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BIOMIMETIC HABITATS FOR CELLS: ORDERED MATRIX DEPOSITION AND DIFFERENTIATION IN GINGIVAL FIBROBLASTS CULTURED ON HYDROXYAPATITE COATED WITH A COLLAGEN ANALOGUE

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Abstract

In tissues, cells attach and migrate on collagen. Interaction with collagen modulates cell proliferation and differentiation. We propose that similar environments may be constructed by immobilizing collagen-derived cell-binding peptides in a three-dimensional (3-D) template. We identified a cell binding domain of type I collagen and showed that a 15-residue synthetic analogue (P-15) binds cells with high affinity. In the present study, we compared the behavior of human gingival fibroblasts (HGF) in culture on hydroxyapatite (HA) and on HA-P-15. HGF cultured on HA formed sparse monolayers with little order and deposited a scant matrix. HGF on HA-P-15 formed highly oriented 3-D colonies and deposited copious amounts of fibrous and membranous matrix. Northern analysis showed increased expression of osteogenic markers type I collagen, alkaline phosphatase, osteonectin and transforming growth factor β-1 on HA-P-15. Alizarin red stained the matrix of HGF cultures on HA-P-15 suggesting neo-mineralization. Gingival fibroblasts are a source of reparative cells in periodontal tissue repair. Our studies support this concept and suggest that HA-P-15 matrices may be effective as endosseous grafts. When seeded with appropriate cells HA-P-15 matrices may serve as tissue engineered bone substitutes.

Key Words: Biomimetic environments, collagen analogue, cell-binding peptide, P-15, hydroxyapatite-P-15 complex, gingival fibroblasts, differentiation, three-dimensional colony formation, ordered matrix deposition.

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Introduction

Cell migration and attachment requires scaffolds. In the body cells attach to and migrate on type I collagen and exist in three-dimensional (3-D) compartments formed by collagen and other cells. The flow of chemical and mechanical information between the extracellular matrix (ECM) and cells and between adjacent cells contributes to differentiation (Hay, 1989). Inert scaffolds cannot exchange information with cells via physiological pathways of communication. Our hypothesis is that incorporating the cell-binding activity of type I collagen in a 3-D distribution in bioceramic scaffolds or in polymeric carrier matrices will facilitate the attachment, migration, and differentiation of bone-forming cells. Such a matrix may be expected to serve as a hospitable habitat for cells for tissue engineering and when used in endosseous implants, it may serves as a bioactive scaffold.

We discovered a potent cell binding domain in type I collagen, located in the sequence ^{766}GTPGPQGIAGQRGVV^{780} in the α1(I) chain (Scaria *et al.*, 1989; Qian and Bhatnagar, 1996; Bhatnagar *et al.*, 1997). A synthetic peptide P-15 analogous to this sequence promoted the attachment and proliferation of human dermal and periodontal ligament fibroblasts on natural and synthetic hydroxyapatite (HA) particles and polyglycolide fibers, and allowed cell migration in the lattice of otherwise inhospitable agarose hydrogels (Qian and Bhatnagar, 1996; Bhatnagar *et al.*, 1998, 1999). In previous studies, we observed remarkable phenotypic changes in human dermal fibroblasts grown on bovine bone HA on which P-15 is adsorbed (Qian and Bhatnagar, 1996). In this culture system, human dermal fibroblasts assumed an osteoblast like morphology, deposited copious matrix and stained for alkaline phosphatase. These observations supported the role of P-15 in promoting the binding and migration of cells and suggested that cells may thrive in this environment.

Human gingival fibroblasts include cell populations capable of generating reparative matrices in the periodontium (Pitaru *et al.*, 1994), a composite tissue com-

prised of bone and ligamentous structures. Gingival fibroblasts in culture have been shown to form organized matrices and to orient themselves in structures reminiscent of the dentogingival and transseptal fibers observed *in vivo* (Pitaru and Melcher, 1983, 1987). The attachment of gingival fibroblasts to surfaces *in vitro* is increased by the presence of type I collagen (Giannopoulou and Cimasoni, 1996). Coating of substrates with collagen promotes organized growth of gingival fibroblasts (Lowenberg *et al.*, 1985). In the studies reported here, we have used human gingival fibroblasts as a model to investigate the ability of P-15 to generate biomimetic environments.

Materials and Methods

Peptide synthesis

The peptide P-15, GTPGPQGIAGQRGVV was synthesized by solid phase procedures (Zeng *et al.*, 1997) using 9-fluorenylmethoxycarbonyl protecting groups, except for Gln residues which were coupled with 1-hydroxybenzyl triazole. The peptide was purified to by reverse phase high precision liquid chromatography (HPLC) using a C-18 column in a gradient of acetonitrile and H_2O. The purity of the peptide used in these studies was > 95%. The amino acid sequence was confirmed by sequence analysis.

Hydroxyapatite (HA)

Bovine bone derived porous HA in a particulate form with a particle size of 250-420 μm was obtained from CeraMed Corp. (Lakewood, CO). The HA had a mean pore volume of 0.13 cc/g and a total porosity of 28% based on mercury porosimetery. This preparation of bone derived HA was used because of the manufacturer's certification that deproteination was complete based on Kjeldahl and carbon analyses (ASTM D4129). The purity of HA was further warranted by X-ray diffraction standard (JCPDS 9-432). Microanalytical procedures used in our laboratory confirmed the absence of nitrogenous materials in the HA preparation.

Preparation of hydroxyapatite-P-15 (HA-P-15) complex

Details of preparation and analysis of HA-P-15 composites have been presented previously (Qian and Bhatnagar, 1996). P-15 is adsorbed on HA in a saturable manner. Maximal adsorption of the peptide occurred when particulate HA was incubated for 24 hours in 2X volume of a solution containing 100 $\mu g\ ml^{-1}$ P-15 in phosphate buffered saline (PBS). Incubation was carried out at room temperature with gentle shaking to ensure equilibration of the peptide with all exposed surfaces of the microporous HA. Unadsorbed peptide was removed from the particles by washing in 5X volume of PBS by gently shaking and decanting over a 24 hour period. The HA-P-15 composite was dried *in vacuo* over Drierite (W.A. Hammond Drierite Co., Xenia, OH) and stored at room temperature in moisture-proof containers. HA and HA-P-15 were sterilized under ultraviolet light for cell culture experiments. Analyses for adsorbed peptide were made on the sterilized preparations. In experiments to examine the desorption of P-15, it was found that the complex between P-15 and HA is stable under physiological conditions. Significant amounts of the peptide were eluted only at pH < 4.5 concomitant with the dissolution of the mineral. Between 10-25% of the adsorbed peptide was eluted at pH > 10 in NaOH or in 0.1 M sodium borate buffer, pH 10.8. These conditions are not relevant to physiological systems and, in the present studies, it was assumed that little peptide would be eluted under culture conditions.

Assay for peptide adsorption

The amount of peptide adsorbed on HA was determined by hydrolyzing the HA-associated peptide with 2.0 N NaOH, for 2 hours at 100°C and assaying the amino acid content of the hydrolyzate with the fluorophor o-phthalaldehyde (Jones *et al.*, 1981). This method is specific for amino groups and allows the measurement of picomolar quantities of amino acids. Fluorescence measurements were made in a SPEX (Metuchen, NJ) Fluorolog II spectrofluorometer using excitation at 340 nm and measuring emission at 455 nm. The procedure used in these assays allowed us to measure as little as 1.0 ng (0.72 pmole) P-15 reproducibly with a confidence level of $\pm$ 5% in standard solutions in buffers. All determinations were corrected for background levels of o-phthalaldehyde reactive material in HA which were less than 0.5 pmole o-phthalaldehyde-reactive material/g HA.

Tissue culture

All media and supplies for tissue culture were obtained from University of California San Francisco (UCSF) Tissue Culture Facility. Primary cultures of human gingival fibroblasts were prepared from healthy tissue samples obtained from UCSF Oral Surgery Clinics. The tissues had been excised as part of unrelated clinical procedures. The cultures were maintained in Eagle's minimum essential medium with Earle's salts supplemented with 10% fetal bovine serum. All cells used in these experiments were in the 5-7th passage and were dissociated from confluent cultures.

Fibroblast culture on HA and HA-P-15

In order to promote cell binding to HA, cultures were prepared in silanized glass dishes prepared by coating acid cleaned 60 mm glass petri dishes with Sigma-

cote (Sigma Chemical Co., St. Louis, MO). Except where indicated, 200 mg of untreated HA or HA-P-15 was placed in the dish and covered with 5 ml growth medium containing 5.0 x 10^4 fibroblasts. Microscopic examination revealed that few cells attached to the surface of the silanized glass vessels. The medium and non-adherent cells were aspirated from the cultures after 48 hours and replaced with 5 ml of growth medium. In long term cultures, the medium was changed every three days.

Scanning electron microscopy (SEM)

The cultures were fixed for 1 hour in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2. The samples were washed twice, 10 minutes each time, in cacodylate buffer. The cells were then fixed for 1 hour in 1% osmium tetroxide in sodium cacodylate, and rinsed again in the buffer. Dehydration was accomplished using a graded series of ethanol (30%, 50%, 70%, 95% and 100%). The cells were dried in a Polaron critical point dryer (Electron Microscopy Sciences, Fort Washington, PA) using CO_2. The particulate cultures were mounted on aluminum stubs with two-sided adhesive tape and coated with gold/palladium using a Hummer VII (Anatech Limited, Alexandria, VA) sputter coater. The samples were viewed with a Philips XL-40 scanning electron microscope (Philips Electron Optics, Eindhoven, The Netherlands).

Gene expression

The particulate cultures were placed in microcentrifuge tubes and the cells were lysed with 1.0 ml of Ultrospec (Biochrom Ltd, Cambridge, UK) cell lysing solution for the extraction of informational macromolecules. RNA concentration was determined using the ratio of absorption at 260 nm and 280 nm. Ten μg RNA from each sample was electrophoresed on denaturing 1.3% agarose gels in the presence of formaldehyde. After electrophoresis, the RNA was blotted overnight to positively charged nylon membranes by capillary transfer in 10 x saline sodium citrate (SSC; 3.0 M NaCl, 0.3 M Na citrate, pH 7.0). RNA was fixed to the membrane by baking in an oven for 1 hour at 80°C and then prehybridized for 2 hours in a prehybridization solution (5x SSC, 0.02% sodium dodecyl sulfate, 0.1% N-lauryl sarcosine, 2% blocking reagent, 20 mM Na maleate, pH 7.5). Hybridization was performed at 42°C in the same solution for 18 hours using the following human cDNA probes: (1) transforming growth factor-β (TGF-β), 2.1 kb EcoR1 fragment of human phTGF β-1; (2) type I collagen, 1.8 kb EcoRI fragment of human COL1A1; (3) osteonectin, 1.2 kb EcoR1 fragment of human SPARC. The cDNA probes were labeled with digoxigenin (DIG-11-dUTP) by random priming according to the recommendations of the manufacturer of the kit (Boehringer Mannheim/Roche, Indianapolis, IN). The filters were washed initially twice, for 5 minutes each, at room temperature with 2X wash solution (2X SSC, 0.1% sodium dodecyl sulfate), and finally washed for 30 minutes at 65°C with 0.5X wash solution (0.5X SSC, 0.1% sodium dodecyl sulfate). The hybridization signals were visualized by fluorography. For normalization, comparison was made with ethidium bromide stained bands for 28s and 18s RNAs after initial electrophoresis.

Staining with Alizarin Red for mineralization

Red staining with Alizarin Red is an indicator of mineralization (Keila *et al.*, 1994; Bodine *et al.*, 1997). HGF cultures on HA and HA-P-15 were prepared for staining by removing the medium, washing the cells with PBS, and fixing in 80% ethanol for 5 minutes following which the cells were gently washed with H_2O. The cells were stained in a solution of 1% Alizarin Red S, pH 6.4 for 2 hours. Unreacted stain was removed by destaining in 95% ethanol + 5% concentrated HCl for 10 minutes. The samples were air dried, and were examined and photographed in a light microscope.

Results

Figure 1 shows the growth patterns of HGF on HA (Fig. 1A) and on HA-P-15 (Fig. 1B). Cells were allowed to grow on HA and HA-P-15 particles for 10 days and examined by SEM to determine if there were any morphological differences in cultures grown on the two matrices. HGF grew in dehiscent monolayers on HA and generally formed arrays which were void of order. Most cells appeared to have a flattened and quite angular morphology. Even after 10 days in culture, the number of cells growing on HA was not adequate fully to cover the particle surface. In contrast, HGF growing on HA-P-15 fully covered the surface and appeared to form highly adherent layers. HGF growing on HA-P-15 formed ordered parallel arrays of elongated cells. These differences are much more obvious at a higher magnification (Fig. 2). As seen in Figure 2A, at 10 days, HGF cultures on HA are sparse, and the granular surface of the bone-derived HA is seen clearly. The monolayer of cells appeared to dehisce easily from the surface of HA. In this field, many of the cells appeared to grow in parallel arrays. This is consistent with previous observations on HGF (Pitaru and Melcher, 1983, 1987). In contrast to the monolayers seen on HA, HGF grown on HA-P-15 for 10 days (Fig. 2B) formed a thick multilayered colony in which the layers appeared to be interspersed with an abundant fibrous matrix. The fracture of the colony during preparation for SEM provided an opportunity to inspect the interior of the

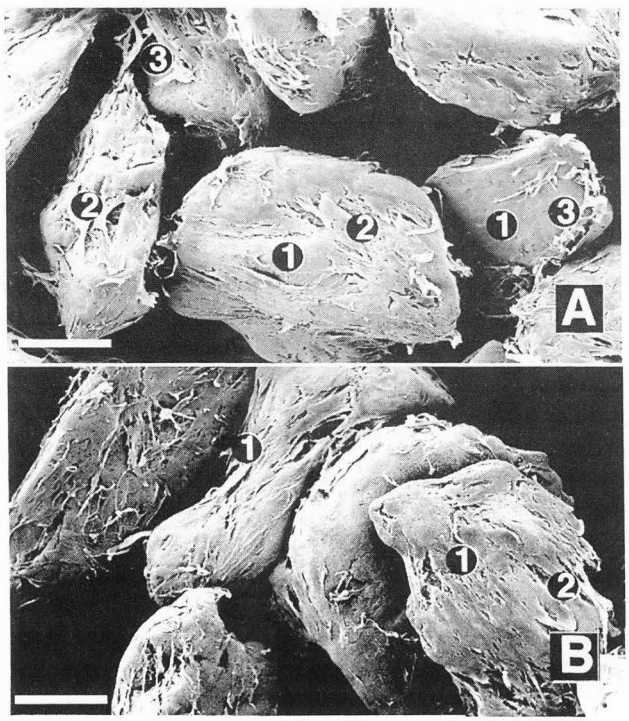

Figure 1. Scanning electron micrographs of human gingival fibroblasts on HA (**A**) and on HA-P-15 (**B**) after 10 days in culture. Many bare areas (**1**) seen on HA particles may have resulted from dehiscing cell layers (**3**). Cultures on HA formed monolayers of irregular shaped cells (**2**). In contrast, HGF formed dense colonies of elongated cells, forming parallel arrays (**1**) on HA-P-15. No bare spots were observed on HA-P-15, and the cell layers appeared to be firmly attached. Viewed from an edge the colonies appeared to be multilayered (**2**). Bars = 225 μm.

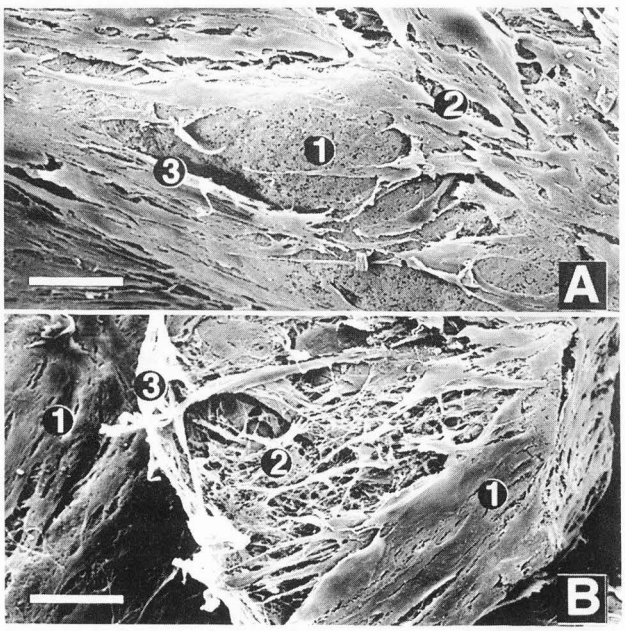

Figure 2. Scanning electron micrographs showing the structure of HGF colonies on HA (**A**) and on HA-P-15 (**B**) of 10 day cultures. Higher magnification showed the differences between HA and HA-P-15 cultures more clearly. (**A**). The bare surface (**1**) of porous bovine HA is clearly seen in the control. Most of the cells on HA formed monolayers of irregular shaped cells (**2**). A dehiscent monolayer is seen at (**3**). Bar = 75 μm. (**B**). HGF cultured on HA-P-15 displayed elongated and well oriented cells (**1**) which formed dense multilayered colonies. Cell layers of cells appeared to be interspersed with fibrous matrix (**2**) some of which appeared to form membranous structures (**3**). Bar = 90 μm.

cellular mass. The elongated, parallel arrangement of cells and the copious matrix are clearly seen.

An interior view of the mass of cells and matrix in 10 day culture of HGF on HA-P-15 showed interspersed layers of matrix and cells (Fig. 3A). The presence of a thick felt of fibrous matrix between cell layers appeared to be a common feature in most fields examined. The field displayed in Figure 3A was selected to show the relationship of matrix to cells. A large number of the matrix fibers seemed to emerge from filopodia. These structures formed connections with the underlying cable like matrix and HA-P-15 surface (Fig. 3B). Much of the matrix appeared to form stretched cables.

Although very little matrix was observed in HGF cultures on HA at 10 days, fibrous matrix was seen in many fields after 22 days in culture (Fig. 4A). At this time point, HA cultures also displayed some areas with stacking of cells, although the cells continued to exist in flattened, highly spread forms. This is similar to the observations with the 10 day cultures on HA-P-15. In contrast, 22 day cultures of HGF on HA-P-15 formed a voluminous 3-D matrix with many aspects (Fig. 4B). Both fibrous and membranous matrices were observed.

It is interesting to observe the formation of membrane-like matrix in HGF cultures on HA-P-15 (Fig. 5). These cultures were maintained for 10 days, a period during which very little matrix was obvious in control cultures on uncoated HA. Figures 5A, 5B and 5C, at successively higher magnifications, display the membrane-like character of matrix formed in a field generated by cells that pulled two HA particles into close contact by tractional forces. The fibers in this matrix appear to be aligned mainly in parallel arrays and also appear to adhere with one another forming sheet-like structures (Fig. 5A). At a higher magnifi-

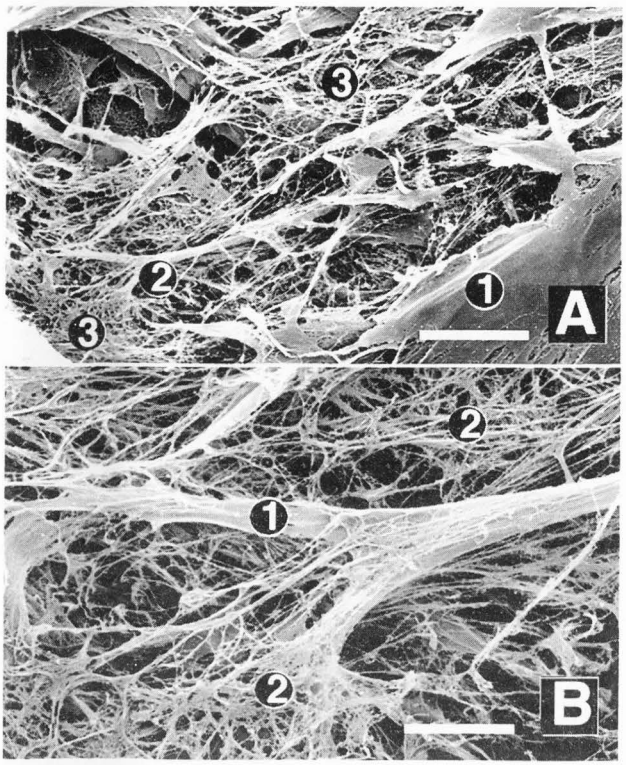

Figure 3. Scanning electron micrographs showing the relationship of collagenous matrix to cells in HGF cultures on HA-P-15 in 10 day cultures. (**A**). Parallel arrays of cells (**1**) in the outermost layer; filopodia (**2**) in layers close to the HA surface appear to be connected with matrix (**3**). Bar = 50 μm. This relationship is more clear in (**B**), a high magnification view of the central part of the field in (**A**). Bar = 12 μm. Matrix appears to be arising from the filopodia (**1**). The matrix is present in the form of highly stretched cables (**2**).

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cation, Figure 5B shows a nest-like space formed by a matrix membrane. Because of the relative dimensions we speculate that this space might have been occupied by a cell that no longer exists. The membranous character of the matrix is seen clearly in Figure 5C obtained from a 20 day culture. The membranes appear to be interconnected with fibrous matrix and form a complex 3-D structure. Whether this structure originally contained cells or other material could not be determined.

In previous studies, we reported the ability of human dermal fibroblasts grown on HA-P-15 to generate sufficient forces to cause clustering of the particles (Qian and Bhatnagar, 1996). Similar clustering was also observed in HGF cultures on HA-P-15. As particles were drawn close together, cells could form tight connections between them as seen in Figure 6. Tightly

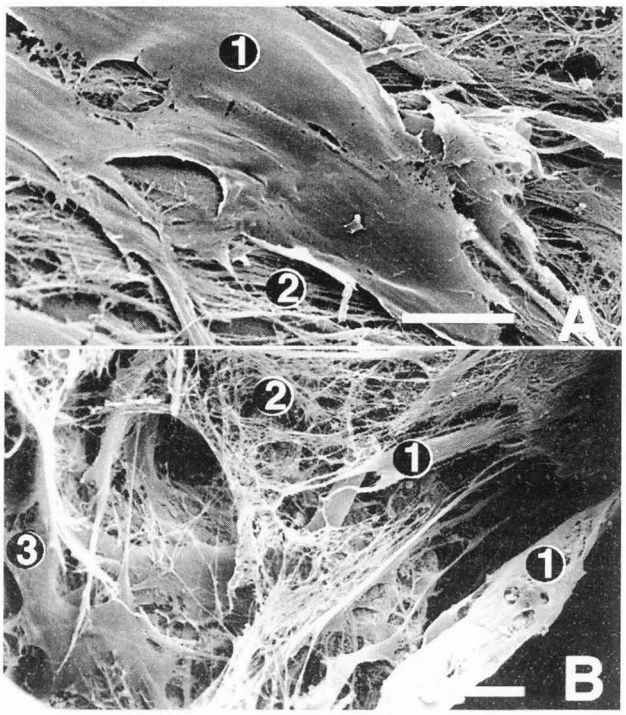

Figure 4. Scanning electron micrographs of matrix in 22 day cultures on HA and HA-P-15 shows different orientation and forms. (**A**). Twenty-two day cultures of HGF on HA formed essentially a closely packed confluent monolayer of cells (**1**). The cell layer rested on an underlying fibrous matrix (**2**). Bar = 20 μm. (**B**). HGF cultured on HA-P-15 for 20 days formed multilayered colonies (**1**) and deposited more abundant matrix (**2**), some of it organized in membranes which formed chamber-like structures (**3**). Bar = 40 μm.

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stretched filopodia and matrix fibers are seen to hold two HA-P-15 particles. The gap between the particles can be seen underneath the bridging elements of filopodia and matrix. Some of these bridge elements also appear to be present within the gap and are parallel to the gap axis.

The ordered arrangement of cells and matrix seen in the various scanning electron micrographs supported the idea that HGF cultured on HA-P-15 may undergo increased differentiation to a phenotype consistent with repair and regeneration of periodontal bone and ligament. We therefore examined the expression of several bone-related markers in HGF cultured after 7, 11, and 14 days in culture on HA and on HA-P-15.

The results from this experiment are shown in Figure 7. Gingival fibroblasts in culture are known to synthesize collagen (Irwin *et al.*, 1994). Although, little difference was seen in the level of expression of collagen after 7 days in culture on HA and HA-P-15,

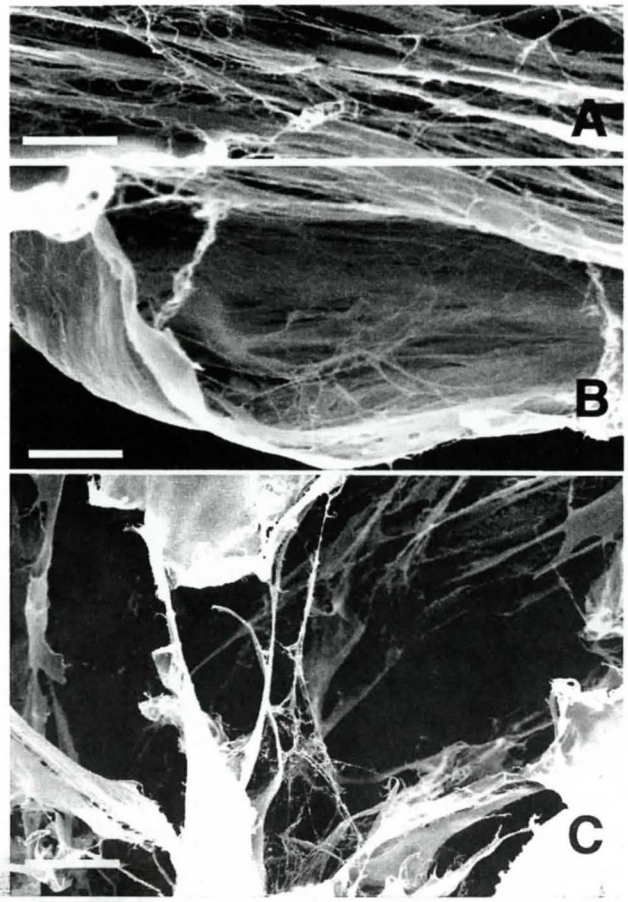

Figure 5. Scanning electron micrographs showing 3-D arrangement of matrix in 10 day HGF cultures on HA-P-15. (**A**). Some of the matrix formed parallel cables which appeared to be interspersed with elongated cellular structures, possibly filopodia. Bar = 25 μm. (**B**). Nest-like structure made up of a sheet of parallel matrix fibers. Bar = 12 μm. (**C**). 3-D compartments made up of matrix. Bar = 40 μm.

greater expression was observed in 11 day and 14 day cultures on HA-P-15. The synthesis and deposition of collagen is regulated by TGF β-1 (Ignotz and Massague, 1986; Irwin *et al.*, 1994). As seen in Figure 7, cultures on HA-P-15 expressed increased levels of TGF β-1. Although we did not measure the amount of TGF β-1 secreted in the culture, it may be speculated that the increased matrix resulted from both the presence of larger numbers of cells and increased amounts of the growth factor.

Detectable levels of alkaline phosphatase have been reported in monolayer cultures of human gingival fibroblasts (Ogata *et al.*, 1995). As HGF are considered to be a source of reparative cells in the periodontium (Pitaru *et al.*, 1994), it is likely that in permissive,

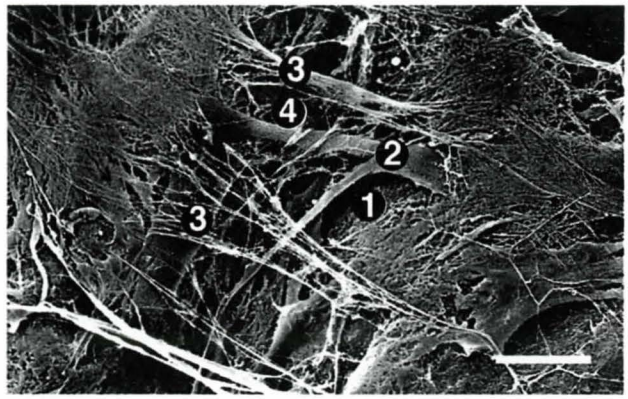

Figure 6. Tractional forces involving filopodia and emanating fibrous matrix contribute to clustering of HA-P-15 particles by HGF. This scanning electron micrograph shows the mechanical coupling between cellular elements and HA surface. Underlying microgranular surface of HA (**1**) appears to be firmly connected to the very taut filopodia (**2**). Many matrix cables (**3**) are part of the cell-matrix construct holding two separate particles in close proximity. The gap between the two particles is seen as a dark space (**4**). Bar = 40 μm.

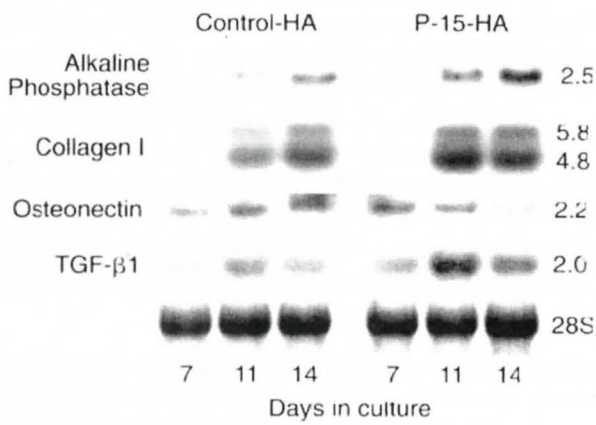

Figure 7. Northern blots showing expression of genes related to osteogenic differentiation.

differentiation promoting environments, these cell may display osteogenic markers. The presence of both alkaline phosphatase and osteonectin in HGF cultured on HA (Fig. 7) is consistent with the observation that the mineral provides an osteoconductive environment (Damien and Parsons, 1991). In a previous study, we reported the induction of alkaline phosphatase in human neonatal dermal fibroblasts cultured on HA-P-15 (Qian and Bhatnagar, 1996). Thus, it is not surprising that the levels of both alkaline phosphatase and osteonectin

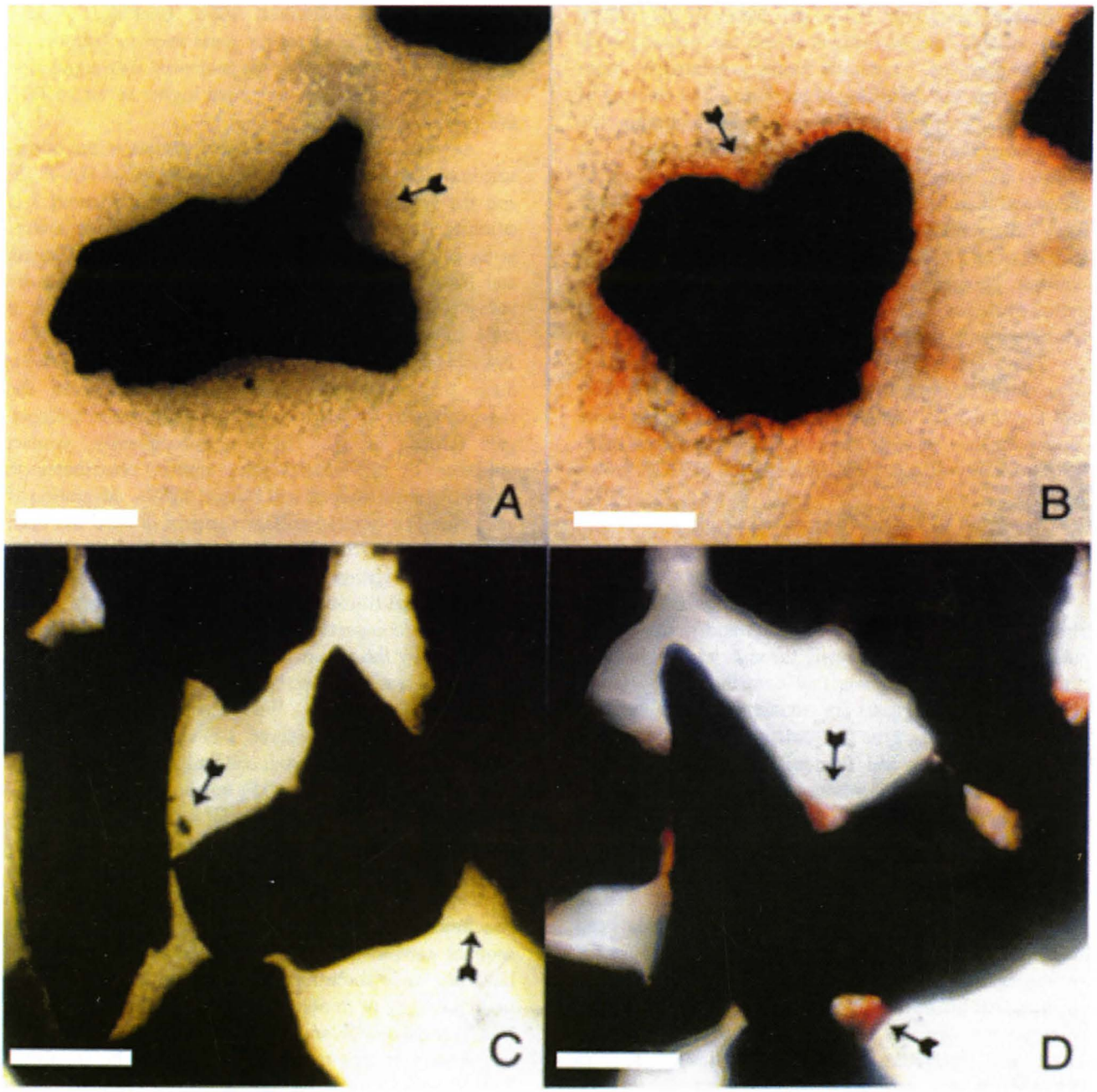

Figure 8. Evidence for mineralization using Alizarin Red staining. (**A**). Little staining is observed in peri-particle area in HGF culture of HA. (**B**). A halo of red stain around HGF on HA-P-15 particle suggests local mineralization. While very little stain is seen in inter-particle cellular bridges in HGF on HA (**C**), intense stain in such bridges (**D**) in cultures on HA-P-15 strongly suggests mineralization. Bar = 600 μm.

induction were elevated in HGF cultured on HA-P-15.

The increased expression of osteogenic markers in HGF cultured on HA-P-15 suggested the possibility of in vitro bone formation. Staining of matrix with Alizarin Red dye is considered to be an indicator of mineralization (Keila *et al.*, 1994; Bodine *et al.*, 1997) characterized by the deposition of Ca^{2+} (Stanford *et al.*, 1995). While very little staining with the dye was seen in HGF cultures on HA as well as on HA-P-15 in early (3-10 day) cultures, marked differences were seen in 14 day cultures (Fig. 8). Figures 8A and 8B show staining around isolated HA and HA-P-15 particles. A halo of

red stain consistent with Ca^{2+} deposition is seen in HGF matrix associated with HA-P-15. Many of the cells in these cultures formed bridges across the gaps between adjacent particles. Tractional forces generated by these cells result in the clustering of particles. The staining of intercellular bridges was examined by focussing the microscope in planes above the culture dish surface. In Figures 8C and 8D, the culture dish surface is out of focus and therefore the background appears to be blank. Very little staining was seen in the inter-particle bridge matrix when HGF were cultured on HA (Fig. 8C). In contrast, the matrix associated with the inter-particle cellular bridges on HA-P-15 displayed staining behavior consistent with the presence of Ca^{2+} (Fig. 8D).

These observations are consistent with the induction of an osteogenic phenotype in HGF cultured on HA-P-15 and together with our observations on osteogenic gene induction, they strongly suggest that the presence of P-15 remarkably affects the differentiation of HGF grown on HA.

Discussion

The construction of a biomimetic environment for tissue engineering, and for implants and grafts should take into account the channels through which chemical and biomechanical signals that contribute to differentiation and morphognesis are conducted. Cells attach to and migrate on type I collagen by haptotactic mechanisms (Guido and Tranquillo, 1993; Tranquilo *et al.*, 1993). All anchored cells exist in 3-D compartments made up of an extracellular matrix comprised of a network of collagen among components. In this environment, cell-cell and cell-matrix interactions are mediated by highly specific receptors. These interactions facilitate the exchange of chemical and mechanical signals. Inert scaffolds lack mechanisms for providing flow of information to and from cells. We propose that incorporating P-15, the cell-binding activity of type I collagen in a 3-D distribution will facilitate the attachment, migration, and differentiation of cells in biologically inert templates. Such a matrix may be expected to serve as a hospitable biomimetic habitat for cells for tissue engineering and it may serve as a bioactive scaffold for the construction of implants. Matrices containing the immobilized peptide may be expected to emulate the properties of collagen in promoting cell attachment, migration, and differentiation.

Although we did not measure cell numbers in the present studies, morphological observations suggest the presence of larger numbers of cells in association with HA-P-15 compared to uncoated HA. These observations are consistent with our previous studies which showed increased association of cells with HA in the presence of P-15 (Qian and Bhatnagar, 1996; Bhatnagar *et al.*, 1999). The increased matrix mass seen in cultures on HA-P-15 is a reflection of the increased cell population and may also reflect the presumed higher levels of TGF β-1 production.

An interesting aspect of these studies is the observation of well organized matrix in cultures on HA-P-15, and the apparent organization of cells in oriented 3-D colonies. Contact guidance by the extracellular matrix is an important mechanism of morphogenesis (Stopak and Harris, 1982). Generation of tractional forces by many cells is said to be the key to the organization of cells and matrices in ordered structures (Harris *et al.*, 1981; Huang *et al.*, 1993; Tranquillo *et al.*, 1993). Such tractional forces have been implicated in the reorganization of matrices by cells cultured in collagen gels (Huang et al., 1993). Subsequently, contact guidance by the extracellular matrix contributes to morphogenesis (Stopak and Harris, 1982). Migration of cells along an ordered collagen matrix *in vitro* has been elegantly demonstrated by Guido and Tranquillo (1993), who used birefringence to observe the organization of human dermal fibroblasts along oriented collagen gels in which the compaction of gels by tractional forces generated by the populating cells was eliminated. Our SEM observations clearly show a highly organized arrangement of HGF. We speculate that the highly elongated profile and ordered 3-D arrangement of HGF on HA-P-15 may arise from cells migrating along a field of collagen-like ligand (P-15) on the surface of a rigid matrix (HA) in a manner similar to that suggested by Tranquillo *et al.* (1993). Furthermore, the SEM observations also show a copious organized matrix rarely seen in monolayer cultures. The overall effect of the initial attachment and elongation of HGF may include the subsequent tractional organization of the matrix. While we have not made force measurements in the present studies, clustering of mineral particles and the stressed matrix fibers and cellular processes seen in Figures 3A, 3B and 6 suggest that tractional forces may be involved. This is seen in a spectacular fashion in Figure 6 which shows two HA-P-15 particles tied together by highly stressed fibers and cellular processes.

The concept that mechanical signals may be involved in HGF growing on HA-P-15 is further supported by the increased expression of TGF β-1. The expression of TGF β-1 is increased in osteogenic cells under conditions of mechanical loading or stimulation (Klein-Nulend *et al.*, 1993; Raab-Cullen *et al.*, 1994; Neidlinger-Wilke *et al.*, 1995). The release of growth factors in tissues under mechanical forces is an important mechanism of skeletal homeostasis. Mechanical loading also contributes to the expression of other bone related genes

including alkaline phosphatase (Raab-Cullen *et al.*, 1994).

The increased level of osteogenic markers is likely to be related to the increased expression of TGF β-1 and collagen, and to the morphologically observed increase in a fibrous collagen matrix. TGF β-1 is a modulator of cellular phenotype and has been implicated in osteo-differentiation (Long *et al.*, 1995; Yamaguchi, 1995; Cheifetz *et al.*, 1996). Collagen and TGF β-1 play synergistic roles in modulating cell differentiation even as collagen itself modulates the induction of the growth factor (Streuli *et al.*, 1993). TGF β-1 promotes the deposition of collagen (Ignotz and Massague, 1986; Irwin *et al.*, 1994). Type I collagen regulates osteogenic differentiation (Shi *et al.*, 1996; Lynch *et al.*, 1995) and other studies suggest that collagen synthesized under the influence of TGF β-1 is a significant effector of this process (Takeuchi *et al.*, 1996). Our observations showing the increased expression of collagen and TGF β-1, as well as the large amount of collagenous matrix are consistent with these findings. Our studies support the concept that P-15 may facilitate the coupling of cellular tractional forces to the relatively bulky mineral particles. The increased expression of TGF β-1 may result from mechanical stressing of cells. Increased TGF β-1 may result in increased production of collagen and its organization into an ordered matrix. Collagen and other components of the extracellular matrix are known to act synergistically with growth factors in promoting differentiation (Kirk and Kahn, 1995; Mizuno and Kuboki, 1995; Kostenuik *et al.*, 1996; Takeuchi *et al.*, 1996; Taipale and Keski-Oja, 1997). The collagenous matrix also serves as a reservoir for TGF β with the growth factor forming a physical complex with fibers (Taipale *et al.*, 1996). Our studies support this relationship. As seen from the gene expression data, the increased expression of TGF β-1 at 7 days in culture resulted in subsequent increases in collagen expression and synthesis, also seen clearly in the scanning electron micrographs. The synergistic effects of the growth factor with the large amount of collagenous matrix may have contributed to the increased expression of alkaline phosphatase and osteonectin.

Our observations support a role for P-15 in promoting attachment, ordered 3-D colony formation and differentiation of HGF. The formation of ordered 3-D colonies and organized matrix is consistent with a mechanism involving mechanical force signals. The physiology of cells is profoundly affected by applied mechanical forces and displacements. Mechanical stimulation stimulates bone formation and inhibits bone resorption in cultured bone tissue and cells (Kubota *et al.*, 1993; Klein-Nulend *et al.*, 1997). Intermittent compressive forces stimulated the release of an autocrine growth-factor from bone, suggesting that mechanical forces may modulate skeletal remodeling by affecting the production of local growth factors (Klein-Nulend *et al.*, 1993). If P-15 acts as a force-conducting ligand for cells, this would explain the increased expression of TGF β-1 in HGF cultures on HA-P-15.

We are interested in developing biomimetic environments for cells. An accurate biomimetic habitat for cells would provide an environment in which cells may experience the full range of biochemical and biophysical signals that regulate their behavior in their authentic physiological environment. The role of biochemical signals, such as TGF β-1, in modulating cell differentiation and morphogenesis is well recognized. In the physiological environment, cell behavior is also regulated by exogenous and biogenic mechanical forces (Ingber *et al.*, 1994; Ingber, 1997). Force signals are especially important in tissues that are subject to mechanical loading, such as the periodontium, alveolar bone and weight-bearing skeletal structures every where in the body. One of the major mechanisms that couples mechanical signals in these tissues to an intracellular apparatus for the regulation of cell behavior involves the conduction of mechanical signals via an extensive network of collagenous extracellular matrix (Ozerdem and Tozeren, 1995; Chiquet *et al.*, 1996: Maniotis *et al.*, 1997). The junction between the ECM, its receptor integrins, and the cells' cytoskeleton plays a crucial role in cell differentiation and morphogenesis by serving as the agent for transducing mechanical forces into chemical and biochemical work. Type I collagen is a major component of the matrices of load bearing tissues such as the periodontium. In its physiological solid state, collagen is one of the most important protein carriers of mechanical stimuli. Interactions of cells with collagen play a key role in cell migration, differentiation and tissue morphogenesis (Green *et al.*, 1995; Lynch *et al.*, 1995; Shi *et al.*, 1996). The interaction between a force-transmitting ligand region of collagen with its receptors is likely to result in a very high affinity bond. The strength of adhesion between the "ligand" and its receptor can be expected to be a major determinant of the ability of the cell to respond to mechanical stimuli.

Our studies suggest that the synthetic peptide P-15, an analogue of the cell binding domain of collagen may serve as a force transducing ligand in place of collagen. It is interesting to consider this property of P-15 in the context of tissue engineering. The placement of cells in 3-D arrays in synthetic matrices in apposition to immobilized P-15 will mimic cells' native force environment, facilitate exchange of mechanical signals and promote cell differentiation. Such environments may be considered to be bioactive habitats rather than inert scaffolds. These studies raise the interesting possibility of fabrication of hospitable and bioactive habitats for cells for use

in tissue engineering and as components of implantable devices.

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Discussion with Reviewers

O.R. Beirne: Why was a control group of HA com-

bined with a 15 amino acid long non-specific peptide not evaluated?
Authors: In previous studies, we showed that the cell binding activity of P-15 is lost on inverting the central --IA- sequence to -AI- (Bhatnagar *et al.*, 1997). The inability of the peptide to address the receptor in a specific manner disables the mechanisms involved in cell matrix connection. It is for this reason that all experiments reported here are based on the bioactive peptide.

Our objective is to generate hospitable, biomimetic habitats for cells for use in tissue engineering. The interaction of cells with their mechanical environment plays a crucial role in cell differentiation and morphogenesis. Collagen forms a rigid network of highly insoluble fibrous collagen and as discussed in the manuscript, it is a major force carrier. The junction between collagen and highly specific, high affinity collagen-binding receptors (presumably integrins) with the cell's cytoskeleton comprises the apparatus for the conversion of mechanical work into chemical energy. P-15 is an analogue of the region of collagen addressed by cells in this manner, and our studies clearly show that it plays a role in connecting the cells to their mechanical environment.

O.R. Beirne: If HA-P-15 causes expression of an osteogenic phenotype, why is the level of expression of osteonectin decreasing in comparison to the control group?
Authors: The expression of osteonectin appears to peak post-proliferation *in vivo* (Cowles *et al.*, 1998), and *in vitro* (Choi *et al.*, 1996; Nohutcu *et al.*, 1997) osteogenic differentiation. An interesting observation in this context was reported by Virolainen *et al.* (1995) who observed decreased expression of osteonectin mRNA after 14 days, in a repair site grafted with autologous bone whereas unfilled defects showed continued increase.

O.R. Beirne: How does change in TGF β-1 expression during culture compare with the induction of osteogenic phenotype?
Authors: The increase in the expression of TGF β-1 in gingival fibroblasts grown on HA-P-15 is consistent with earlier observations. The expression of TGF β-1 was shown to be increased along with other osteogenic markers such as type I collagen, and alkaline phosphatase during osteogenic differentiation (Long *et al.*, 1995; Yamaguchi, 1995; Cheifetz *et al.*, 1996).

O.R. Beirne: Have the authors considered examining expression of other TGF factors such as BMP-2 (bone morphogenetic protein 2), or BMP-7 in this model?
Authors: In continuing studies, we will examine the expression of these growth factors as well as other osteogenic markers using PCR techniques. Many of the osteogenic proteins are expressed transiently in a temporal program, and warrant an extensive investigation to establish their presence.

Reviewer II: The notion that gingival fibroblasts may contribute to healing of subcrestal wounds is puzzling.
Authors: Previous studies (Pitaru and Melcher, 1983, 1987; Pitaru *et al.*, 1994) support the concept that gingival fibroblasts may contribute to repair. Our studies support this concept and demonstrate that gingival fibroblasts may undergo differentiation into an osteoblast-like phenotype in permissive environments.

Reviewer III: Lower magnification "overview" type micrographs perhaps by phase or Nomarsky light optics are required.
Authors: We used SEM because of the difficulty in observing opaque, large-3D samples by light microscopy.

Reviewer III: There is a need to better define fibrous versus membranous matrix.
Authors: The differences between fibrous and membranous matrix were made on the basis of fine structure of the matrix. Some matrices show a rather amorphous structure. We refer to these as membranous matrices. Other matrices have a much more obvious fine structure and appear to be composites of fibers, and are referred to as fibrous matrices.

Reviewer III: The authors have provided no proof that a cell was present in the micrograph shown (Fig. 5C). The micrograph is equally consistent with fine cell membrane ruffles or thin pseudopodia.
Authors: Our conclusion is based on the dimensions of the "nest" which are consistent with a cell.

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