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Laboratory Investigations

Mechanical Stimulation by Intermittent Compression Stimulates Sulfate Incorporation and Matrix Mineralization in Fetal Mouse Long-Bone Rudiments Under Serum-Free Conditions

Č. Bagi* and E. H. Burger

Department of Oral Cell Biology, Akademisch Centrum Tandheelkunde Amsterdam—Vrije Universiteit, Amsterdam, The Netherlands

Summary. Mechanical stimulation evoked by intermittent hydrostatic compression (IC) in a closed culture system has been shown to stimulate calcification of fetal long-bone rudiments in the presence of serum [6]. We have studied effects of IC on sulfate metabolism and matrix mineralization under serum-free conditions, in short-term (24 hours) cultures of mineralizing long-bone rudiments in alpha minimum essential medium (MEM) + 0.2% bovine serum albumen (BSA). Exposure to IC for 24 hours stimulated radiosulfate incorporation into the papain-digestible pool in the noncalcifying epiphyses and, to a larger extent, in the calcifying diaphysis. The percentage release of ^{35}S from prelabeled rudiments was stimulated in the epiphyses, but inhibited in the diaphyses. The changes in sulfate metabolism of matrix mineralization, in hypertrophic cartilage, and the diaphyseal bone collar, were judged from the increase in length of the diaphysis. This study shows that under serum-free conditions, mechanical stimulation by IC increases sulfate content while stimulating mineralization in calcifying cartilage of fetal long-bone rudiments. Mechanical stimulation seems to be an important regulator of cartilage calcification.

Key words: Intermittent compression — Proteoglycans — Calcification — Organ culture.

The normal turnover of skeleton is modulated by physical strains and by bone-seeking hormones. Glucksman [1] was the first to show that many of the structural effects resulting from physical strain are due to its direct action on the skeleton, as bone tissue cultures *in vitro* responded directly to physical strains. It seems that in the living body, load or muscular activity serve as an extracellular stimulus which is transmitted to cells where it may modulate their genetic program for growth and differentiation. Rodan et al. [2, 3] demonstrated that mechanical perturbation of cartilage and bone-cell membrane induces changes in both cyclic nucleotides and in ionic fluxes. Veldhuijzen et al. [4] pointed out the importance of an intermittent compressive force (ICF) of physiological magnitude evoked by hydrostatic pressure on cartilage cell proliferation. Using high-density chondrocyte cultures, van Kampen et al. [5] reported that proteoglycan (PG) synthesis was significantly increased in cultures exposed to intermittent compression (IC) as compared with control cultures, but IC also improved the aggregating capacity of PG and the coherence of PG with other matrix components. Using organ cultures of fetal mouse metatarsal bone rudiments, Klein-Nulend et al. [6, 7] showed that IC significantly stimulated the calcification process in the matrix of hypertrophic cartilage. In addition, 5 days culturing under IC increased the degree of sulfation of the chondroitin sulfate (CS) chains in calcified and noncalcified cartilage, but reduced the total amount of newly incorporated sulfate per tissue volume unit in the calcified diaphysis [7]. The authors conclude that the effects on matrix mineralization and sulfate metabolism are probably linked, and that loss of chondroitin sulfate chains is part of the

Present address: University of Utah, Radiobiology Division, Bld. 586, Salt Lake City, UT 84112, USA.

Send reprint requests to Cedo Bagi, M.D., Ph.D. at his present address.

complicated process of cartilage calcification. However, as their data are based on 5-day cultured bone rudiments, short-term effects of IC on sulfate metabolism were not clearly identified. In addition, as they used serum in the culture medium, it remains unclear if the effect of IC resulted from a direct interference with matrix metabolism, or from an enhanced responsivity under the influence of IC serum factors such as IGF. Therefore, in the present study, we investigated the effects of IC on sulfate metabolism and matrix mineralization in calcifying long-bone rudiments cultured for 24 hours under serum-free conditions.

Materials and Methods

Tissue Culture

Cartilaginous long-bone rudiments of the metatarsus of 17-day old Swiss albino mouse embryos were aseptically harvested, carefully dissected and freed of adhering tissue without disturbing the perichondrium, and rinsed briefly in Puck's phosphate-buffered saline solution (Gibco, Paisley, Scotland). Rudiments were cultured in concave-bottomed 48-well polystyrene culture dishes (Costar, Cambridge, MA., USA), containing 150 μl of culture medium per well in humidified (98%) incubator (5% CO_2 in air) at 37° for 24 hours. The culture medium consisted of alpha minimum essential medium (MEM) without nucleosides (Gibco) supplemented with 10% heat-inactivated rat serum (RS), or 0.2% bovine serum albumin (BSA, Merck), and without antibiotics. In some experiments, rudiments were cultured for 96 hours with a change of medium after 48 hours.

Metatarsal rudiments from each embryo were paired so that in all experiments each animal served as its own control.

Intermittent Compression

The apparatus for applying IC on metatarsal bone rudiments *in vitro* has been described previously [6, 7]. In brief, IC was generated by intermittently compressing the gas phase (5% CO_2 in air) of a closed culture chamber (humidity 98%) which contained the culture dishes and which was placed in 37°C incubator. Pulse frequency was 0.3 Hz and the hydrostatic pressure applied to the bone rudiments was 132 g/cm^2, which resembles the calculated maximal physiological pressure resulting from muscle contractions in the fetuses [7].

Determination of Bone Rudiment Growth and Mineralization

The length and growth of the whole bone rudiment (total length), and the mineralization of the diaphysis were measured before culturing (time 0), after 24 hours (time 1), 48 hours (time 2), and finally after 96 hours (time 4) in control (Co) or experimental conditions (IC) in alpha MEM supplemented with RS or with BSA. For all measurements we used a linear eye piece micrometer (Zeiss) at ×40 magnification.

^{35}S-Sulfate Incorporation into Glycosaminoglycans

To study newly synthesized sulfated glycosaminoglycans (GAGs), the incorporation of ^{35}S-sulfate into calcifying cartilage was measured. Rudiments were labeled for the last 3 of the 24-hour culture period in fresh medium (alpha MEM supplemented with 0.2% BSA) containing 5 μCi of $Na_2{}^{35}SO_4$/ml (specific activity 1 mCi/mmol; The Radiochemical Centre, Amersham, UK). Subsequently, the incubation medium was decanted and the rudiments were rinsed three times for 10 minutes in Puck's saline solution to remove exchangeable radioactivity. All rudiments in the experimental (IC conditions) and Co (normal conditions) group were divided into two subgroups. In subgroup A, the amount of ^{35}S-sulfate was measured in the whole rudiment including the perichondrium; in subgroup B, the rudiments were cleaned of adherent perichondrium by rolling them on a glass slide in a drop of collagenase solution for a few seconds (2 mg/ml; 156 U/mg, Cls 11, Worthington Diagnostic System Inc., which was tested previously for specificity) [7], and rinsed in Puck's saline solution without serum. Subsequently, the periosteum-free rudiments (so-called 'stripped bones') were carefully divided in a calcified medial part (diaphysis—composed of cartilage, calcified cartilage, and bony collar) and noncalcified proximal and distal parts (epiphyses). Calcified and noncalcified parts were analyzed separately for ^{35}S-sulfate content. Samples from all subgroups were digested in 2 ml fresh medium with 0.3% papain at 60°C for 24 hours [8, 9]. To this digest, 0.5 ml of 10% cetyl pyridinium chloride (CPC), Merck) in 0.2 M NaCl was added. After 18 hours at 22°C, the precipitate (of CPC and GAGs) was washed three times with a solution of 0.1% CPC, a treatment that reduced the activity in tissue blanks to the background level. The precipitate was dissolved in 0.5 ml formic acid (23 N) and the amount of ^{35}S-sulfate was determined with a liquid scintillation counter.

^{35}S-Sulfate Release

In the ^{35}S-sulfate-releasing experiments, pregnant mice were injected intraperitoneally with 1 mCi of ^{35}S-sulfate/100 g body weight at day 16 of pregnancy; 24 hours later the mice were killed, fetuses were removed, and their metatarsal rudiments were aseptically harvested. One group of rudiments was used for ^{35}S-sulfate determination in the diaphyseal and epiphyseal parts of rudiments without culturing. Other rudiments were cultured for 24 hours under control or IC conditions. After 24 hours of culture period, rudiments were divided into two subgroups: A, total bone with periosteum and B, periosteum-free rudiments divided in diaphyses and epiphyses. All rudiments were separately prepared for ^{35}S-sulfate determinations as described previously. The culture media were also collected and used for ^{35}S-sulfate content measurements.

Statistics

Statistical evaluation of the data was performed using Student's *t* test for paired observations.

Results

At the time of dissection, the diaphysis of metatarsal rudiments consisted of a core of cartilage sur-

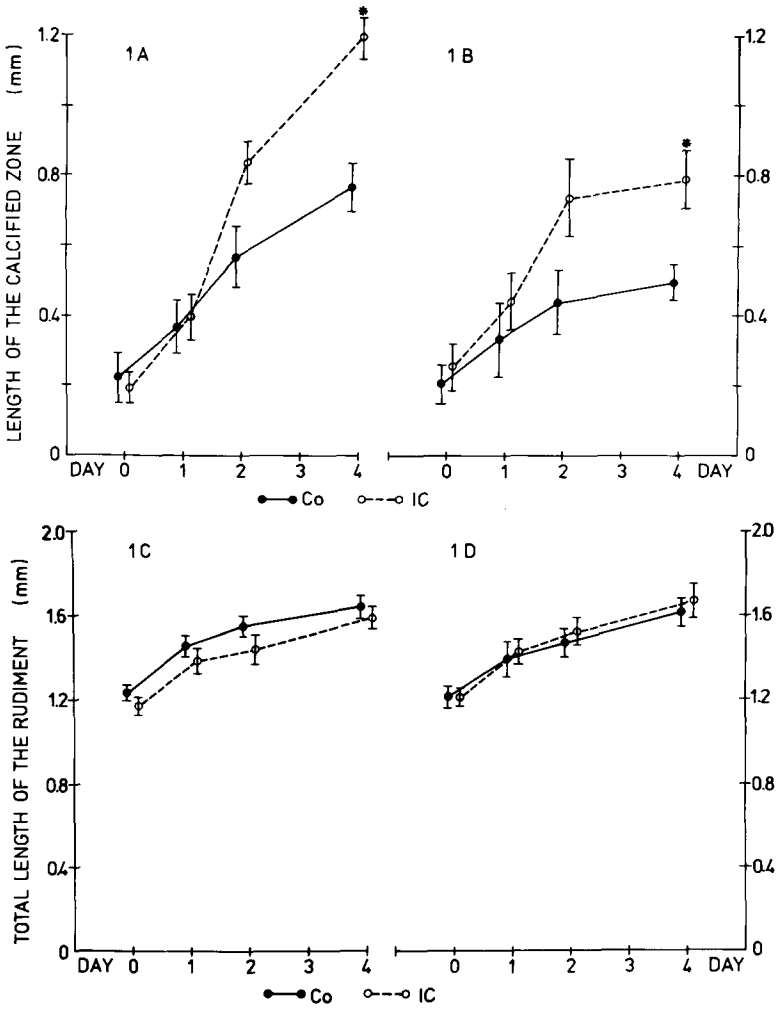

Fig. 1. The increase in length of the calcified zone (diaphysis) **(A,B)** of the 17-day-old metatarsal bone rudiments after 4 days culturing in control conditions (Co) or with application of intermittent compression (IC). The cultures were supplemented with 10% RS **(A)** or with 0.2% BSA **(B)**. Values are $\bar{x} \pm$ SEM, n = 18. $^*P < 0.001$. The increase in total length of the 17-day-old metatarsal bone rudiments **(C,D)** after 4 days culturing in control conditions (Co) or with application of intermittent compression (IC). The cultures were supplemented with 10% RS **(C)** or with 0.2% BSA **(D)**. Values are $\bar{x} \pm$ SEM, n = 18.

rounded by a thin calcified bone collar. Excavation of the diaphysis had not yet started [10].

Calcification proceeded in organ cultures, both in the presence and absence of serum, but was greater in serum containing medium (Fig. 1A,B), IC stimulated calcification in both media, and to a similar extent (IC/Co = 1.5 with serum; IC/Co = 1.6 without serum) after 4 days culturing. Both Co and experimental (IC) rudiments increased some 33% in total length during the experimental period of 4 days in cultures with or without 10% RS (Fig. 1C,D). Thus, growth in length of metatarsal rudiments was not affected by IC. In the absence of serum, we could detect a stimulatory effect of IC on calcification even after 24 hours culturing (Table 1).

To study if the effect of IC on matrix mineralization was accompanied by effects on matrix metabolism, we measured macromolecular ^{35}S-sulfate incorporation in whole rudiments including perichondrium, as well as in epiphysis and diaphysis after removal of the perichondrium. Table 2 shows that 24-hour treatment with IC stimulated sulfate incorporation in GAGs by some 20% in whole, nonstripped metatarsal rudiments.

When the noncalcified epiphyses and the calcified diaphysis were analyzed separately after removal of the perichondrium, important differences were found. IC stimulated sulfate incorporation in both compartments, but its effects were much greater in the diaphysis than in the epiphysis (diaphysis: IC/Co = 2.0; epiphysis: IC/Co = 1.20; Fig. 2A,B).

IC also changed GAG degradation, measured as the release of incorporated ^{35}S from prelabeled rudiments (Table 3). Culture for 24 hours under control conditions reduced the amount of label in the diaphysis to 38% of the noncultured values, and in the epiphysis to 73% (Table 3). Treatment with IC inhibited the release of label from the diaphysis (IC/Co = 1.52) while stimulating ^{35}S release from the

Table 1. The effect of 24-hour culture (serum free) in the presence (IC) or absence (Co) of intermittent compression, on the increase in length of the calcified diaphyseal part of long-bone rudiments

Exp. no.	Diaphyseal length (μm) before culture	Δ before-after culture			
		Co	IC	IC/Co	(n)
1	250 ± 12	42 ± 8	75 ± 10[a]	1.8	12
2	128 ± 8	12 ± 3	32 ± 7[a]	2.7	17
3	207 ± 8	8 ± 3	31 ± 5[a]	3.9	18

[a] $P < 0.01$, Student's t test; values are $\bar{x} \pm$ SEM

Table 2. The effect of 24-hour culture (serum free) in the presence (IC) or absence (Co) of intermittent compression, on macromolecular sulfate incorporation in whole nonstripped metatarsal rudiments

Exp. no	^{35}S (cmp/bone rudiments)			
	Co	IC	IC/Co	(n)
1	612.6 ± 32.7	739.1 ± 16.5[a]	1.21	8
2	1466.5 ± 52.6	1756.6 ± 75.1[a]	1.20	6

[a] $P < 0.01$, Student's t test; values are $\bar{x} \pm$ SEM

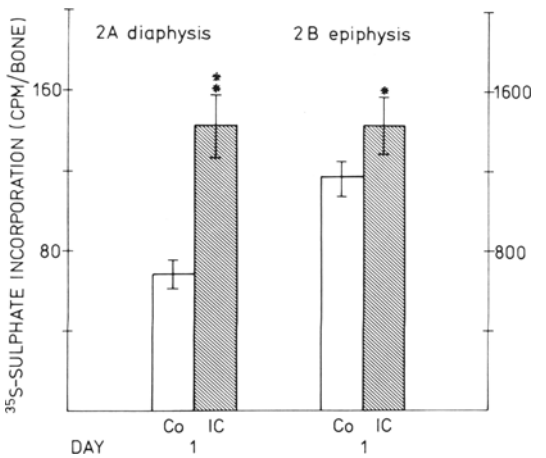

Fig. 2. The incorporation of ^{35}S-sulfate in diaphysis (**A**) and epiphyses (**B**) of 17-day-old periosteum-free ("stripped") metatarsal rudiments after 24 hours culturing in control conditions (Co) or with applications of intermittent compression (IC). The labeling period was the last 3 hours of the culture period. Values are $\bar{x} \pm$ SEM, n = 18. $^{**}P < 0.001$, $^{*}P < 0.05$.

epiphyses (IC/Co = 0.79) when compared with controls.

Discussion

It is generally assumed that nonmineralizing tissues normally contain sufficient levels of naturally occurring calcification inhibitors to preclude them from undergoing inappropriate calcification, and that mineralization can only proceed at a site of potential calcification when such inhibitors are enzymatically destroyed or otherwise removed, or if appropriate nucleators are brought together to initiate hydroxyapatite deposition [11].

About 50% of the cartilage organic matrix is a gel with a special kind of macromolecular organization consisting mainly of viscous hydrophylic cartilage PG which is synthesized locally by chondrocytes, and which provides the molecular basis for its remarkable resilience and the ability to resist compression [12–17]. There is much evidence for one of the major hypotheses that PG inhibits hydroxyapatite formation *in vitro* [18–22] or mineralization *in vivo* [23, 24] and must be degraded or otherwise modified before its onset [25]. On the other hand, mechanical forces have been shown to affect metabolic activities associated with bone remodeling [26, 27] and also, that mechanical stimulus is one of the major factors in the control of cartilage matrix synthesis [2–4, 28, 29].

Investigating the influence of IC on calcification of growth plate cartilage using organ cultures of 17-day-old fetal mouse long-bone rudiments, we found that IC of physiological magnitude had no effect on general the growth of the rudiments during a culture period of 24 hours (Fig. 1C,D), and that growth of the rudiments was not affected by rat serum. As opposed to total length, the length of the calcified diaphyseal zone of rudiments was significantly increased by IC (Fig. 1A,B), independent of the presence of serum. These results are in good agreement with those of Klein-Nulend et al. [6], who used slightly younger rudiments cultured for 5 days in the presence of 10% RS.

With the pulse-labeling technique we demonstrated that IC significantly increased the incorporation of sulfate into GAGs of metatarsal rudiments (Table 2). Following the distribution of the increased incorporation of the radioactive sulfate in calcified diaphyses and noncalcified epiphyses, it has been shown that IC has a stronger effect on calcified diaphyseal parts (Fig. 2A; $P < 0.001$) in the first 24 hours of culturing than on noncalcified epiphyseal parts (Fig. 2B; $P < 0.05$), although, the effect on epiphyseal parts has been significantly

Table 3. The effect of 24-hour culture (serum free) in the presence (IC) or absence (Co) of intermittent compression on GAG degradation by calcifying and noncalcifying cartilage

	^{35}S Retained in tissue GAG (cpm/bone rudiments)			
	Noncultured	Co	IC	IC/Co
Diaphysis	625.2 ± 73.2	238.5 ± 21.1	361.5 ± 34.7[a]	1.52
Epiphyses	1760.2 ± 83.2	1276.5 ± 79.5	1007.8 ± 49.5[b]	0.79
	^{35}S Retained in culture medium			
	- - -	445.7 ± 21.6	607.8 ± 25.5[a]	1.36

[a] $P < 0.01$, [b] $P < 0.05$, Student's t test. Values are $\bar{x}$ ± SEM, n = 12

higher compared with controls. Our results are in accord with findings of van Kampen et al. [5] who also found that mechanical stimuli increase sulfate incorporation in chicken chondrocyte aggregates, and with findings of Klein-Nulend et al. [7] who reported that IC significantly increased the degree of sulfation of CS chains in mouse bone rudiments after 5 days in culture, but except increased size of the CS chains in epiphyses, no effect was found on the hydrodynamic size of the PG aggregates or monomers. No other changes of structural characteristics of the macromolecules were observed. According to earlier findings of Inerot and Heinegard [30] that the ratio of chondroitin sulfate C6/C4 increases with increasing age, normally only C4 is synthesized in fetal cartilage. In line with these observations they found a very low C6/C4 ratio [7], which is somewhat higher in the diaphysis.

We also found that stimulation of early mineralization and expansion of the rudiment by IC is parallel with a greater number of sulfate groups in the matrix. Shepard and Mitchell [31] clearly show that there is a selective increase in the concentration of PGs in the hypertrophic zone of the cartilage growth plate, exactly in the same regions where mineral clusters are formed.

The data reported here from our ^{35}S-sulfate release experiments (Table 3) show that under the influence of IC more than one-quarter of the ^{35}S-sulfate-labeled PGs were removed from the tissue in media during culture period of 24 hours, mostly from noncalcified epiphyseal parts of the rudiments, but parts that will be calcified in the near future. Also, the time course of the labeled PGs corresponds with a marked increase in length of the calcified zone of IC-treated rudiments, suggesting that this change refers to mineralization. Our results correlated well with results of Scherft and Moskalewski [32] who showed that PGs are not removed before calcification, but are degraded from the uncalcified portions of the matrix.

The question raised is how to accept at first glance two opposite results: that IC charge increases incorporation of radioactive sulfate in PGs of metatarsal rudiments; and that the same mechanical stimulus promotes the release of the radiolabeled PGs, mainly from noncalcified rudiment parts? However, the increased incorporation of sulfated PGs at the beginning of intensive calcification ensures an enhanced need for Ca^{2+}; actually they precede accumulation of Ca^{2+} serving as its storage [33]. At this interval of time the sulfated PGs can simultaneously act as inhibitors of calcification [18–22] and may be removed and/or changed from noncalcified epiphyseal parts before the onset of mineralization in those parts. According to earlier findings that bone, hypertrophic cartilage, and cartilage contains a distinct type of PG [34, 35], it is also possible that the large cartilage proteoglycan was replaced by two small proteoglycans under the influence of IC, which results with release of labeled sulfate from epiphyseal parts of rudiments in media. The diaphysis/epiphysis ratio of labeled PG in metatarsal rudiments prior to culturing shows that about 36% of the sulfated PG was present in the calcified diaphyses (Table 3). Following the 24-hour interval of culturing, the same distribution of the sulfated PG was found in rudiments after IC treatment, whereas the control group revealed only 19% of the PG being contained in diaphyses. Thus, retained distribution content of ^{35}S-sulfated GAGs between calcified and noncalcified rudiment parts suggests that the applied mechanical stimulus is maximally close to physiological conditions *in vivo* in the sense of mechanical charge.

The mechanical stimulus within physiological limits has been shown as a fine regulatory mechanism influencing essentially the distribution and amount of sulfated PG in the process of calcification, changing simultaneously and optimally physiological properties of the cartilage rudiments and adjusting them for their mechanical needs within a very short interval of time. In recent years it has become evident that the control of proliferation and differentiation of connective tissues is under the influence of local factors and/or growth factors [36]. A paracrine mechanism that involves some of these growth factors might help explain the many discrep-

ancies in hormone action that are observed in different model systems, including those differences between *in vivo* and *in vitro* responses [37, 38]. As the mechanical stimulus on rudiments was not mediated by serum factors, such stimulus may induce production of local factors that modulates PG metabolism. IC changes the metabolism of sulfate PG, acting in varying modes calcified diaphysis and noncalcified epiphyses, thus enabling a better simulation of physiological conditions in *in vitro* experiments. In this way we can better observe regulatory effects of mechanical stimulation on calcification processes of epiphyseal growth plate cartilage.

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