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Collagen and hyaluronic acid based polymeric blends as drug delivery systems for the release of physiological concentrations of growth hormone

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Two synthetic polymers, poly(vinyl alcohol) (PVA) and poly(acrylic acid) (PAA) were blended, in different ratios, with two biological polymers, collagen (C) and hyaluronic acid (HA). These blends were used to prepare two different materials, sponges and hydrogels, which were loaded with growth hormone (GH). The GH released, was monitored *in vitro* using a specific enzyme-linked immunoadsorbent (ELISA) assay. The results show that GH is released in a dose-dependent manner, from HA/PAA sponges and from HA/PVA and C/PVA hydrogels. The amount of GH released was proportional to the percentage of the natural polymer (HA and C). The release of GH from HA/PAA sponges was constant with time, whereas in HA/PVA hydrogels it was linear for the first 3 days followed by a slower release. The GH release pattern in C/PVA hydrogels was different, with a slow release for the first 3 days followed by a more rapid release. The concentrations of GH released from the materials were within a physiological range and sufficient to have a local effect on cellular proliferation. The effects of GH were tested *in vitro* using primary human osteoblast-like cells (HOBS) and measuring cell proliferation and alkaline phosphatase (ALP), a biochemical marker of HOB cell differentiation.

1. Introduction

The success of synthetic polymers as biomaterials is mainly related to their wide range of mechanical properties, processing methods and low production costs, but their interaction with living tissues is a major problem. On the other hand, many biological polymers of interest as biomaterials (namely, collagen, elastin and glycosaminoglycans) possess good biocompatibility but their mechanical properties are often inadequate. An interesting set of new materials have been made by combining synthetic polymers with naturally occurring polymers [1–3]. We have investigated the potential use of such polymer blends as drug delivery systems for the release of GH. Our aim was to measure the GH release and to determine if the rate of GH release from these materials was sufficient to have a local effect on cellular growth. Bone remodelling is a process that occurs throughout life and is dependent on cellular activity; this is controlled by numerous systemic and local factors [4, 5]. Although it has been well documented that GH is a major regulator of skeletal growth, and that it stimulates longitudinal bone growth in a dose-dependent manner [6, 7], there is increasing evidence that GH has a direct and indirect effect via insulin-like growth factors on bone cell proliferation and differentiation [8–10].

2. Materials and methods

2.1. Hyaluronic acid/poly(acrylic acid) sponges

Solutions of 5% hyaluronic acid (supplied as sodium salt by Fidia Advanced Biopolymers SpA-Italy) and 5% PAA with molecular weight 250 000 (Aldrich) were prepared in water, dissolved at 50 °C. The two solutions were blended in three different HA/PAA ratios: 20/80, 40/60, 60/40. The samples were then lyophylized. After crosslinking by thermal treatment at 130 °C under vacuum for 24 h, GH was added. One sponge was used as a control and GH was added to the other four to produce final concentrations of 25, 50, 75 and 100 mIU GH per ml of HA. The samples were lyophylized again and the release of GH was monitored *in vitro*.

2.2. Collagen/poly(vinyl alcohol) hydrogels

1.5 g of collagen was dissolved in 100 ml of 0.5 M acetic acid, in an ice bath, to obtain a final collagen

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solution of 1.5%. 10 g of PVA was added to 100 ml of distilled water and dissolved in an autoclave for 1 h at 120 °C to obtain a final concentration of 10% PVA. The two solutions were blended in three different C/PVA ratios: 30/70, 20/80, 10/90. One well was used as a control, and GH was added to the other four to produce final concentrations of 25, 50, 75, 100 mIU GH per ml of collagen. After GH addition, samples underwent eight cycles of freeze–thawing to obtain hydrogels. Each cycle, with the exception of the first one, consisted of 1 h at − 20 °C and 30 min at room temperature. The first cycle differed from the others due to a longer standing time at − 20 °C (overnight).

2.3. Hyaluronic acid/poly(vinyl alcohol) hydrogels

A 5% hyaluronic acid solution was made in distilled water at 50 °C. 5 g of PVA was added to 100 ml of distilled water and dissolved in an autoclave for 1 h at 120 °C to obtain a final concentration of 5% PVA. The two solutions were blended in three different HA/PVA ratios: 30/70, 20/80, 10/90. One well was used as a control, and GH was added to the other four to produce final concentrations of 25, 50, 75, 100 mIU GH per ml of hyaluronic acid. After GH addition, samples underwent eight cycles of freeze–thawing to obtain hydrogels. Each cycle, with the exception of the first one, consisted of 1 hour at − 20 °C and 30 min at room temperature. The first cycle differed from the others due to a longer standing time at − 20 °C (overnight).

2.4. Elution studies

The GH eluate from the samples were monitored *in vitro*. The hydrogels and sponges were each placed in 3 ml of phosphate-buffered saline (PBS) in individual universals at 37°C. The elution fluids were removed at regular time intervals (every day for 7 days and then every 2 days for a further 7 days), stored at − 20 °C. The eluate was replaced with fresh PBS and the universals returned back to 37 °C.

2.5. GH assay

Elution fluids were assayed for the GH using ELISA, as previously described [8]. The microtitre plates were read using a 96-well fluorescent plate reader (MR. 700 Dynatec Microplate Reader). Optical density was measured at 490 nm with a reference wavelength of 650 nm. The standard used was 22K rhGH (Novo-Nordisk, Gentofte, Denmark).

2.6. Cell culture

The primary HOBS were isolated from femoral heads obtained from patients undergoing surgery for total joint replacement. Trabecular bone fragments were dissected under sterile conditions, washed several times in calcium and magnesium-free PBS and cultured in complete Dulbeccos Modified Eagles Medium (DMEM, supplemented 10% foetal calf serum, 1% non-essential amino acids, 1% ascorbic acid, 1% L-glutamine, 2% hepes and 50 U/ml penicillin, 0.05 mg/ml streptomycin), in a humidified atmosphere with 5% CO_2 at 37 °C, for a period of 4–5 days, with one media change during this time, thus allowing the removal of any non-adherent cells. The bone fragments were then digested using a collagenase (100 U/ml) and trypsin (0.02%) digest mixture and were incubated for 20 min at 37 °C in this solution, in order to allow the release of bone cells from within the bone fragments. Following digestion the "osteoblast-rich" supernatant was transferred to a sterile universal and spun down to obtain a cell pellet. The cells were washed several times in complete DMEM to remove all traces of the digestion media. The cells were counted and seeded to obtain an appropriate number of cells for the different proliferation assays.

2.7. Cell proliferation

Dose-dependent stimulation of HOBS was assessed using the bromodeoxyuridine (BrdU) assay (Amersham); this assay determines the number of S-phase cells. The cells were seeded (40 000/well) in a 96-well plate in complete medium. After a 24 h incubation the medium was replaced with serum-free medium containing different physiological concentrations of GH (3.125–100 ng/ml). (The conversion factor for GH is 100 ng = 0.3 mIU.) BrdU (200 ml) was added to all the wells and incubated for a further period of 2 h. The medium was drawn off and retained for biochemical analysis, the cells were then fixed and a further incubation with a monoclonal anti-BrdU followed. The incorporation of BrdU by the cells was detected using a peroxidase-labelled anti-mouse IgG_{2a} substrate. The reaction was stopped and read spectrophotometrically at 410 nm.

2.8. ^{3}H-thymidine incorporation

HOB cells were seeded as above and grown in culture for 24 h, after which the medium was replaced with serum-free medium (SFM) to arrest cell growth. The medium was then changed and the cells incubated in the presence of GH at concentrations of 0–50 ng/ml (generously donated by Novo/Nordisk A/S Denmark). ^{3}H-thymidine (1 mCi/ml, Amersham) was added for a further 24 h incubation. After washing in DMEM containing cold thymidine (5 mg/ml), the cells were papain digested (1 ml/ml) and an aliquot was counted in the scintillation counter.

2.9. Assay for alkaline phosphatase

ALP production by the cells stimulated with GH was measured using the substrate p-nitrophenyl phosphate dissolved in diethanolamine buffer pH 9.8 (Diagnostica Merck) on a COBAS BIO centrifugal analyser.

3. Results

3.1. Hyaluronic acid/poly(acrylic acid) sponges

The rates of GH release from samples prepared using the three different HA/PAA blends were linear. Interestingly, those sponges with higher hyaluronic acid

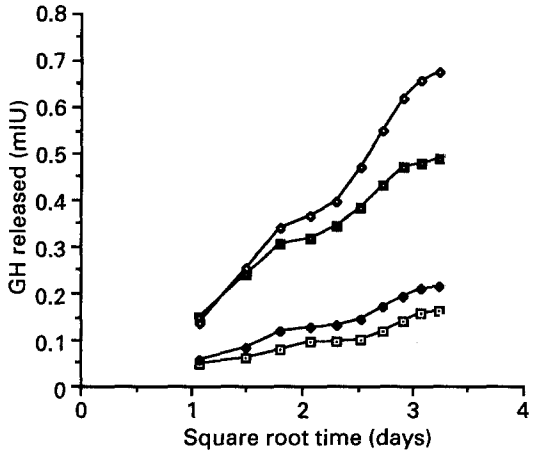
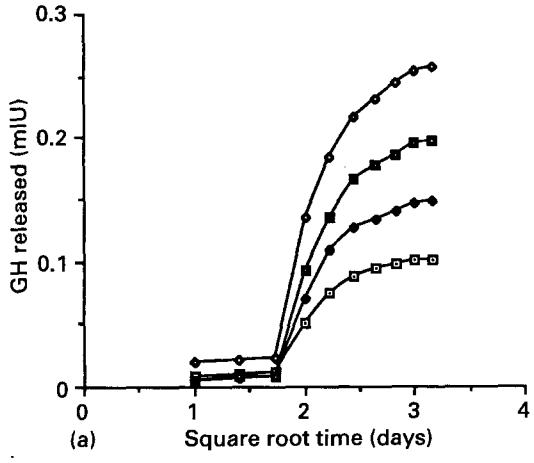


Figure 1 Release of GH from HA/PAA sponges (60/40) loaded with four different concentrations of GH (◇ 100 mIU; ■ 75 mIU; ◆ 50 mIU; ⊡ 25 mIU).

content but the same GH concentration released more GH, i.e. there was significantly more GH released from samples prepared using the 60/40 (HA/PAA) blend than the samples prepared using the 40/60 (HA/PAA) blend ($p < 0.05$–paired t-test) and in turn samples prepared using the 20/80 (HA/PAA) blend ($p < 0.05$–paired t-test). In addition, there was more GH released from the sponges with higher initial GH concentrations. Fig. 1 shows the release curves for GH from sponges with the same hyaluronic acid content (60%) but different GH concentrations.



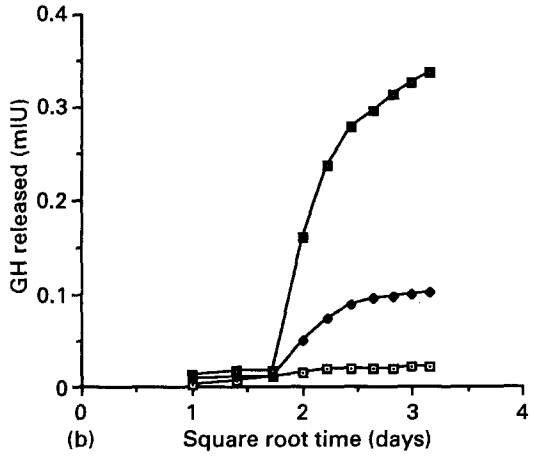


Figure 2 (a) Release of GH from C/PVA hydrogels (20/80) loaded with four different concentrations of GH (key as for Figure 1). (b) Release of GH from different C/PVA hydrogels loaded with 25 mIU GH (C/PVA = ■ 30/70; ◆ 20/80; ⊡ 10/90).

3.2. Collagen/poly(vinyl alcohol) hydrogels

GH was released from the C/PVA hydrogels with a slow lag phase during the first 3 days followed by a faster burst of GH release. The initial GH concentration did not affect the pattern of release but did, however, affect the total amount released. There was a direct relationship between the amount of GH incorporated in the hydrogels and the total amount of growth hormone released. Fig. 2a shows the release curves for GH from hydrogels with the same collagen content (20%) but different GH concentrations. It was also observed that increasing the collagen content of the hydrogels loaded with the same amount of GH (25 mIU), increased the total amount of GH released (Fig. 2b). Samples from the 30/70 blend released significantly more GH ($p < 0.05$, paired t-test) than samples from the 20/80 blend, which in turn, released significantly more GH ($p < 0.05$, paired t-test) than samples from the 10/90 blend.

3.3. Hyaluronic acid/poly(vinyl alcohol) hydrogels

During the first 3 days GH was released from the HA/PVA hydrogels in a constant linear manner which subsequently reached a plateau (Fig. 3). The 30/70 blend polymer released significantly more GH ($p < 0.05$, paired t-test) than the 20/80 blend and these in turn released significantly more GH ($p < 0.05$, paired t-test) than the 10/90 blend. The initial GH concentration did not affect the pattern of release but did affect the total amount released.

3.4. Effect of GH on HOB cell proliferation and ALP production

GH stimulated proliferation of the HOB cells in a dose-dependent manner. Maximal stimulation occurred between GH concentrations of 70 ng/ml and

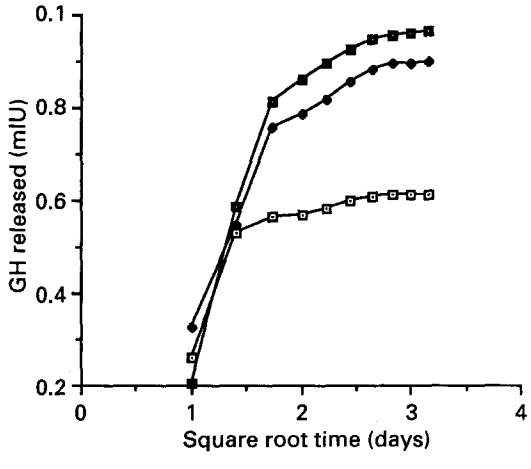


Figure 3 Release of GH from different HA/PVA hydrogels loaded with 25 mIU GH (HA/PVA = ■ 30/70; ◆ 20/80; ⊡ 10/90).

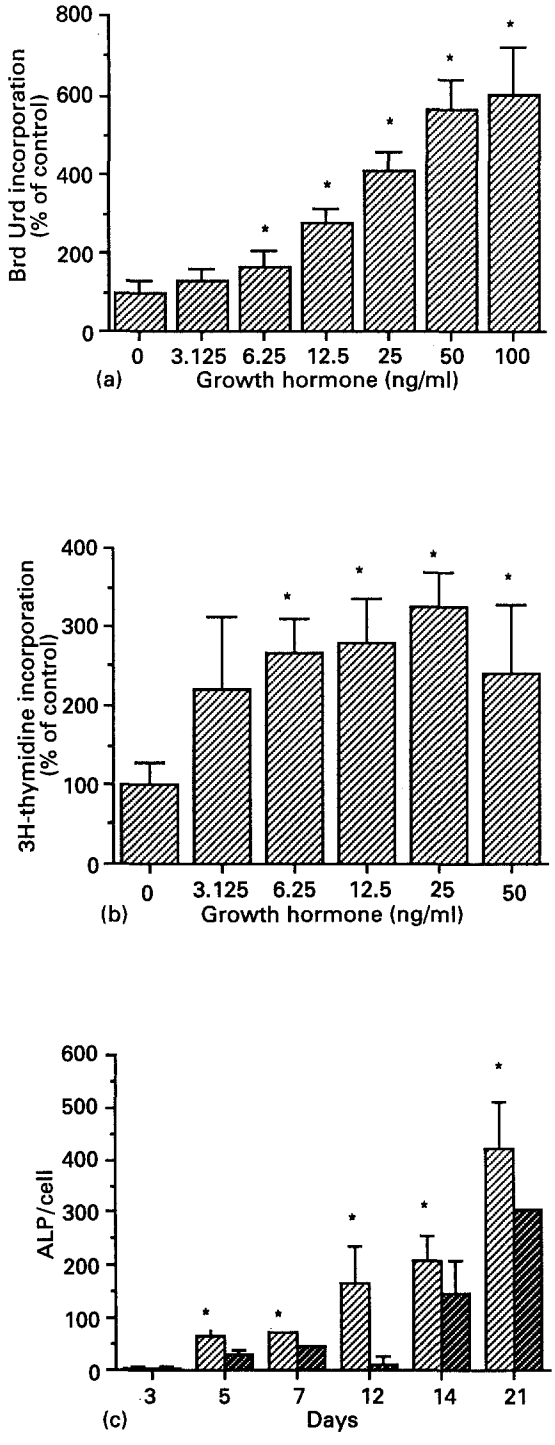


Figure 4 (a) Incorporation of BrdU in cellular DNA of HOB cells (results expressed as % control) stimulated with different doses of GH (*$p < 0.05$, significant). (b) Incorporation of ^{3}H-thymidine in HOB cells in response to different doses of GH (*$p < 0.05$, significant). (c) Production of ALP by HOB cells in the presence of GH (*$p < 0.05$, significant increase in ALP production) (▨ control; ▨ GH).

100 ng/ml as indicated by both the BrdU assay (Fig. 4a) and ^{3}H-thymidine incorporation (Fig. 4b). GH had a significant effect on the production of ALP at days 7 and 12 post-seeding ($p < 0.05$) (Fig. 4c).

4. Discussion

The development of controllable, long-term, effective, release systems for the delivery of growth hormone and other growth factors may improve wound healing and tissue repair in a variety of biomedical applications. Systemic treatment with GH has been shown to cause an increase in bone formation, and direct stimulation of chondrocytes [12–15]. The effects of GH have been shown to go beyond the control of longitudinal bone growth to the level of bone turnover [16–20]. Our results have indicated that HOB cells can be stimulated by GH in a dose-dependent manner, and at physiological dose ranges of the hormone, an increase was seen in the uptake of BrdU, ^{3}H-thymidine incorporation and the production of ALP in response to GH. The polymer systems we have investigated could be used to release GH and/or other growth factors directly to a target site, for a sufficient time period, in the appropriate concentration. We have shown that HA/PAA sponges proved to be an excellent delivery system for GH. They are able to release GH at physiological concentrations that can stimulate the proliferation of cells. The release of GH was linear with time and the GH released was directly related to the initial amount incorporated in the sponges. Examination of the scanning electron micrographs of both the surface and the internal structure of the sponges has shown porous structures composed of sheets of the polymers with interconnecting channels [21]. This type of structure may facilitate the transport of GH from the polymers into the surrounding environment as a result of an increased surface area. The hydrogels also proved to be useful for GH delivery. In C/PVA hydrogels there was an initial lag phase followed by a burst of GH release, whereas the HA/PVA hydrogels exhibited a rapid release followed by a plateau. Although HA/PVA hydrogels released more GH than C/PVA hydrogels, the amount of GH released from the latter was within a more physiological range. In addition all the blends of polymers studied indicated that by increasing the biological component (collagen or hyaluronic acid) it was possible to increase the amount of GH released, thus indicating that this could be used as a control mechanism. In conclusion, we have successfully combined natural and synthetic polymers to produce new systems for the delivery of GH and other growth factors in physiological quantities.

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