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Tensile Forces Enhance Prostaglandin E Synthesis in Osteoblastic Cells Grown on Collagen Ribbons

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Summary. An experimental system has been developed to examine the prostaglandin (PG) production induced by tensile mechanical forces in bone cells cultured on collagen ribbons. Fetal rat calvaria cells (osteoblast-enriched) were grown on collagen ribbons. The collagen ribbons were stretched under culture conditions in a machine that recorded force and displacement. Repeated stretching of the collagen ribbons (8 times, 5–10%, over 2 hours) increased the rate of prostaglandin synthesis approximately 3.5-fold over that of cells on nonstretched ribbons. This system should provide a suitable method to quantitatively study the effect of mechanical forces on various parameters of PG synthesis.

Key words: Mechanical force — Bone cells — Prostaglandins.

It has been proposed that prostaglandins, ubiquitously present in mammalian tissues [1, 2], act as local regulators of bone metabolism (for review see ref. 3). There is evidence suggesting that bone remodeling and bone growth induced by mechanical forces *in vivo* are prostaglandin-mediated [4, 5]. Moreover, *in vitro* studies have shown that mechanical stress, or detachment from their substratum, stimulates prostaglandin synthesis in fibroblasts and embryonic rat calvaria cells [6, 7].

To further study cellular responses to mechanical forces, we have developed a system for exposing bone cells in culture to precisely measured mechanical stress. This report describes the system and illustrates the effect of repeated stretching on PGE production by osteoblastic bone cells.

Materials and Methods

Cell culture medium, serum, and antibiotics were purchased from GIBCO, Grand Island, NY. Culture plastic ware from Costar, Cambridge, MA or Falcon, Oxnard, CA; Collagenase, hyaluronidase and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma, St. Louis, MO. Radioactive PGE_2 was purchased from New England Nuclear; PGE-specific monoclonal antibody was generously provided by Dr. Edward Lally, University of Pennsylvania. Collagen ribbons were from Ethicon Co., NJ. Dated, pregnant rats were purchased from Charles Rivers, Wilmington, MA.

Preparation of Bone Cells

Embryonic rat calvaria cells were obtained by sequential collagenase/hyaluronidase digestion from 19–20 day fetal rat calvaria, according to the method of Wong and Cohn [8]. Cells released between 20 and 40 min of enzyme digestion were collected and characterized as an osteoblast-enriched cell population by PTH response and alkaline phosphatase activity. The cells were centrifuged at 5,000 rpm for 5 min and were plated at 47,000 cells/cm^2 in F-12 medium supplemented with 5% horse serum, 2% fetal bovine serum (FBS), and 100 μg/ml kanamycin. After 1 week, cells were seeded onto collagen ribbons.

Preparation of Collagen Ribbons

Collagen ribbons (dry thickness 45 μm) were cut to 5 cm lengths and sterilized by exposure to UV light overnight. The teflon frame (Fig. 1) was sterilized by autoclaving and the collagen strips were then clamped into the frame under aseptic conditions, leaving a distance of approximately 3 cm between the clamps. The frames were incubated at 37°C for 2 h in a 100 mm Petri dish containing 100% FBS, to allow binding of attachment macromolecules (fibronectin and others) to the collagen ribbons [9]. The calvaria cells were trypsinized, counted, and seeded at a density of 6×10^5 cells/ribbon directly onto the collagen ribbons. The cultures were maintained in F-12 plus 10% FBS and 100 μg/ml kanamycin for 2 weeks.

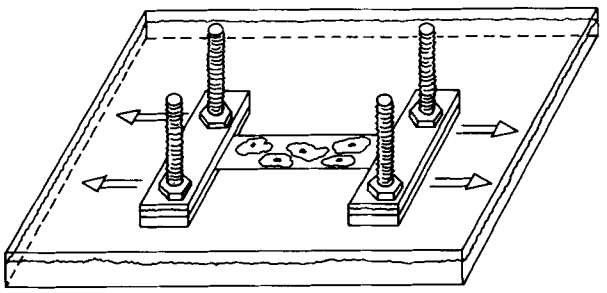

Fig. 1. Diagram of collagen ribbon clamp in medium bath. Collagen ribbons were fixed between two clamps. Cells were seeded and cultured on the ribbon, which was then stretched via screws bolted to the device described in Methods. The collagen ribbon was immersed in a culture dish containing medium, 2% FBS, and 100 μg/ml kanamycin.

Stretching Machine

The force-generating apparatus was adapted from a variable speed infusion pump (Compact Syringe Pump, Model 975, Harvard Apparatus, Natick, MA). The collagen strip, mounted in the frame shown in Fig. 1 was stretched between a fixed bar attached to the body of the infusion pump and the piston-moving mechanism. Force and displacement transducers were attached to the apparatus and linked to recorders. The cell-covered collagen ribbon was immersed in F-12 medium with 2% FBS and 100 μg/ml kanamycin during stretching. The entire apparatus was housed in an incubator maintained at 37°C, with a 5% CO_2/95% air atmosphere.

PGE Radioimmunoassay

At given times during the experiment, PGE was measured in duplicate samples in medium aliquots. 0.2 ml samples of medium were removed and incubated with 10,000 cpm radioactive PGE_2 and 0.1 ml monoclonal antibody against PGE, diluted 1/5,000 in 0.01 M Tris HCl, 0.14 M NaCl, pH 7.6 at 37°C for 1 h. An equal volume of $(NH_4)_2SO_4$ (saturated at 37°C) was added and incubation continued for 45 min. After centrifugation at 2,500 rpm for 15 min, the supernatant was counted. This rapid procedure yields reliable measurements between 100 and 2,500 pg, which is adequate for the PGE levels found in the bone cell medium. The sensitivity of the assay can be increased about threefold by diluting the antibody 1/15,000 and incubating the reaction mixture overnight. The antibody does not discriminate between PGE_1 and PGE_2, requires about 10-fold higher concentrations of 6 keto $PGF_{1\alpha}$ or $PGF_{2\alpha}$ to compete with PGE, and does not cross-react with PGA_1 and PGB_2.

DNA Assay

Following each experiment, ribbons were removed and cell content quantitated by DNA measurements. Each collagen ribbon was sonicated for 15 sec. in 1 ml of 50 mM Tris HCl (pH 7.50) and aliquots were assayed using a fluorometric method [10].

Preparation of SEM

Collagen ribbons were fixed in 0.5% glutaraldehyde for 30 min, then transferred to a 0.1 M cacodylate buffered solution of 2% glutaraldehyde and 1% osmium tetroxide, for 2 h. The samples were dehydrated by using a series of ascending alcohol concentrations, were critical-point dried, coated with gold, and examined in an Hitachi 3010 at 20KV.

Results

Growth of Bone Cells on Collagen Ribbons

Rat calvaria bone cells seeded onto collagen ribbons attach and proliferate to cover most of the ribbon surface in 14–16 days (Fig. 2). Cells cultured under these conditions have remained viable up to 30 days. The bone cells remain in monolayers and seem to align along the collagen fibrils.

Physical Properties of Collagen Ribbons Containing Cells

Figure 3 shows stress-strain curves of collagen ribbons containing bone cells. The general pattern of these curves is typical of many natural collagenous tissues, for example, rat tail tendon or other tendons, skin and periodontal ligament [11, 12]. The Young modulus estimated from the linear portion of the curve was 12.2 ± 1.36 MPa. In most experiments, repeated stretching of the ribbon (up to 8 times) yielded identical curves (Fig. 3B). However, in some cases, hysteretic effects were evident (Fig. 3A), probably due to improper alignment of the ribbon in the stretching device and slippage. In addition, the presence of cells did not alter the stress-strain curves of the collagen ribbons.

PGE Production by Bone Cells

The rate of prostaglandin synthesis is shown in Fig. 4. Control cells cultured on the ribbons and held in the apparatus without stretching produced PGE in a linear fashion over 2 h, at an average rate of 17.5 ± 2.3 pg PGE/μg DNA/min. The experimental ribbons were subjected to 8–10 cycles of slow stretching (2–3 mm over 15 min each) and rapid relaxation, during a period of 2 h. The mean rate of prostaglandin synthesis in the stretched group was 64.5 ± 5.1 pg/μg DNA/min, which was significantly higher than in the control group ($P < 0.01$, Student's t test).

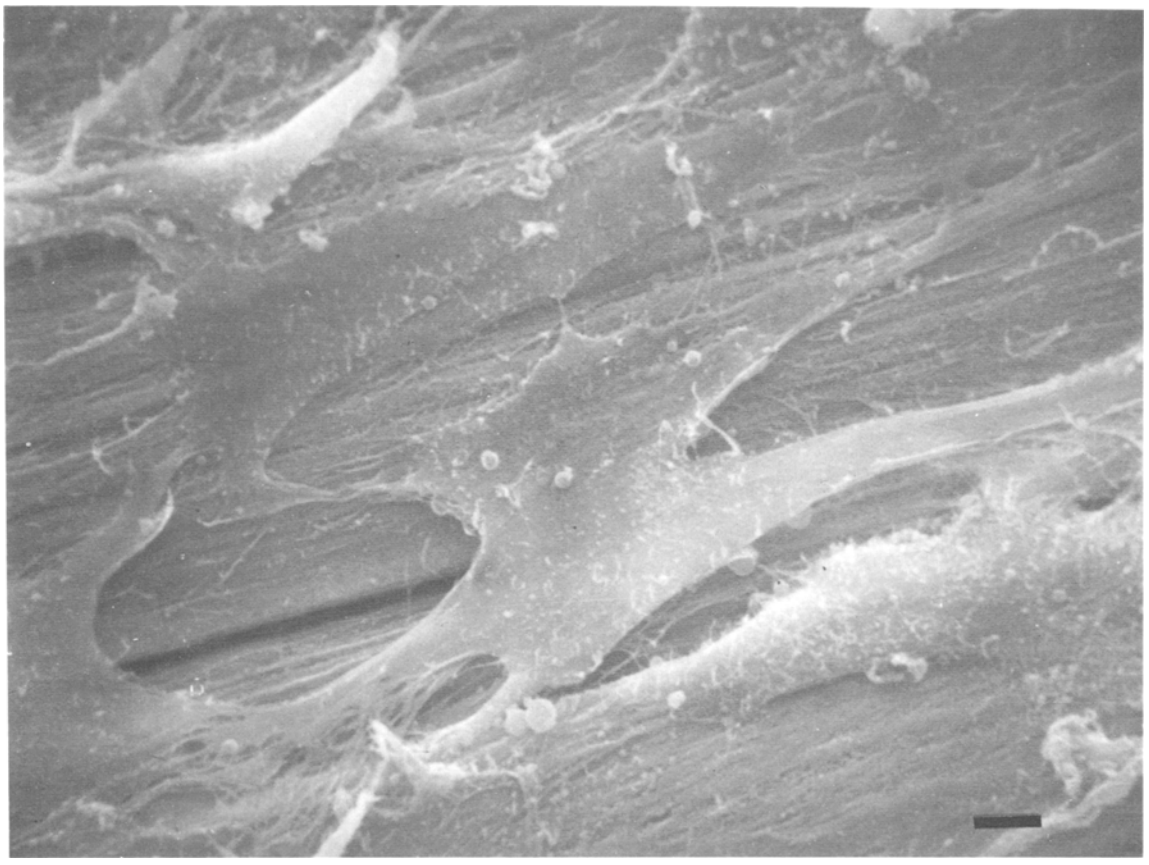

Fig. 2. Scanning electronmicrograph (SEM) of bone cells cultured on collagen ribbon. Confluent primary cultures of bone cells were trypsinized and seeded on collagen ribbons. On day 16 after seeding, the ribbon was prepared for SEM, as described in Methods. Bar = 7 μm.

Discussion

It is well known that mechanical forces can influence the shape of bones during growth. One hundred years ago, this phenomenon was postulated as Wolff's law, which states that bone will change its shape to best withstand the mechanical forces exerted upon it [13]. The mechanism for this adaptation is not yet fully understood, but recent advances in cell biology have generated several testable hypotheses regarding this phenomenon.

It is well documented that the bone remodeling process involves complete replacement of small segments of tissue brought about by osteoclastic resorption of old tissue followed by deposition of new tissue. The latter is carried out by a different group of cells, the osteoblasts. There are probably two major rate-limiting steps which determine the activity of these two groups of cells: their recruitment from a precursor pool, and the intensity and duration of their differentiated function. Both are under the

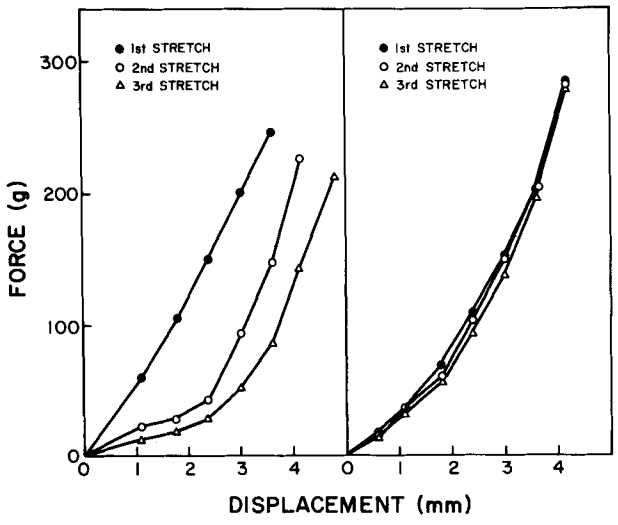

Fig. 3. Stress-strain curves of collagen ribbons containing bone cells. Bone cell-covered ribbons were stretched and force and displacement were measured as described in Methods. (**A**) Frames were mounted slightly off 90° angles relative to long axis of ribbon. (**B**) Frames were mounted perpendicular to long axis of ribbons.

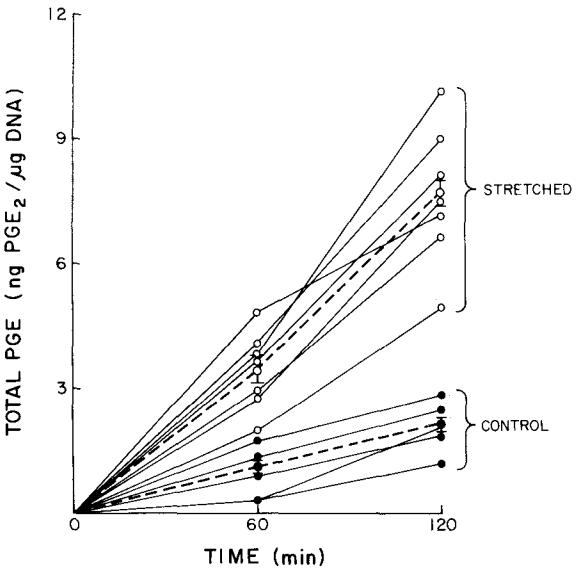

Fig. 4. Prostaglandin E production by bone cells: effect of stretching. Collagen ribbons containing 12–16 day cells were mounted in the stretching device immersed in 10 ml Ham F-12 medium with 2% FBS and 100 µg/ml kanamycin. The ribbons were either stretched (— O —) or kept stationary (— ● —) as described in Methods. PGE was measured in duplicates in media aliquots and DNA in the whole ribbon. Each *line* represents an independent experiment and the *dotted lines* are means ± SEM for the groups, which are significantly different at $P < 0.01$.

control of the environment which includes hormones, growth factors, nutritional and obviously mechanical influences.

The translation of environmental stimuli into cellular signals has been extensively studied in a wide variety of cells and seems to be restricted to a small number of mechanisms which, according to current knowledge, include (1) opening of ion channels (frequently, calcium channels); (2) activation of adenylate cyclase; and (3) triggering of phospholipid breakdown (probably phosphatidyl inositol). These transduction processes are not mutually exclusive and are linked inside the cell by complex feedback processes which include activation of protein kinases and modulation of intracellular calcium fluxes.

About 8 years ago, Rodan et al. [14] and independently, Davidovitch et al. [15], suggested that mechanical stimuli effects on cartilage and bone may be mediated by changes in cyclic nucleotides, one of the ubiquitous mechanisms listed above. Initial evidence collected on bone exposed to mechanical perturbations *in vivo* and cartilage cells exposed to compressive forces in culture supported these assumptions [16]. The *in vitro* studies suggested that cartilage cells did not require matrix to respond to mechanical perturbations, but the presence of matrix modified the response [14]. A possible mechanism for producing changes in cyclic AMP accumulation was suggested by the study of Somjen et al. who showed that stretching of the substrate on which bone cells grew enhanced prostaglandin synthesis [7]. Hong and Levine [6] had shown that mild mechanical removal of fibroblasts from the surface on which they grew produced substantial stimulation of prostaglandin synthesis.

Prostaglandins are likely mediators of mechanical effects on bone for several reasons. Studies on osteosarcoma cell lines and rat calvaria bone cells indicate that prostaglandin synthesis is associated with the osteoblastic phenotype [17]. Prostaglandins are typical intercellular mediators which can provide both a localized response and signal amplification necessary for the mechanical modulations of bone remodeling. Prostaglandin E_2 is one of the most potent bone resorbers *in vitro* [18, 19], and recent studies indicate that prostaglandins also enhance bone formation by acting on a different group of cells (Raisz and Martin) [3]. The anabolic effects of the prostanoids include stimulation of cell proliferation as well as increased collagen synthesis. From the information currently available, one can visualize the following simplified sequence of events for mechanical regulation of bone formation or remodeling: (1) stretching of attachment points in a sensitive subpopulation of osteoblasts results in prostaglandin synthesis (primarily prostaglandin E_2); (2) PGE_2 can initiate both bone resorption, if PGE levels are high enough, and bone formation, in appropriate populations of responsive cells; (3) bone resorption is itself followed by bone formation via a "coupling" mechanism.

The findings presented in this study document the first step in this sequence. This experimental system provides the opportunity to study the quantitative relationship between stress, displacement and prostaglandin synthesis, and the biochemical pathway for prostaglandin production in these cells.

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