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Pulsed Electromagnetic Fields Promote Collagen Production in Bone Marrow Fibroblasts via Athermal Mechanisms

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Summary. Primary and passaged cultures of fibroblasts (RBMFs) raised from the bone marrow stroma of young rabbits were treated with pulsed electromagnetic fields (PEMFs) from the start of each culture until 1 week after they became confluent. The PEMF treatment had no effect on cell proliferation, estimated by phase contrast microscopy, by ^{3}H-thymidine incorporation into DNA, or by total DNA assay. Collagen production, estimated by conversion of ^{3}H-proline to ^{3}H-hydroxyproline in nondialyzable material was markedly elevated in postconfluent cultures, but not in cultures that had only just reached confluence. About 65% of ^{3}H-hydroxyproline was in low molecular weight form, and a correlation between collagen breakdown and cyclic AMP (cAMP) levels in RBMFs was demonstrated by adding dibutyryl cAMP or prostaglandin E_2 (PGE_2) to the culture medium concurrently with ^{3}H-proline. The PEMF apparatus caused an insufficient temperature rise (less than 0.1°C) to account for these results. We propose that the rise in collagen production is consistent with the hypothesis that PEMFs act by reducing cAMP levels in RBMFs, and that thermal effects are insignificant.

Key words: Pulsed electromagnetic fields — Fibroblasts — Collagen — cAMP.

Pulsed electromagnetic fields (PEMFs) have been used extensively to treat nonunions of bone and related disorders [1–3], and although the resumed healing process has been described in these studies, the basic interaction between PEMFs and skeletal tissue remains the subject of speculation. The application of electrical stimuli to connective tissue *in vitro* has suggested that cAMP metabolism may be modified [4–6], although differences in the electrical parameters of the stimuli used render direct comparison of these results difficult. It might be anticipated that changes in cAMP levels would modify the synthetic activity of connective tissue cells, and previous work has suggested that PEMFs increase the collagen content of embryonic chick tibiae [7] and glycosaminoglycan synthesis in chondrocyte cultures [8]. We have demonstrated recently that collagen production by embryonic tendon fibroblast cultures can be substantially increased by PEMF treatment [9], and that cAMP changes are implicated in these results. In the present work we extend these studies by applying PEMFs to a different population of mammalian, bone-derived cells. Fibroblasts (RBMFs) derived from rabbit bone marrow stroma [10], described as determined osteoprogenitor cells, were selected for this study because they offer the distinct advantage that they can maintain their functional capacity during serial culture over many passages [11].

Collagen production is the major function of fibroblasts [12] and may be important for the physiological role of RBMFs, which is to provide support for the hemopoietic elements of bone marrow and, perhaps, to contribute to fracture repair [10]. It may be that the clinical action of PEMFs involves a bias of tissue metabolism towards collagen formation, and that this facilitates the healing process. If such an effect proved to apply generally to fibroblasts in culture, then *in vitro* models might be developed that would enable the mechanism of action of PEMFs to be explored further.

It has been suggested that local heating might be adequate to explain the effects of PEMFs [13, 14]; the present work addresses this question by mea-

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surement of the heating effect of the PEMF apparatus under culture conditions and by comparison of the effect on collagen production of small variation in culture temperature with that of PEMFs.

Materials and Methods

Pulsed electromagnetic fields were generated by a BiOsteogen apparatus, providing a waveform similar to that used clinically [1–3], but driving circular coils which we have described previously [9, 23]. Cell cultures were placed within the coils so that the magnetic field was tangential to the growth surface. PEMFs were applied for alternate 6-hour periods throughout each experiment.

Cell cultures were established as described by Ashton et al. [15], using 10% fetal calf serum in HEPES-buffered EMEM without non-essential amino acids, in accordance with recent work on intracellular collagen breakdown [16]. Passaged cells were derived from confluent control cultures by collagenase (0.1% in EMEM) digestion, and inoculated at 3×10^5 cells per 25 cm^2 flask. For cyclic AMP (cAMP) measurements, passaged RBMFs were raised in 30-mm diameter tissue culture dishes seeded at the same density, and maintained in polycarbonate vessels (Gallenkamp Ltd, Loughborough, England). Cultures were placed in a warm air room at 37 ± 0.5°C, and whereas passaged cells were usually confluent after about 1 week, primary cells grew more slowly. Temperature was monitored continuously using a type T thermocouple immersed in medium in a culture flask placed among the others, so that control and PEMF-treated cultures could be compared. Cell density was monitored during each experiment by phase contrast microscopy.

Collagen production by RBMF cultures was estimated by adding ^{3}H-proline (2.5 μCi/ml) to the complete medium during the last 24 hours of culture. The cell layer was scraped into cold 0.5 M acetic acid, ultrasonicated briefly, pooled with the used culture medium, and an aliquot dialyzed against cold acetic acid. The resultant suspension and a nondialyzed aliquot of the cell layer and medium were hydrolyzed (6 M HCl, 105°C, 24 hours), and ^{3}H-hydroxyproline separated from ^{3}H-proline by ion exchange chromatography [17]. The ^{3}H-hydroxyproline counts present in dialyzable and nondialyzable form enabled short-term degradation of newly synthesized collagen to be estimated as well as collagen production. Collagen types were examined by polyacrylamide gel electrophoresis of pepsin-resistant salt precipitates of the pooled cell layer and medium. By using the delayed reduction technique [18] to cleave the disulfide crosslinks in type III collagen, we were able to identify type III by its altered electrophoretic mobility. We have described these methods more fully elsewhere [9]. To establish the effect of elevated cAMP levels on collagen production, dibutyryl cAMP (1 mM) or PGE_2 (1 μM) were added to the culture medium concurrently with ^{3}H-proline, and samples were processed as above.

Cell proliferation was compared by phase contrast microscopy after each medium change and by measuring the incorporation of ^{3}H-thymidine into DNA. The RBMFs were labeled for 4 hours immediately after the last PEMF treatment period at confluence, and in parallel cultures 1 week later. Cell layers were washed free of label with EMEM, digested with papain, and ^{3}H-thymidine estimated [19]. The DNA was assayed fluorometrically in a portion of the digest [20].

Incubation temperature was investigated for its effect on collagen production in control cultures maintained for 2 weeks in separate incubators set in the range 35.7 to 38.9°C. ^{3}H-proline metabolism was examined as above.

Cyclic AMP levels were measured at various time points during the last day of culture using a competitive binding assay [21] on acid ethanol (2 ml concentrated HCl in ethanol) extracts of cell layers.

Collagenolytic activity in used culture medium was estimated [22] using ^{14}C-labeled collagen fibrils as the substrate for untreated and trypsin-activated medium from the last change in three experiments.

Scintillation counting was either carried out on a machine, which provided dpm directly, or we checked that scintillation quenching was the same in each group of samples before statistical analysis was undertaken.

Results

Cell proliferation was not affected by PEMF treatment; estimation of cell numbers by microscopy during the early stages of culture showed no effect on primary or passaged RBMFs, although more variation occurred in primary cultures. Total DNA levels measured in just-confluent cultures and 1 week later were not altered by PEMF treatment, and although thymidine incorporation was less consistent, there was no significant change [23].

Collagen production was altered by PEMF treatment only in postconfluent cultures: no difference was found if RBMFs had just reached confluence. In postconfluent cultures the total conversion of proline to hydroxyproline was slightly elevated (about 7%) in each experiment after PEMF treatment; overall this effect proved significant. A more marked effect was found on the nondialyzable component of ^{3}H-hydroxyproline-containing material: three of four experiments showed a significant increase (about 25%) in PEMF-treated cultures, and overall this effect was also significant (Table 1). No change in collagen type was found. The relative amount of nondialyzable hydroxyproline fell significantly when dibutyryl cAMP or PGE_2 were added to the culture medium (Table 2). Collagenolytic activity in the used medium from these experiments was undetectable.

Temperature differential of the order of 0.1°C occurred randomly between the control and PEMF culture positions in the warm room, and a rise of about 0.05°C occurred when the PEMF apparatus was active. There was, therefore, a small systematic difference in temperature between control and PEMF-treated cultures. In a separate experiment to examine the effect of small variation, total conversion of proline to hydroxyproline proved almost insensitive to incubation temperature (Fig. 1), and the proportion of macromolecular material fell significantly from control values of about 40% only at the

Table 1. Percentage change in ^{3}H-proline metabolism in PEMF-treated RBMFs

1A: Total conversion of ^{3}H-proline to ^{3}H-hydroxyproline					
Passage no.:	Primary	1	3	15	Mean effect
Just confluent:	2.6	−0.1	3.8	2.2	2.1 ± 1.6%
Postconfluent:	7.0[a]	5.0	5.3[a]	10.1	6.9[a] ± 2.3%
1B: Nondialyzable ^{3}H-hydroxyproline					
Passage no.:	Primary	1	3	15	Mean effect
Just confluent:	20	2	4	−4	5.5 ± 10.2%
Postconfluent:	31[b]	18[b]	22	25[a]	24[a] ± 5.5%

Table 1 shows the effect of PEMF on collagen metabolism in RBMFs when the cell layers were just confluent and after a further week of PEMF treatment. 1A shows the effect on total conversion of ^{3}H-proline to ^{3}H-hydroxyproline, and 1B on the proportion that was nondialyzable. Data are expressed as % increase relative to control cultures. Student's t tests were performed on raw data (cpm), and the 95% confidence interval is shown for the mean effects. There were four replicates in each group

[a] $P < 0.05$

[b] $P < 0.01$

Table 2. The effect of elevated cAMP on ^{3}H-hydroxyproline distribution in postconfluent RBMFs

Agent	Total ^{3}H-hypro	Nondialyzable ^{3}H-hypro
Dibutyryl cAMP	−7.4%[a]	−21%[a]
Prostaglandin E_2	−4.2%	−20%[a]
PEMF treatment	+6.9%[a]	+24%[a]

Table 2 shows the negative correlation between elevated cAMP and collagen metabolism, in terms of hydroxyproline production, and includes mean PEMF data for comparison. Data are expressed as percentage difference between cultures treated with the specified agent and controls. There were five replicate cultures in each group, and statistics were performed on ^{3}H dpm by ANOVAR.

[a] $P < 0.05$

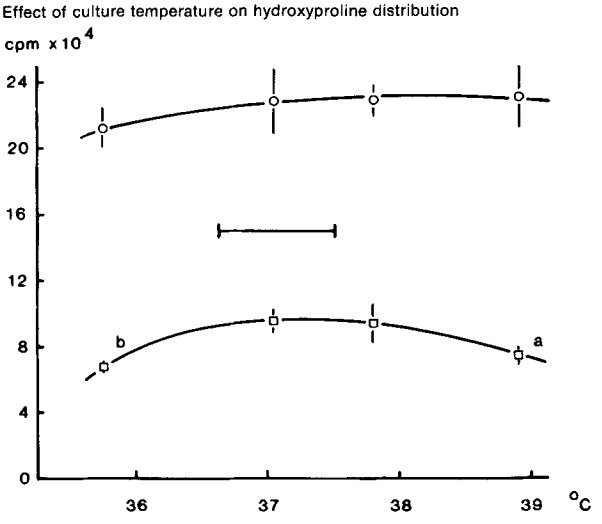

Fig. 1. The effect of incubation temperature on conversion of ^{3}H-proline to ^{3}H-hydroxyproline. The upper curve shows total conversion, and the lower trace shows cpm present in nondialyzable material. Data presented are means of four replicates ± SEM. Significance levels were determined by ANOVAR: a = $P < 0.05$, b = $P < 0.01$, relative to mid-range temperatures. The horizontal bar shows the range of random variation in culture temperature during PEMF experiments.

extremes of the range covered, i.e., 35.7 and 38.9°C. Cell density, by microscopical examination, increased up to 37.8°C and fell slightly at the highest temperature.

Cyclic AMP levels were lower at each time point after PEMF treatment, and four of five measured differences in two experiments were significant. The results from one of these are shown in Table 3.

Discussion

This work suggests that PEMFs promote collagen production in RBMFs to a significant extent, and that the cells are not responsive until after confluence has been reached, as in our previous experiments on embryonic chick tendon fibroblasts [9]. The observed increase in macromolecular hydroxyproline-containing material was not accompanied by a proportionate rise in total conversion of proline to hydroxyproline, which suggests that increased cell numbers were not responsible for the effect. This was confirmed by the similar cell growth rate observed in control and PEMF-treated cultures; although it has been suggested that low amplitude sinusoidal PEMFs may enhance thymidine incorporation into DNA in human fibroblasts [24], we can find no evidence from the present work on RBMFs or in embryonic tendon fibroblasts [9] for a general effect on cell proliferation.

The observed rise in temperature caused by PEMF treatment was minimal, and random variation between control and PEMF culture positions was small. It is unlikely, therefore, that temperature

Table 3. Cyclic AMP levels in RBMFs after chronic PEMF treatment

Time point (hours):	0	2	6	
Control:	7.05 (0.13)	6.84 (0.07)	6.15 (0.06)	pmoles/dish
PEMF-treated:	6.84[a] (0.11)	5.85[b] (0.05)	5.49[a] (0.05)	pmoles/dish

Table 3 shows mean cAMP levels (± SEM) per culture dish (i.e., about 10^6 cells) in postconfluent RBMF cultures treated with PEMFs for 1 week after confluence. Samples were taken at the end of a treatment cycle (time zero) and again at 2 and 6 hours. There were at least five samples per group, and Student's *t* test was used to establish significance between groups at the [a]($P < 0.05$) or [b]($P < 0.01$) level

differences contributed to the observed effects of PEMFs on collagen production. An examination of the effect of incubation temperature on collagen metabolism in RBMFs (Fig. 1) suggested that controls would need to be at least 1°C cooler than PEMF-treated cultures to account for the effect shown in Table 1, a difference that would easily have been resolved by the measuring equipment used. Our previous work on the effect of PEMFs on lactic acid production in RBMFs supports this conclusion [23]. These data demonstrate that PEMFs can activate athermal mechanisms *in vitro*.

Collagen production proved to be susceptible to manipulation of cellular cAMP levels, however, and the use of dibutyryl cAMP or PGE_2, which elevate cAMP levels in RBMFs [25], showed that elevated cAMP can lead to a small reduction in total conversion of proline to hydroxyproline and a more marked fall in the macromolecular component of this parameter. The magnitude of the latter (about 20%, Table 2) is similar to that described by previous workers using different fibroblast populations [26, 27], and is of opposite direction to the changes produced by chronic PEMF treatment.

The results presented here support our previous conclusion [9, 25] that collagen production can be elevated in fibroblasts by suppression of cAMP-dependent processes after PEMF treatment. Similarly, no changes were observed in the collagen types expressed by RBMFs, which suggests that the effect of PEMFs is quantitative rather than qualitative, and that PEMFs might exert control on collagen production at a post-translational stage. The effect of PEMF on collagen production was independent of the passage number of the cells: primary RBMFs showed a similar response to those in their fifteenth passage. Collagen phenotype in control cultures was stable during long-term serial passage culture. Type I collagen formed the major component, with about 10% of type III and traces of type V [25]; this finding is in agreement with previous work on bone marrow-derived cells [28, 29].

Intracellular degradation of collagen or procollagen molecules has been identified as a cAMP-dependent pathway by which the cell controls collagen production prior to its secretion from the cell [30]; collagenolytic activity in the used RBMF culture medium was not detectable by appropriate techniques, and suitable controls indicated that no artifactual collagen degradation occurred during the sample handling procedure. The observed results are consistent, therefore, with a fall in cAMP levels in the cells after PEMF treatment, and a resultant rise in net collagen production following reduced intracellular breakdown of newly synthesized collagen.

Measurements made on postconfluent cultures, i.e., at a time when collagen production might be expected to be responsive to PEMF, suggest that a chronic reduction in cAMP levels occurs after PEMF treatment. This finding is consistent with that of Luben et al. [6], who showed reduced cAMP levels in PEMF-treated osteoblasts in response to parathyroid hormone. We suggest that in our experiments and Luben's, PEMF treatment may have caused the hormone:receptor:adenylate cyclase complex to become desensitized to hormone challenge. We have shown that substantial prostaglandin production occurs in RBMF cultures [25], which renders the cell less sensitive to exogenous hormones (Farndale and Murray: in preparation). The PEMFs appear to reduce sensitivity further, and their action might be explained in terms of these suggestions: if PEMFs cause skeletal tissue cells to become less responsive to hormones that normally stimulate tissue resorption, then a net increase in tissue formation may result. A detailed examination of the effects of PEMFs on cAMP metabolism is called for.

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