JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 6 (1995) 739–744

The growth of chondrocytes using Gelfoam® as a biodegradable scaffold

J. S. STANTON, V. SALIH, G. BENTLEY
Institute of Orthopaedics (IRC in Biomedical Materials), Royal National Orthopaedic Hospital, Stanmore, Middlesex HA7 4LP, UK

S. DOWNES
Department of Human Morphology, The Medical School, Queen's Medical Centre, Nottingham NG7 2UH, UK

Successful articular cartilage resurfacing must overcome several problems: the implant must easily fit the defect, it must be stable within the defect before full incorporation of repair tissue has occurred, and the reparative tissue must closely approximate the structure of normal hyaline cartilage. To this end, several natural and synthetic components have been used, both *in vivo* and *in vitro*, to provide a scaffold. These include isolated chondrocyte allografts, intact cartilage allografts, periossteal grafts, reconstructed collagen sponges, hydrogels and carbon fibres. However, promising results have been reported using three dimensional scaffolds in culture with isolated chondrocytes with subsequent implantation. This preliminary *in vitro* study utilizes Gelfoam® (a purified gelatin sponge) as such a scaffold. The biocompatibility of Gelfoam with both chondrocytes and osteoblast cells was first confirmed. The ability of chondrocytes to replicate and differentiate within Gelfoam scaffolds was assessed biochemically by measurement of the DNA content and glycosaminoglycans (GAG) production over 25 days in culture. The distribution of the cartilagenous matrix produced was observed by light microscopy, and the constituents of this matrix were assessed using specific antibodies and immunolocalization.

1. Introduction

Osteoarthritis, both primary and secondary, is a major cause of disability and morbidity in a wide age range of the population, although we have gone some way to an effective treatment of major joint arthritis in the older patient by way of total joint replacement. There remains no effective treatment for articular cartilage defects and lesions in the younger patient. Any cartilage repair tissue tends to undergo metaplasia to fibrocartilage [1] and since arthroplasty may only last five to ten years with subsequent revisions being less successful, a more conservative operative procedure would be of benefit.

One possible treatment involves the resurfacing of an articular cartilage defect, thereby preventing secondary osteoarthritis and allowing preservation of the rest of the joint. Successful resurfacing must overcome several problems: the implant must be easily adapted to fit the defect, it must be stable within the defect during the reparative process (a simple injection of chondrocytes into the defect would seem unsuitable [2, 3]), and the final reparative tissue must closely approximate the structure of normal hyaline cartilage. This is an active area of research using both natural components, such as isolated chondrocyte allografts [4], allografts of intact cartilage [5], fascial membranes and periosteal grafts [6], and synthetic materials such as carbon fibre patches [7], ceramics [8], ivalon sponges [9] and reconstructed collagen sponges [10]. These artificial materials are typically porous, relatively inert and act as scaffolds around and within which three-dimensional reparative tissue may form [11]. Although these materials address the initial problems of successful resurfacing, the ultimate repair tissue is mostly fibrocartilagenous in nature.

The synthetic approach has been taken further by culturing chondrocytes within porous scaffolds *in vitro* until the cells have synthesized extra-cellular matrix with the scaffold. The operative procedure would involve implanting this hybrid when sufficient cultured cartilage has been formed. As the chondrocytes expand *in vivo*, they need to maintain their phenotype which can be facilitated by the structural support of the scaffold, and with time, the neo-cartilage continues to grow and less support is needed. Thus a biodegradable scaffold would be the optimum system with the rate of degradation timed to coincide with the increasing 'self-support' of the new matrix. This approach has been performed experimentally but unfortunately implantation of these hybrids has often been into non-weight bearing parts of nude mice [12] using the host as little more than 'medium' to feed the chondrocytes within the scaffold.

0957–4530 © *1995 Chapman & Hall*

The materials used as the scaffold have varied from 'natural' synthetic materials such as collagen discs [13], collagen gel [14] and carbon fibre mesh [15] to manufactured polymers such as polyglycolic acid and polydiaxononc [16]. Chondrocytes have been applied to the materials in a variety of ways, using small volumes of high concentrations of cells and dispersing the cells within the materials by partial vacuum [13], by gentle agitation, by allowing cells to enter a compressed scaffold which is allowed to expand or by simple gravity [17]. Maintenance has been achieved either by frequent changes of medium or by continuous pulsed perfusion [16].

Gelfoam® (Upjohn Co., USA), manufactured from purified porcine skin gelatin, usually used as a haemostat in the fields of ENT and neurosurgery, has been used as a scaffold for culturing bovine pulmonary endothelial cells [18]. We have looked at its use as a biodegradable scaffold within which to culture chondrocytes with a view to future transplantation into cartilagenous defects.

2. Materials and methods

2.1. Insolation of chondrocytes

Hyaline cartilage was obtained from the proximal surface of bovine metacarpalphalangeal joints, washed in sterile phosphate buffered saline (PBS) before transferring to complete Dulbecco's modification of Eagle's medium (DMEM) with 20% fetal calf serum (FCS), incubated overnight, and examined for any evidence of infection. The cartilage was finely minced and sequentially digested using pronase, 700 U/ml (BDH, UK) and collagenase type II, 300 U/ml (Sigma, UK). The suspension was then filtered via a 70 μm nylon filter and centrifuged for 5 min. at 2000 rpm. The cell pellet was resuspended in fresh medium and washed twice more. Cell yield was obtained using a haemocytometer.

2.2. Assessment of biocompatibility

a) with bovine chondrocytes; i) A 4% agarose solution was mixed with an equal volume of complete DMEM containing bovine chondrocytes at a concentration of 4×10^6/ml, 1 ml of this mixture was added to each well of a 24 well plate. These plugs were cultured for five days in medium containing varying proportions of fresh DMEM and DMEM which had had gelfoam soaking in it overnight in order to allow any elutants to be washed out. Chondrocyte viability was assessed by cutting a thin strip from the centre of each plug and staining each strip with trypan blue. Stained and unstained cells were counted under phase contrast.

b) with human osteoblast cells; Human osteoblast (HOB) cells (derived from the trabecular bone of femoral heads obtained at surgery for total hip replacement) were cultured as a monolayer in the absence of Gelfoam and in the presence of Gelfoam. 50 μl of DMEM containing 3×10^5 HOB cells were added to ten 35 mm petri dishes, each containing 1 ml of complete DMEM. Five dishes had a 10 mm $\times$ 10 mm $\times$ 5 mm piece of Gelfoam added and were maintained for 14 days. The remaining dishes acted as negative controls. Sampling of media aliquots took place at days 2, 5, 7, 9, 12 and 14, and those dishes not sampled had medium changed at these intervals. After trypsinizing the monolayers, 50 μl aliquots of the cell suspension were taken for DNA analysis.

2.3. Construction of the Gelfoam/chondrocyte hybrid

With Agarose: Gelfoam was cut into approximately 7 mm^2 pieces and compressed in a petri dish filled with complete DMEM. The saturated pieces would then be transferred to a dry petri dish and once again compressed using forceps so that they were no longer saturated and had excess air bubbles removed. Each piece was then placed in the bottom of each well of a 24 well plate, and 2×10^6 cells in 50 μl of DMEM were pipetted dropwise onto the material which expanded to absorb the cell mixture. These were freshly isolated chondrocytes in order that no de-differentiation might take place [19]. The sponge/ chondrocyte suspension was then covered in 1 ml of 1% agarose and when this had set, 1 ml of complete DMEM was added. The agarose plugs were maintained at 37 °C in a humidified atmosphere of 5% carbon dioxide. The medium was changed regularly and the Gelfoam/chondrocyte hybrids were sampled for GAG production and DNA content at various time points up to day 25.

Without Agarose: The ability of Gelfoam to support chondrocytes without the additional support of agarose was assessed to confirm that any maintenance of phenotype and production of matrix components by chondrocytes maintained in culture (as described above) was not merely due to presence of agarose. The production of glycosaminoglycans (GAG) was measured under four different conditions. The test condition involved adding a 100 μl aliquot of DMEM containing 1×10^7 chondrocytes/ml to pieces of Gelfoam placed in a 96 well plate, then one hour later adding 100 μl of fresh complete DMEM as maintenance medium. The first control involved the same number of cells but suspended in a 100 μl suspension of 1: 1 complete DMEM and 2% agarose. This was added to the same sized piece of Gelfoam and maintained in the same way. The second control was similar to the first but excluding the use of Gelfoam, and the final control involved adding 100 μl of 2% agarose to Gelfoam, omitting the addition of any chondrocytes, then maintained in the same way. Sampling of the hybrids took place on days 5, 7, 10 and 14 and GAG production was measured.

2.4. Histological evaluation

Specimens for histology were fixed in 4% paraformaldehyde in 0.02 M phosphate buffer. Specimens were dehydrated through a graded series of ethanols, cleared in xylene and wax embedded. Sections (7 μm)

were cut on a Leitz microtome and mounted on slides. Sections were stained with Haematoxylin and Eosin and Safranin 'O'.

2.5. Immunolocalization

Specimens for immunolocalization were mounted on cork discs in cryomountant (Cryo-M-Bed, Brights Instrument Co.) and frozen by immersion in liquid nitrogen. Cryostat sections (10 μm) were cut and mounted on slides, then stored at − 20° C. The frozen sections were examined for the following matrix components: chondroitin-4-sulphate, chondroitin-6-sulphate, keratan sulphate, and collagen II.

The sections were hydrated into 100 mM Tris buffer pH 7.2 containing 150 mM NaCl and 0.5% bovine serum albumin (Sigma, UK). Sections examined for the presence of proteoglycans were digested with chondroitinase ABC (0.5 I.U./ml, Sigma, UK). Sections examined for the presence of collagen II were treated with chondroitinase and hyaluronidase (3 I.U./ml). Sections were washed three times in Tris buffer between steps. Specific primary monoclonal antibodies were then added to each section (one per section), and incubated in a humidified atmosphere. After washing, a fluoroscein-conjugated rabbit anti-murine serum (F261, Dako. Ltd., dilution 1:30) was applied. Positive controls consisted of frozen sections of both human and bovine cartilage, and negative control consisted of omissions of the primary antibody with subsequent use of non-immune mouse serum. Sections were then mounted under glass cover slips in glycerol/PBS containing 1,4-didiazobicyclo [2.2.2.] octane, (DABCO, 25 mg/ml, Sigma, UK) to retard photobleaching then stored in a darkened environment.

2.6. Primary monoclonal antibody details

Collagen type II. Primary monoclonal antibody CIICI (Developmental Studies Hybridoma Bank, University of Iowa, USA). Chondroitin-4-sulphate (2-B-6) and chondroitin-6-sulphate (3-B-3). Primary monoclonal antibodies 2-B-6 and 3-B-3 (Kennedy Institute of Rheumatology, London U.K.). Keratan sulphate. Primary monoclonal antibody 5D4 (ICN Flow). Non-immune mouse serum. NIS (Developmental Studies Hybridoma Bank, University of Iowa, USA).

2.7. Biochemistry

2.7.1. Measurement of Glycosaminoglycans (GAG) production

The GAG content was quantified using a modification of the DMMB (Dimethylmethylene blue) method of Farndale *et al.* [20]. The maintenance medium was removed and excess agarose was trimmed off the Gelfoam/chondrocyte hybrid so as to only measure the GAG produced by chondrocytes within the Gelfoam and not from those cells suspended in the surrounding agarose. Each agarose plug was treated with 1 ml of papain digest buffer for 1 h prior to digestion with 20 μl agarose (Sigma, UK, 1000 I.U./ml), and 5 μl papain (Sigma, UK, 20 I.U./mg protein) at 70 °C overnight. 40 μl of digest were then added to individual wells of a 96 well plate followed by 250 μl of DMMB solution. Absorbance was then measured at 595 nm using a Bio-Rad microplate reader (3550).

2.7.2. Measurement of DNA content

DNA content was measured using a fluorimetric method. Using the contents prepared for GAG measurement, 100 μl samples were added to 400 μl 0.01 M NaCl, 100 μl 0.04 M Tris-HCl buffer and 100 μl of 4'6-diamidino-2-phenylindole.2HCl (DAPI). The fluorescence was then measured at 460 nm (Perkin-Elmer fluorimeter, LS-2B), and the DNA content calculated by comparison with the fluorescence of known standard concentrations of DNA.

3. Results

3.1. Biocompatibility

Gelfoam was found to be biocompatible with both chondrocytes and human osteoblast cells. Substances which may have eluted from Gelfoam appeared to have no effect on the viability of the chondrocytes after five days in culture (Table I). The presence of Gelfoam appeared to exhibit no inhibitory effect on the expansion of human osteoblast cells in monolayer culture (Fig. 1).

3.2. Culture of Gelfoam/Chondrocyte/Agarose Hybrids

There appeared to be minimal degradation of the Gelfoam after four weeks in culture, either macro- or microscopically. Following seeding, there was a good random distribution of cells throughout the Gelfoam scaffold, and by day seven there were isolated cells throughout the scaffold dividing and producing matrix as determined by safranin 'O' staining. After this stage, the majority of the cellular activity was confined to the upper surface layer, with islands of tissue observed by day 18 (Fig. 2) – there was abundant matrix with multiple lacunae containing well-differentiated chondrocytes. On H&E staining at this

TABLE I The influence on cell viability of increasing concentrations of DMEM containing elutant from Gelfoam used to maintain chondrocyte/agarose hybrids in culture for five days as measured by trypan blue exclusion.

% Fresh DMEM/ % DMEM + elutant	% Viability (± SD)
100/0	91.1 (± 0.95)
80/20	91.6 (± 0.76)
60/40	92.4 (± 2.90)
40/60	96.2 (± 2.86)
20/80	94.7 (± 3.57)
0/100	91.2 (± 1.35)

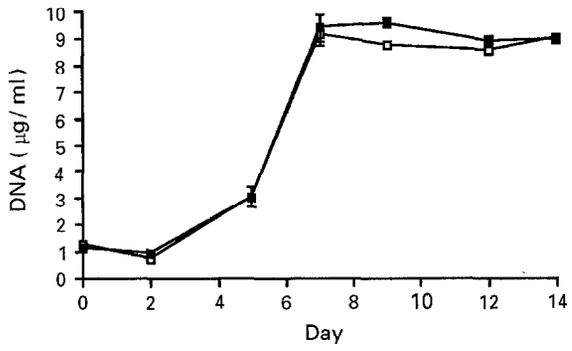

Figure 1 The effect of Gelfoam on monolayer culture of human osteoblast cells assessed by total DNA content. —■—: DNA – G; —□—: DNA + G.

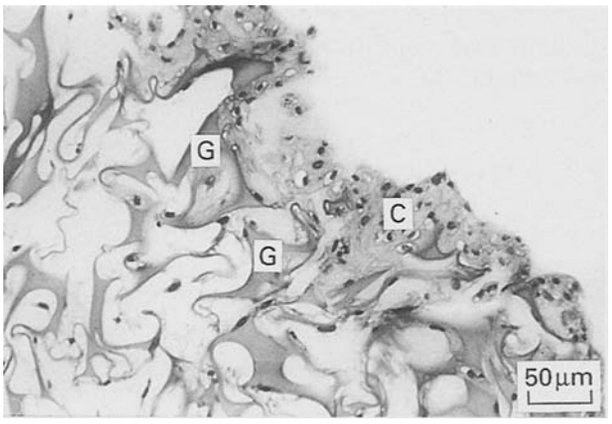

Figure 3 Chondrocytes showing rounded morphology at edge of Gelfoam scaffold after 18 days. C = cells; G = Gelfoam. HE stain.

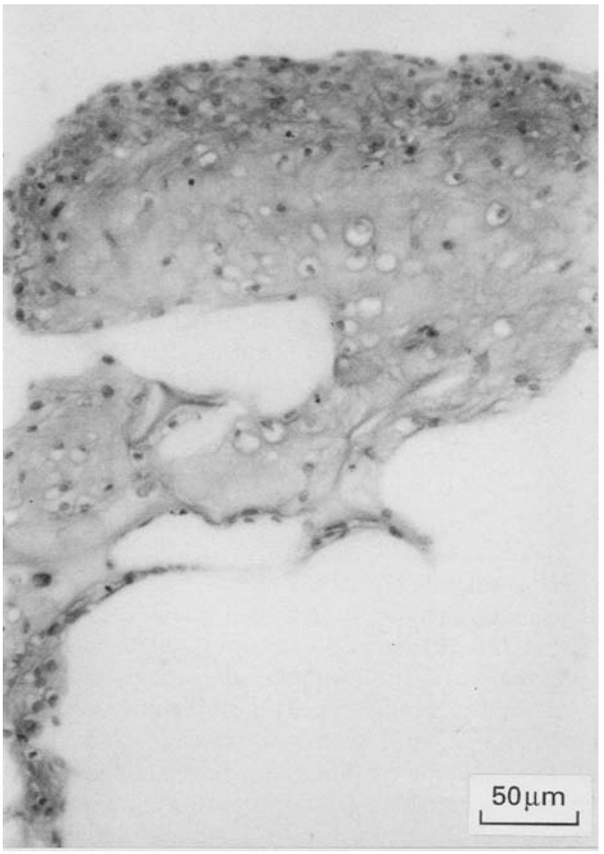

Figure 2 Chondrocyte 'island' at edge of Gelfoam scaffold showing extensive matrix after 18 days. Safranin 'O' stain.

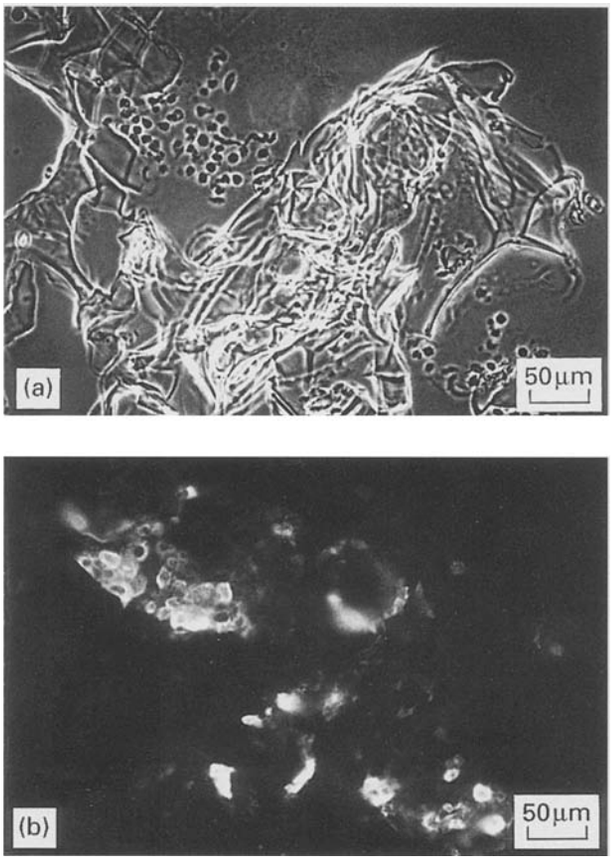

Figure 4 Immunolocalization of collagen II in chondrocytes cultured on Gelfoam after 12 days: (a) phase contrast; (b) immunofluorescence. (Scale bar = 50 µm).

stage, there were still chondrocytes visible throughout the matrix, but the cells in the middle of the matrix appeared less rounded than those at the surface (Fig. 3).

Collagen II was present by day 12 in a peri-cellular position (Fig. 4). The proteoglycans chondroitin-6-sulphate and keratan sulphate were seen in abundance, their distribution following the strands of Gelfoam, rather than being confined to a peri-cellular position and being more abundant in the surface layers. Chondroitin-4-sulphate was less abundant though similarly distributed. Positive controls showed staining for all antigens, negative controls did not show fluorescence.

There was a steady rise in GAG production from time zero (Fig. 5) – there is an apparent plateauing of GAG production at about day 16. The DNA content at day one was approximately the same as that contained by one aliquot of seeding medium plus that of a one sixth piece of 12-7 Gelfoam. This indicates that the pieces of Gelfoam absorb almost 100% of the cells seeded on them, in contrast to other work [15]. The DNA content rises fairly steadily until it plateaus at about day 12 (Fig. 6), with a DNA content double (6–7 µg/ml) that at time zero (3 µg/ml), indicating that the chondrocytes exhibited the expected low rate of cell division.

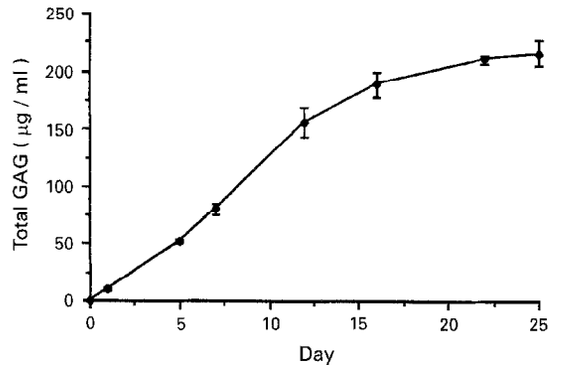

Figure 5 Total glycosaminoglycans production by Gelfoam/Chondrocyte/Agarose hybrids cultured *in vitro* over 25 days (error bars = ± SE, $n = 6$).

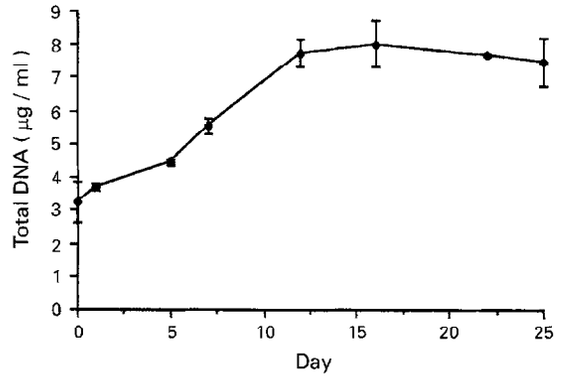

Figure 6 Total DNA content of Gelfoam/Chondrocyte/Agarose hybrids cultured *in vitro* over 25 days (error bars = ± SE, $n = 6$).

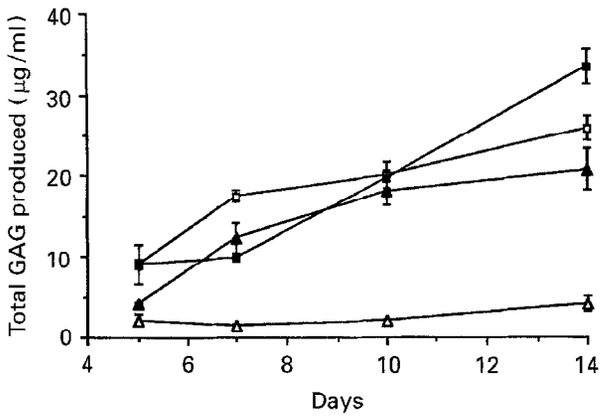

Figure 7 Total glycosaminoglycans production by bovine chondrocytes under different culture conditions (error bars = ± SE, $n = 6$). —□—: Test – Gelfoam + cells; —▲—: Control 1 – Gelfoam + agar + cells; —■—: Control 2 – Agar + cells; —△—: Control 3 – Gelfoam + agar.

3.3. Culture of Gelfoam/Chondrocyte Hybrids in the absence of agarose

There was very similar total GAG production by the bovine chondrocytes under different test conditions (Fig. 7) – this indicates that in the absence of agarose, Gelfoam supports the culturing of chondrocytes with formation of matrix components. Safranin 'O' staining showed that the absence of agarose had little effect on the distribution of chondrocytes within the Gelfoam scaffold, cartilage matrix being produced once again mainly on the surface layers.

4. Discussion

The method used to seed chondrocytes within the Gelfoam produced initial uniform seeding throughout the sponge, almost all chondrocytes being absorbed as indicated by DNA measurement. During subsequent culture, cells in the periphery of the cell/sponge hybrid retained a rounded morphology as well as those towards the centre. However, this was seen under both culture conditions i.e. in both the presence and absence of surrounding agarose, indicating that maintenance of chondrocytic phenotype was not merely due to agarose suspension culture at the periphery of the hybrid. Positive staining for cartilage matrix by Safranin 'O' was seen from day seven, somewhat earlier than when collagen discs were used for culture [13]. The absence of an agarose supporting layer may have lead to a washing out of matrix components during medium changes, but this was not found to be the case, and histological staining under both our culture conditions were very similar, with subsequent GAG production also showing no significant difference.

GAG production by chondrocytes cultured in Gelfoam correspond to results from other authors [13], the plateauing of GAG production occurring at day 16 being due to a reduction in total GAG formation. In our study however, even after day 16 there remains a steady slow increase in GAG within the hybrid, indicating that cartilage matrix is still being formed. Correlating this with the immunolocalization studies, the majority of the GAG produced appears to be in the surface layers of the hybrid. The best indicator of hyaline cartilage matrix production, collagen II, was found to be present in abundance in the surface layers by day 12, far earlier than when polyglactin is used as the scaffold.

In its clinical use as a haemostat, Gelfoam degrades in the matter of a few days presumably under the effect of enzymes present within blood. In our culture experiments there was minimal degradation (by naked eye and microscopic inspection) of Gelfoam after four weeks. This is important as the time for transplanting a Gelfoam/chondrocyte hybrid into a cartilage defect may be after two to three weeks in culture, and a stable scaffold is required so that the hybrid may maintain its position during integration in the tissue. We simulated the intra-articular environment by immersing pieces of Gelfoam in bovine synovial fluid, and again, degradation was only slightly more pronounced. Transplanting a Gelfoam hybrid into a full thickness cartilage defect would bring the Gelfoam into contact with both blood and synovial fluid components, thus hastening its degradation, and encouraging intrinsic cartilage repair [21].

We believe that in order to resurface joints with cultured chondrocytes we need a stable scaffold enabling chondrocytes to maintain their differentiated state, which then biodegrades preventing any subsequent inflammatory reaction. Gelfoam supports the culture of differentiated chondrocytes and is biocompatible with human osteoblast cells. Further, Gelfoam is biodegradable in the clinical setting but shows no sign of degrading *in vitro*, thus making implantation easier.

References

1. G. BENTLEY, *Critical Reviews in Biocompatibility* **5** (1989) 245.
2. P. D. BENYA, in "Methods in Cartilage Research", (Academic Press, 1990) p. 85.
3. M. BRITTBERG, A. LINDAHL, A. NILSSON, C. OHLSSON, O. ISAKSSON and L. PETERSON, *N. Engl. J. Med.* **331** (1994) 889.
4. M. CENTRA, R. E. RATYCH, G. L. CAO, J. LI, E. WILLIAMS, R. M. TAYLOR and M. ROSEN, *FASEB J.* **6** (1992) 3117.
5. M. C. COBEY, *Clin. Orthop.* **54** (1967) 139.
6. S. DOWNES, R. S. ARCHER and M. V. KAYSER, *J. Mater. Sci.* **5** (1994) 88.
7. R. W. FARNDALE, C. A. SAYERS and A. J. BARRETT, *Conn. Tiss. Res.* **9** (1982) 247.
8. L. E. FREED, J. C. MARQUIS, A. NOHRIA, J. EMMANUEL, A. G. MIKOS and R. LANGER, *J. Biomed. Mat. Res.* **27** (1993) 11.
9. D. A. GRANDE, M. I. PITMAN, L. PETERSON, D. MENCHE and M. KLEIN, *J. Orthop. Res.* **7** (1989) 208.
10. T. W. GREEN, *Clin. Orthop. Rel. Res.* **124** (1977) 237.
11. S. F. HULBERT and J. J. KLAWITTER, *Mater. Res. Bull.* **7** (1972) 1239.
12. A. K. JEFFREY, G. W. BLUNN, C. W. ARCHER and G. BENTLEY, *J. Bone. Joint. Surg.* **73B** (1991) 795.
13. H. J. MANKIN, *N. Engl. J. Med.* **331** (1994) 940.
14. R. J. MINNS, D. S. MUCKLE and J. A. BETTS, *Orthop. Int. Ed.* **1** (1993) 414.
15. A. J. NIXON, A. E. SAMS, G. LUST, D. GRANDE and H. O. MOHAMMED, *Amer. J. Vet. Res.* **54** (1993) 349.
16. D. ROBINSON, M. EFRAT, D. MENDES, N. HALPERIN and Z. NEVO, *Bull. Hosp. Joint Dis.* **53** (1993) 75.
17. J. M. RUBAK, *Acta Orthop. Scand.* **53** (1982) 175.
18. M. SITTINGER, J. BUJIA, W. W. MINUTH, C. HAMMER and G. R. BURMESTER, *Biomaterials* **15** (1994) 451.
19. D. P. SPEER, M. CHVAPIL, R. G. VOLZRG and M. D. HOLMES, *Clin. Orthop. and Re. Res.* **144** (1979) 326.
20. C. A. VACANTI, R. LANGER, B. SCHLOO and J. P. VACANTI, *Plast. Reconstr. Surg.* **88** (1991) 753.
21. S. WAKITANI, T. KIMURA, A. HIROOKA, T. OCHI, M. YONDEA, N. YASUI, H. OWAKI and K. ONO, *J. Bone Joint. Surg.* **71B** (1989) 74.

Received 29 June
and accepted 4 July 1995