfated proteoglycans released into the media by control and PEMF-treated cultures also were similar. There was no difference in average length of pre-existing sulfated glycosaminoglycans retained by the explants or released into the medium in control and PEMF-treated cultures (Fig. 9), nor in their degree of sulfation or charge density (Fig. 10). These data demonstrate further that PEMF treatment suppresses the degradation of sulfated proteoglycans by embryonic chick cartilage explanted to culture without affecting their molecular structure or behavior.

EFFECTS OF PEMF ON HISTOLOGICAL ORGANIZATION OF CARTILAGE

The histological appearance of sternal cartilage either freshly dissected or cultured for 48 h in the absence or presence of PEMF treatment is shown

in Fig. 11. Immediately following isolation, chick sternal cartilage showed the characteristic appearance of developing hyaline cartilage [Fig. 11(a)]. Chondrocytes were flattened on the surface of sternum and rounded and uniformly distributed at deeper levels. The matrix was intensely and uniformly stained with safranin O throughout the full thickness of the keel of the sternum. After 48 h of control culture, the deep chondrocytes were slightly randomly distributed and matrix staining was both weaker and heterogenous [Fig. 11(b)]. Following 48 h culture in the presence of PEMF, the morphology and distribution of chondrocytes did not change and the entire extracellular matrix staining was intense, although discrete deep areas were less uniformly stained [Fig. 11(c)]. Safranin O binds stoichiometrically to either chondroitin sulfate or keratan sulfate in an equivalence ratio very close to one: one molecule of safranin O binds to each negatively charged group of either polyanion [55]. The extracellular concentration of the dye is proportional to the concentration of glycosaminoglycan. Intense and uniform staining of cartilage extracellular matrix with safranin O in histological section of PEMF-treated sternum was in agreement with the quantitative measurement of glycosaminoglycans in the matrix and confirmed the finding that PEMF treatment conserves *in vitro* the extracellular matrix integrity of embryonic chick cartilage in terms of proteoglycan composition.

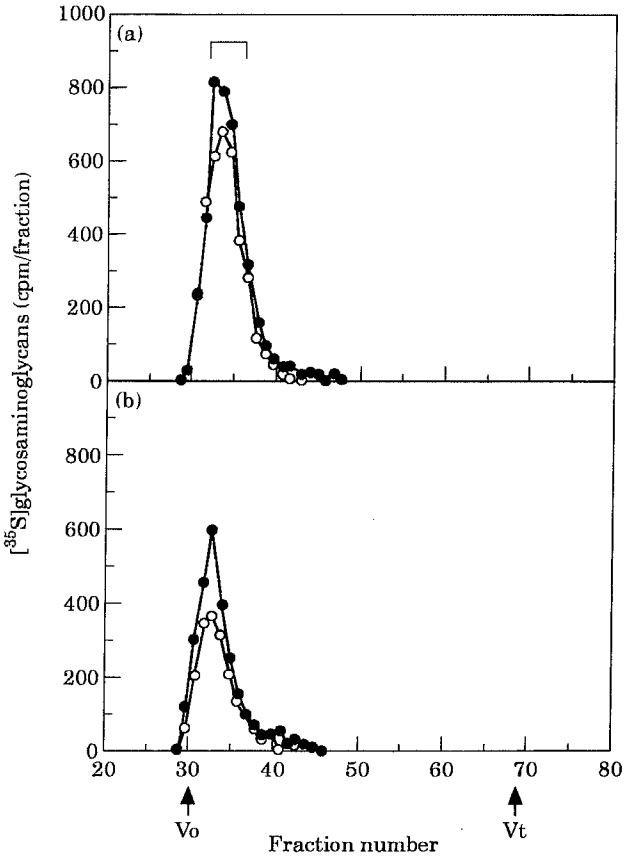

FIG. 9. Sephacryl S-200 chromatography of pre-existing [^{35}S]-sulfated glycosaminoglycans. Pooled proteoglycan fractions were digested with papain and applied to Sephacryl S-200 columns. [^{35}S]-sulfated glycosaminoglycans from control (open circles) and PEMF-treated (solid circles) cultures were eluted at room temperature with 0.5 M sodium acetate, pH 7.0, at a rate of 1.5 m/h. Fractions were analyzed for radioactivity. V_o and V_t indicate the void volume and the total bed volume of the columns, respectively. (a): extract, (b): medium.

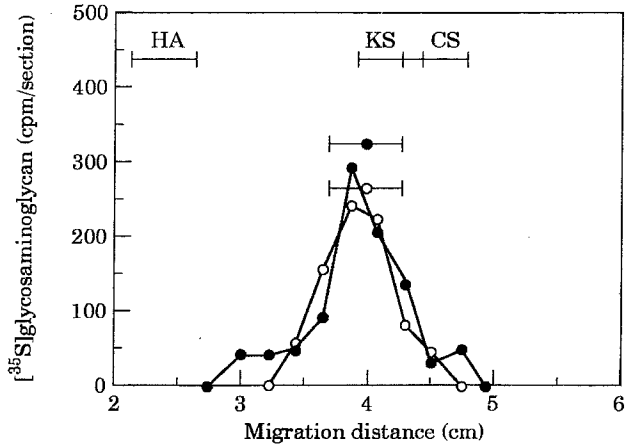

FIG. 10. Cellulose acetate electrophoresis of newly synthesized [^{35}S]-sulfated glycosaminoglycans. Pooled glycosaminoglycan fractions were dialysed, lyophilized and subjected to electrophoresis. Samples from control (open circles) and PEMF-treated (solid circles) cultures were run on cellulose acetate strips in 0.1 M KH_2PO_4/0.1 M HCl, pH 2.0, with a potential gradient of 5 V/cm for 3 h. Hyaluronic acid, chondroitin sulfate and keratan sulfate were included as standards. Strips were either stained with alcian blue, denoted by the bars, or sectioned and analyzed for radioactivity.

Discussion

Cultured cartilage explants provide an efficient model system with which to investigate the influence and mechanism of action of various factors on the synthesis and degradation of extracellular matrix components [56]. Organ culture of cartilage has numerous advantages over cell culture of isolated chondrocytes: in explant culture the extracellular matrix is intact, chondrocytes maintain their differentiated state and are not exposed to the extensive proteolytic activity required for their dissociation [57–58].

Using embryonic chick sternal cartilage explanted to culture, we have previously studied the effect of PEMF on the metabolism of sulfated glycosaminoglycans in cartilage extracellular matrix. The results showed that PEMF modulates

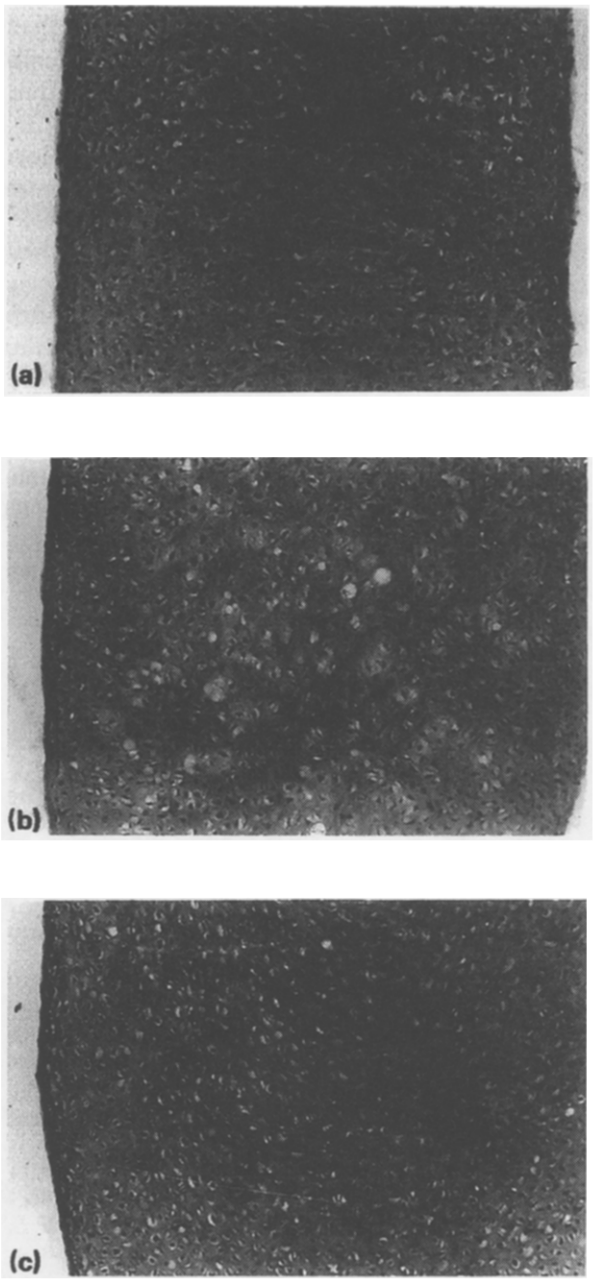

FIG. 11. The histological organization of freshly isolated sternum (a) compared with sterna explanted to culture for 48 h in the absence (b) and presence (c) of PEMF. Tissues were processed to paraffin wax and serial 6 μm sections stained with safranin O and Mayer's haematoxylin (×250).

sulfated glycosaminoglycan turnover by embryonic chick sternal cartilage explanted to culture [29–31]. Treatment with PEMF reduced degradation of pre-existing sulfated glycosaminoglycans, the synthesis of new sulfated glycosaminoglycans and the release of glycosaminoglycans from the explants. Each of these effects was significant within 48 h and was enhanced in the presence of retinoic acid [29–30].

The effects of PEMF on embryonic chick sternal cartilage explanted to culture had also been shown to be influenced by both the signal form and the frequency of treatment [30–31]. In this study, we have focused on the optimal treatment regimen of one PEMF signal [31] and investigated its effects further. We have shown that 3 h treatment per day for 2 days significantly increases the recovery of pre-existing [^{35}S]-sulfated proteoglycans retained by the explants and this is matched by a reduced synthetic rate of [^{35}S]-sulfated proteoglycans. We have also shown both quantitatively and qualitatively that PEMF treatment promotes deposition of glycosaminoglycans in the matrix. Since DNA content of the cultures was not affected by the PEMF exposure, the observed effects of PEMF are one of direct modulation of chondrocyte activity rather than a result of enhanced cellular proliferation.

A consistent finding in the present study is that PEMF treatment prevented the release of both unlabeled glycosaminoglycans and labeled newly synthesized and pre-existing sulfated glycosaminoglycans from the matrix. It seems that PEMF did not act to slow down the rate of turnover of glycosaminoglycan by suppressing chondrocyte activity since proteoglycan catabolism is a well-known chondrocyte-mediated process [43, 59–60]. In addition, PEMF stimulated a significant amount of glycosaminoglycan deposition within the cartilage matrix. Therefore, the decrease in the rate of [^{35}S]-sulfate incorporation may not be by direct inhibition of PEMF on the pathway of glycosaminoglycan synthesis but by a secondary reaction regulated by reduced release of this component from the matrix. This proposal is supported by a general understanding of the regulation of cartilage extracellular matrix. Studies indicate that the homeostatic maintenance of proteoglycan molecules is dependent upon their co-ordinated synthesis and degradation by chondrocytes. The activation of proteoglycan synthesis by loss of this molecule from the tissue has been widely observed in organ cultures of embryonic chick cartilage [36] and young or mature mammalian articular cartilage [60–63] and in human and experimental osteoarthrosis [64–66]. In contrast, addition of proteoglycan to the cultures inhibited its synthesis [60]. As reported for many cartilage explant cultures, our unpublished experiments in the same culture system maintained for up to 8 days revealed that the embryonic chick sternal cartilage underwent initial adaption to culture. During the culture period, synthesis of glycosaminoglycans was markedly stimulated to replace initial rapid loss of this component from the tissue. The synthesis then

gradually lowered to a constant level in response to the lowered and stabilized release of glycosaminoglycans. This observation further supports the proposal that the prevention of release of glycosaminoglycans from the tissue by PEMF treatment resulted in the decrease in synthesis of the molecules. A different PEMF signal has also been found to conserve newly synthesized proteoglycans within the matrix of bovine articular cartilage explanted to culture [67]. Thus, unlike agents such as retinol [68] and interleukin 1 [47] which induce a net depletion of cartilage proteoglycan by decreasing synthesis and increasing release of sulfated glycosaminoglycans, PEMF conserves developing cartilage extracellular matrix.

It can be considered that the [^{35}S]-sulfate label in pre-existing sulfated proteoglycans extracted from the explants is uniformly incorporated after incubation for 10 days *in ovo* and 2 days *in vitro*. The pre-existing [^{35}S]-sulfated proteoglycans thus are interpreted to represent native components laid down in the sternal extracellular matrix. Chromatography of 4 M guanidinium chloride extracts on Sepharose CL-2B demonstrated that the PEMF does not affect either the average size of pre-existing proteoglycan monomers or their ability to aggregate with hyaluronate. Chromatography of papain digests of 4 M guanidinium chloride extracts on Sephacryl S-200 followed by cellulose acetate electrophoresis revealed that pre-existing glycosaminoglycan chain length and degree of sulfation also are unaffected.

The apparent decrease in sulfated proteoglycan synthesis in the presence of PEMF could result from a decreased number of normal proteoglycan molecules. Alternatively, there could be fewer sulfated glycosaminoglycan chains per monomer, or the chains could be shorter or under-sulfated. The ability of sulfated proteoglycan to aggregate with hyaluronic acid also could be perturbed. The present studies show that the proteoglycans synthesized in the presence of PEMF exhibit the same molecular size and that their ability to bind to hyaluronic acid and charge density is not affected by PEMF treatment.

The molecular size of either pre-existing or newly synthesized [^{35}S]-sulfated proteoglycans released to the nutrient medium in the absence or presence of PEMF is consistently smaller. Material released to the nutrient medium is largely unable to aggregate with hyaluronic acid indicating that it possibly constitutes degradation products. However, the chain length of [^{35}S]-sulfated glycosaminoglycan recovered with the medium or explant is not significantly different from one another, indicating that there is no significant degradation of glycosaminoglycan chains. These proteoglycan fragments mainly diffuse out of the matrix, possibly because the monomers have a non-functional binding region [43].

Treatment for 3 h/day with the PEMF employed clearly conserves the sulfated proteoglycan content of embryonic chick sternal cartilage explanted to culture without affecting sulfated proteoglycan structure or function. If such an effect also occurs in articular cartilage, PEMF could exert beneficial therapeutic potential for the treatment of cartilage disorders, such as osteoarthrosis, in which proteoglycan loss is usually progressive [65, 69]. However, it is recognized that the effects of PEMF are highly signal and tissue-specific [70]. Thus, further evidence is needed to illustrate whether the normal or osteoarthritic articular cartilage in mammals will respond to the present signal and treatment regimes in the same way.

Acknowledgment

We are indebted to Electro-Biology, Inc. for their support of this work.

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