Preface

There is an urgent need to develop new approaches to treat conditions associated with the aging global population. The surgeon's approach to many of these problems could be described as having evolved through three stages:

Removal: Traditionally, diseased or badly damaged tissues and structures might simply be removed. This was appropriate for limbs and non-essential organs, but could not be applied to structures that were critical to sustain life. An additional problem was the creation of disability or physical deformity that in turn could lead to further complications.

Replacement: In an effort to treat wider clinical problems, or to overcome the limitations of amputation, surgeons turned to the use of implanted materials and medical devices that could replace the functions of biological structures. This field developed rapidly in the 1960s and 1970s, with heart valve and total joint replacement becoming common. The term "biomaterial" was used increasingly to describe the materials used in these operations, and the study of biomaterials became one of the first truly interdisciplinary research fields. Today, biomaterials are employed in many millions of clinical procedures each year and they have become the mainstay of a very successful industry.

Renewal: Although there were impressive results associated with the use of biomaterials and medical devices, problems remained. The human body did not always accept the presence of a foreign substance, and this could ultimately cause the failure of a medical device. Despite extensive research, long-term failure of medical devices and the need for subsequent revision surgery is a major problem. Throughout the entire history of biomaterials research and development, we have always known that the best materials in the body are healthy human tissues. This has in turn encouraged a reappraisal of our approach to the treatment of trauma and the general degeneration of the aging individual. Surgeons would now like to use healthy living tissues to replace diseased ones, a process best described as "renewal." The emerging field of tissue engineering, in which living tissue is grown in a laboratory before subsequent therapeutic application, is one area of great promise in the search of a solution. Most tissue engineering research, along with the current first generation products, requires some form of biomaterial support or scaffold during culture and/or delivery to the patient.

The scientific discipline of tissue engineering could therefore be described as having originated from (and is still dependent on) the application of biomaterials in medicine. What is perhaps less obvious is that the future of the biomaterials and medical device industry has become closely intertwined with the growth of tissue engineering as a viable therapeutic concept. Tissue engineering has the potential to reduce the need for traditional biomaterials, and may ultimately render many medical devices obsolete.

The recent shift in emphasis away from biomaterials and towards tissue engineering is illustrated by changing patterns of research output. Figure 1 shows a simple analysis of papers published during the last 40 yr of the 20th century. There was a steady growth over that period in the annual number of papers with "biomaterials" as a key word or title word. The phrase "tissue engineering" was not cited in the literature until the mid-1980s and during the 1990s there was an explosion of interest in this emerging field. Indeed, by the dawn of the new millennium there were more papers being published using the term "tissue engineering" than "biomaterials." If research activity provides an insight into the future of technology, then tissue engineering will undoubtedly revolutionize the treatment of disease in the near future.

The application of biomaterials in tissue engineering is a truly interdisciplinary endeavor, involving experts in chemistry, chemical engineering, cell biology, matrix biochemistry, biomechanics, and clinical medicine. In many cases, scientists with a highly focused expertise in one discipline are having to cross boundaries into completely new areas.

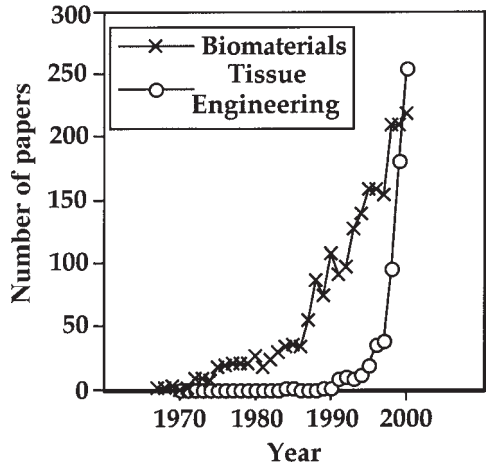

Fig. 1. Publication rates in biomaterials and tissue engineering.

Biopolymer Methods in Tissue Engineering constitutes a major consolidation of the basic methodologies from many of the scientific fields investigating biomaterials into a single volume. Different accounts of several of the approaches included here may be available in other forms elsewhere in the literature, however we hope that our book will serve as a basic laboratory manual allowing tissue engineering scientists not only to access a wide range of techniques in one place, but also to have them described using a standard format.

The chapters are organized into three clear groups. There are nine chapters dealing with the synthesis, processing, and characterization of specific biomaterials. The next four chapters provide details on the successful use of some of these scaffolds for the engineering of tissues. The last six chapters provide a range of techniques that can be used to evaluate the biological quality of tissues that have been engineered on scaffolds. We consider the rigorous assessment of tissue quality to be particularly important since it is often neglected in published accounts of tissue engineering.

We hope that readers of *Biopolymer Methods in Tissue Engineering* will find it a valuable reference manual for day-to-day use in their laboratories. We are indebted to all the international experts included among the chapter authors who have taken enormous trouble to prepare their important contributions to this volume.

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Fibrin Microbeads (FMB) As Biodegradable Carriers for Culturing Cells and for Accelerating Wound Healing

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1. Introduction

Fibrinogen exerts adhesive effects on cultured fibroblasts and other cells. Specifically, fibrin(ogen) and its various lytic fragments (e.g., FPA, FPB, fragments D and E) were shown to be chemotactic to macrophages, human fibroblasts, and endothelial cells ***(1–3)***. Thrombin has also been shown to exert proliferative and adhesive effects on cultured cells ***(4–7)***. We previously demonstrated that covalently coating inert Sepharose beads with either fibrinogen or thrombin rendered them adhesive to a wide range of cell types. We employed such coated Sepharose beads to screen or rank normal and transformed cells for their haptotactic responses to fibrinogen ***(8,9)***.

Micro-carrier beads made of some plastic polymers or glass provide cells with a surface area on the order of 10^4 cm^2/L for cell attachment, which is one order of magnitude larger than the area available with stack plates or multi-tray cell-culture facilities ***(10)***. From the point of view of transplantation biology, the major disadvantage of such cell micro-carriers is that most of them are not biodegradable or immunogenic. Others have prepared microparticles from plasma proteins, such as albumin or fibrinogen, generally using glutaraldehyde to cross-link the proteins. However, glutaraldehyde is not appropriate for preparing cell-culture matrices because such crosslinking slows down degradation of the matrix or blocks the protein epitopes that may attract cells. Consequently, the use of glutaraldehyde crosslinked micro-carriers has been limited to drug release or imaging ***(11–17)***.

Based on our experience with the attraction of many normal cell types to fibrin(ogen) with minimal effect on their proliferation ***(8,9)***, we fabricated small

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microbeads of fibrin (FMB) that could be loaded with cells and grown as a dense suspension. The FMB were found to be haptotactic to a wide range of cell types. These include normal cells such as primary endothelial cells, smooth muscle cells (SMCs), fibroblasts, chondrocytes, and osteoblasts, and osteogenic bone marrow-derived progenitors, as well as several transformed cells, such as 3T3 and mouse mammary carcinoma lines ***(18,19)***. FMB minimally attached normal keratinocytes and different cell lines of the leukocytic lineage. Cells could be maintained on FMB in extremely high densities for more than 2 wk and could be transferred to seed culture flasks or to be downloaded without prior trypsinization. Light, fluorescent, and confocal laser microscopy revealed that—depending on the cell type tested—beads could accommodate up to a few dozen cells per FMB, because of their high surface area, with minimized contact inhibition.

In a pigskin wound-healing model, we showed that FMB + fibroblasts could be transplanted into full-thickness punch wounds and by the third day after wounding, only the wounds in which fibroblasts on FMB were added showed significant formation of granulation tissue, compared to other treatment modalities, such as the addition of PDGF-BB ***(9)***.

We are interested in developing these new biodegradable fibrin-derived microbeads (FMB), 50–300 μm in diameter, as potent cell carriers. FMB technology enables one to transfer cells in suspension into wounds as "liquid-tissue." The non-trypsinized cells on FMB can download onto the wound bed, repopulate it with cells that can regenerate extracellular matrix (ECM), and stimulate neovascularization. Currently, FMB + cells are being evaluated in a number of animal models in which the intention is to regenerate tissues such as skin or bone *in situ*. We anticipate many uses of the novel FMB technology for cell culturing, wound healing, and tissue engineering.

2. Materials

2.1. Fibrinogen and Thrombin

Fibrinogen prepared by fractionation of pooled plasma is a component of clinical-grade fibrin sealant that is typically virus-inactivated by methods such as solvent detergent (S/D) process ***(20,21)*** with human thrombin (stock 200 U/mL) as previously described ***(20)***. The activity of thrombin is performed by clot time assays calibrated against an international standard (Vitex Inc., New York, NY).

2.2. Culture Reagents

For the experimental work that is described here, the culture-medium components were purchased mainly from Biological Industries (Beit-HaEmek, Israel), and fetal calf serum (FCS) was supplied by GIBCO-BRL (Grand Island, New York, NY). Other equivalents should work the same.

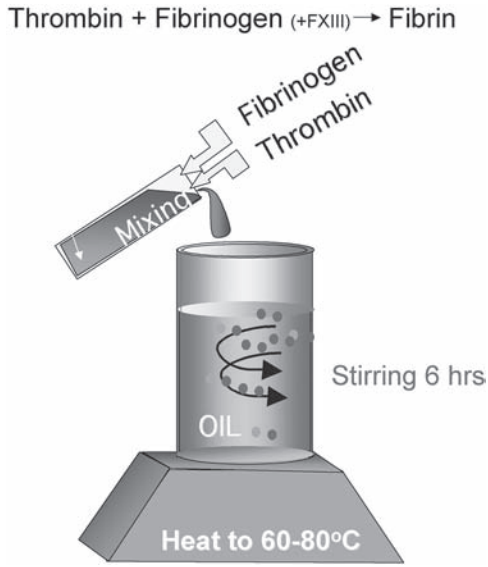

Fig. 1. Cartoon showing the setup for producing FMB by the oil emulsion method.

3. Methods

3.1. FMB Preparation

A typical preparation of FMB is carried out as described in the following steps ***(19)***:

1. Heat 400 mL corn or other type of compatable oil to 60–75°C with high-speed mechanical stirring.
2. Prepare a solution of fibrinogen (25 mL; 35–50 mg/mL) in Tris/saline buffer (pH 7.4) with 5 m*M* Ca^{+2} and mix it with thrombin to 5 U/mL (final concentration) to initiate the coagulation reaction.
3. Add the protein mixture to the heated oil as a flowing gel, and disperse to droplets by vigorous stirring.
4. Under these conditions, the thrombin will promote fibrin polymerization and activate endogenous, relatively heat-stable factor XIII, which can crosslink the fibrin droplets in the heated oil.
5. Continue the mixing and heating at a temperature of ~65–70°C for 5–7 h.
6. Filter off the crude FMB.
7. Sequentially wash with solvent such as hexane and acetone, then air-dry (**Fig. 1**). The resultant FMB will be highly crosslinked, have a low water content, and will be insoluble in water or organic solvents.

8. Wash and resuspend the FMB in 96% ethanol until their use, preferably for at least 24 h. Before using the FMB, wash extensively in sterile phosphate-buffered saline (PBS).
9. The FMB can also be pre-sterilized by gamma irradiation.

3.2. Solubility, Density, and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Test the FMB for solubility in Tris/saline or in 4 *M* urea. The Tris buffer nor the 4 *M* urea should not significantly dissolve the FMB, even after 1 wk at room temperature.
2. To carry out SDS-PAGE analysis, partially digest the FMB using 0.1 *N* NaOH for 1 or 2 h and subject to non-reduced 4–12% gradient SDS-PAGE (Nova, Encino, CA), with fibrinogen as a control. The non-reduced SDS-PAGE of NaOH digests of FMB should show that FMB contains many more crosslinks than observed with normally clotted fibrin, which usually show only γ-γ dimers and loss of α and γ bands as well as a-a multimers (**Fig. 2**).
3. Determine the density of the FMB by layering an aliquot of it onto a sucrose solution of known density. After centrifugation, one can observe that the FMB settles to the bottom or remains on top of the sucrose. Carry out this test using a series of sucrose solutions of different concentrations (and densities), and thereby determine the minimal density of sucrose at which the FMB do not settle at the bottom of the tube to determine their density. Typically, FMB have a density of 1.3 ± 0.05 that enables them to settle down in the bottom of the rotating spinning tubes used for cell growth.

3.3. Cell Cultures

1. Isolate normal human fibroblasts (HF) from skin biopsies of young human subjects as previously described ***(8)***. These cells can be grown for at least 12 passages.
2. Prepare porcine SMCs by separating them from the thoracic aortas of young animals and grow for up to 10 passages.
3. Other cell lines that were tested include the murine fibroblast line (3T3), murine leukemic cell line (P-388), human ovarian carcinoma line (OV-1063), murine mammary adenocarcinoma cells (EMT-6), and murine macrophage-like cell line (J774.2), all of which should be grown and maintained as previously described ***(8,9)***.
4. Maintain all cell cultures at 37°C in a water-jacketed CO_2 incubator, and harvest cells using trypsin/versen solution with 1–2 passages per wk in a split ratio of 1:10 for fast-proliferating transformed cells and 1:4 for normal cell types.

3.4. Assay for FMB Attachment to Cells

Assay for haptotaxis induced by FMB to attached cells in monolayer is done as previously described ***(8)***, and is summarized here. It is similar to the test of the response to fibrinogen-coated Sepharose beads (SB-fib) that was previously described ***(8)***.

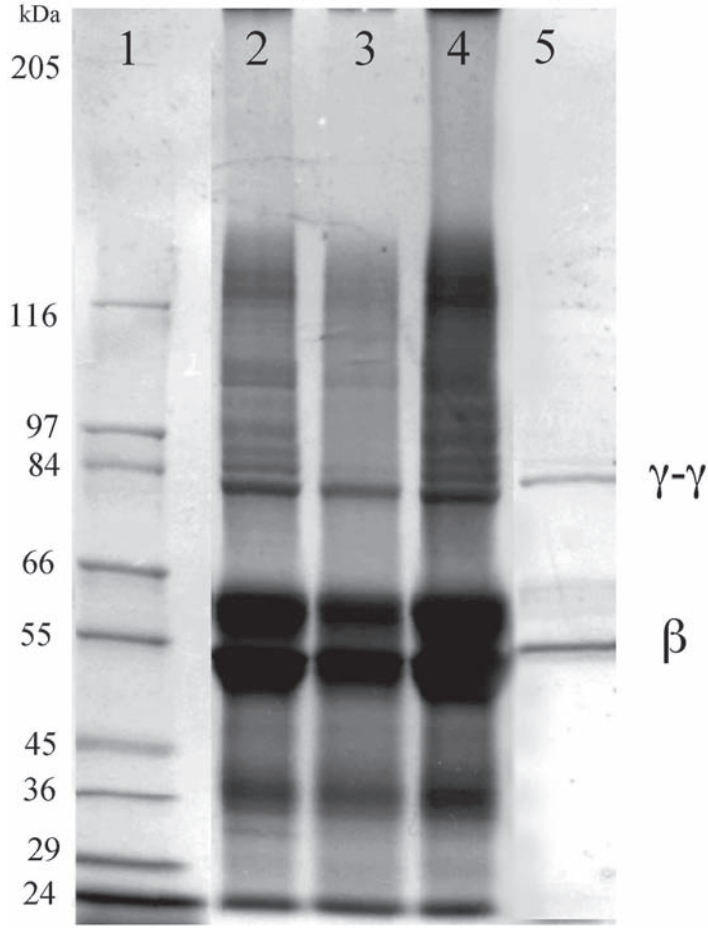

Fig. 2. Non-reduced 4–12% SDS-PAGE of NaOH-degraded FMB ***(2–4)*** and fibrinogen ***(5)***. Note the prevalence of crosslinked fragments in FMB.

1. Add FMB to a growing culture in a 12-well plate and count the attached beads per well periodically by visual inspection with an inverted phase microscope (typically 300 beads but not less then 200 FMB/well) (*see* **Note 1**). Initially, all FMB roll freely over the near-confluent culture.
2. Count the number of FMB anchored to the cell layer at different time intervals from 4 h onward, and calculate the ratio of FMB bound to the cells, relative to their total number. All experiments are done at least with triplicates.
3. FMB attachment to normal and transformed cells should correspond to the cell interactions with fibrin bound to otherwise nonreactive Sepharose beads (SB-Fib)

Table 1
Cell Attachment to SB-Fibrinogen and FMB (%) *

Primary cells	SB-fibrinogen	FMB
Human fibroblasts	>92	>95
Pig fibroblasts	>92	>95
Normal human keratinocytes	<5	<5
Mouse osteoblasts	>95	>95
Pig smooth-muscle cells	75	>95
Human chondrocytes	ND	>95
Bovine endothelial cells	>95	>95
Pig kidney epithelial cells	ND	>95
Transformed Cell Lines		
3T3/NIH fibroblasts	>90	>95
OV-1063 human ovarian carcinoma cells	0	10
EMT-6 murine mammary carcinoma cells	62	94
4T1 murine mammary carcinoma cells	>90	>90
Human melanoma cells	>90	>90
J-774.2 murine macrophage-like cells	0	0

* Sepharose beads with covalently bound fibrinogen (SB-Fib) or FMB were placed on nearly confluent culture, and the percentage of beads attached to the cells at d 1 was counted. Naked SB did not attach (O%).

(**Table 1**). In previous experiments, the FMB have not shown significant attachment to some cell types that grow in monolayer such as normal keratinocytes, OV-1063, and J-774.2 cells; whereas many normal mesenchymal cell types such as normal fibroblasts (human rat or pig) or transformed (3T3) as well as normal SMCs, endothelial cells, and EMT-6 cell line can attach the FMB with equal or greater degree than SB-Fib.

3.5. Loading Cells on FMB

1. Prior to use, suspend FMB in sterile 96% alcohol for at least a few hours, and then rinse extensively with sterile PBS. FMB can also be presterilized by ionizing radiation
2. The cells to be loaded on the FMB are grown in plastic tissue-culture dishes in their normal growth conditions. Prior to reaching confluence, the cells are trypsinized and collected in their growth medium to 50-mL polycarbonate tubes. Typically, up to 1–10 million cells are added per 100 μL FMB suspended in approx 6–10 mL of medium.
3. The tube should be closed by a perforated stopper that is covered loosely with aluminum foil to enable gas exchange with minimal risk of contamination. All tubes con-

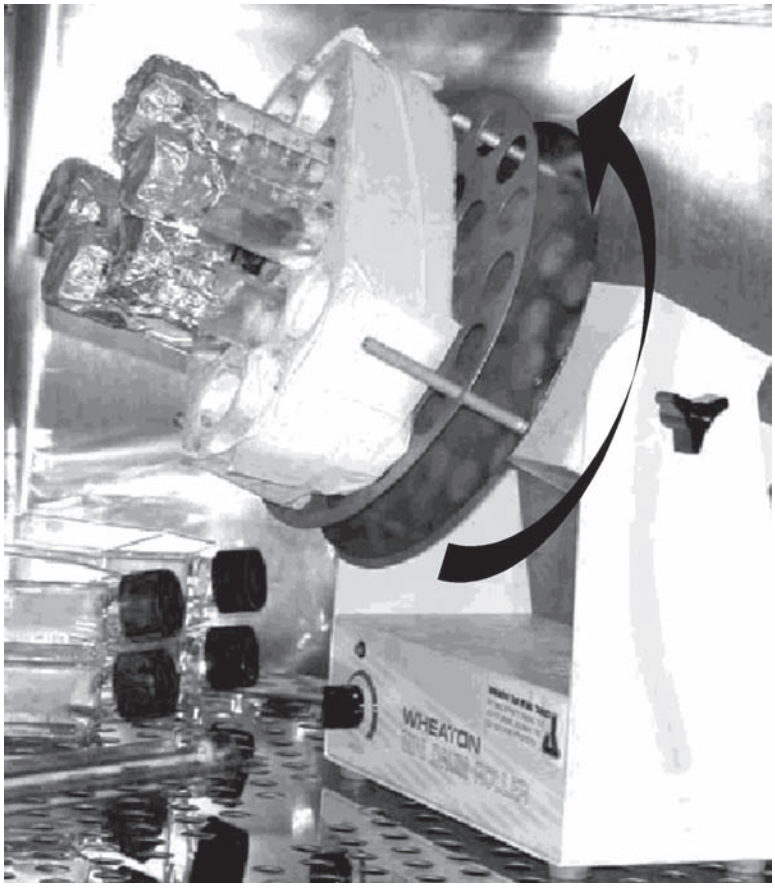

Fig. 3. Rotating cell-culture setup for growing cells on FMB in 50-mL polycarbonate tubes.

taining the FMB + cells should be placed on a rotating device at 10–20 cycles per min at an angle of 20–30°, so that the medium does not reach the stoppers (*see* **Note 2**). The rotating device should be placed in a 7% CO_2 tissue-culture incubator (**Fig. 3**). 48 h after mixing the cells with FMB, the supernatant medium containing unattached cells, as well as small fragments of FMB, should be removed and replaced with fresh medium. The tubes should be kept still for 60–90 s to allow the FMB loaded with cells to sediment before the medium is exchanged. The cells can continue to grow on the FMB in such a rotating device for prolonged periods, up to a few weeks, depending on the cell type and the density of cells on the FMB.

4. Replace the medium frequently, every 2–3 d, depending on cell number on the FMB in the tube.

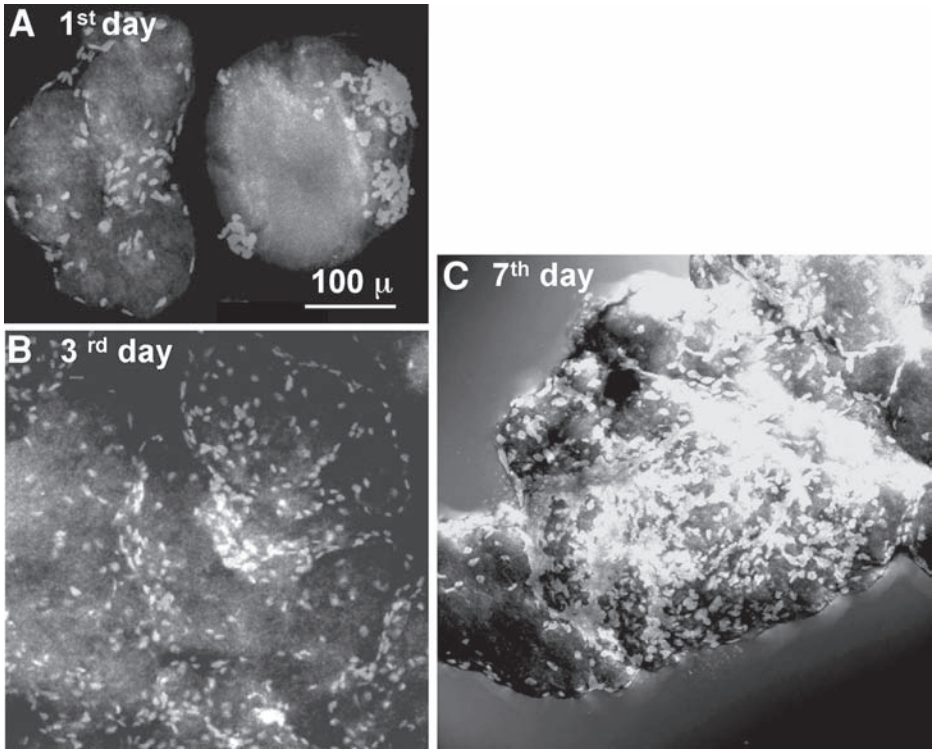

Fig. 4. Endothelial cells seeded and grown on FMB; nuclei fluorescence is seen as light dots. **(A)** 1 d after seeding, **(B)** and **(C)** at 3 and 7 d, respectively. By d 7, the cells secrete ECM that forms aggregates with large number of cells.

3.6. Imaging Cells on FMB

1. Perform light and fluorescent microscopy using a standard fluorescent microscopy system. Micrographs can be taken by single or double (fluorescence and light) exposures.
2. In order to distinguish and localize the cells on FMB, fix the samples in 0.5% buffered glutaraldehyde or 70% alcohol and stain the cell nuclei with propidium iodide (PI) by adding 50 μg/mL PI in darkness for at least 20 min before examination, and rinse with saline.
3. Place the PI stained FMB on a microscope slide with PBS-glycerol 80% and 2% DABCO, and scan with fluorescence microscope or with a computerized confocal laser microscope, typically with double excitation at 410 and 543 nm, to visualize the endogenous fluorescence of the FMB and the PI stained nuclei.
4. For confocal fluorescence microscopy, process the visual composite images (phase and differential interference contrast according to Nomarski) and the

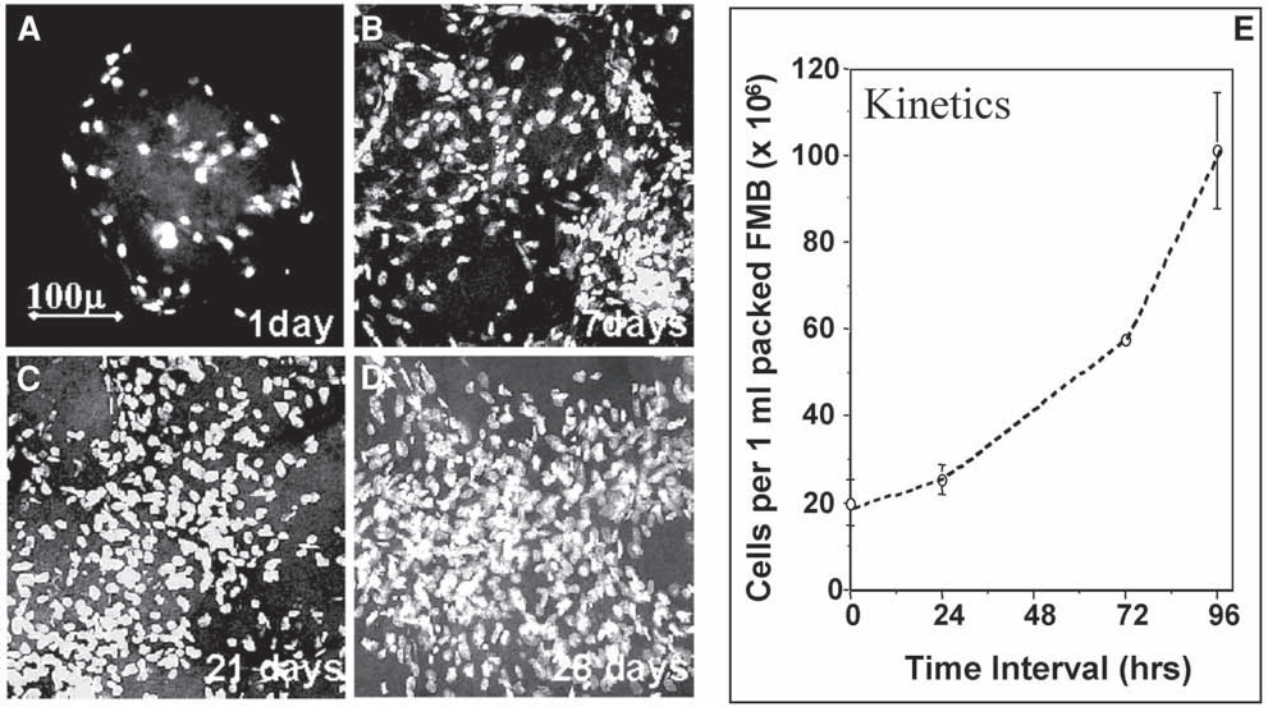

Fig. 5. Human fibroblasts are seeded and grown on FMB as in Fig. 4. About 1 million cells were loaded on 100 µL FMB and cultured in suspension for a period up to 28 d. **(A–D)** Confocal microscopy of samples taken at d 1, 7, 21, and 28 after seeding. The nuclei, revealed by PI (white spots) staining, indicate the increasing cell density over a 4-wk growth period. The Numarsky optics suggest that with time, the single FMB become aggregated and digested to be replaced by secreted new extracellular matrix. **(E)** Cell number on FMB was evaluated by the MTS assay that gave credible results up to d 4–5. Thereafter, the cell number was underestimated because of the inaccessibility of cells buried within the newly synthesized ECM.

fluorescent slice scans for overlap slice summation or three-dimensional (3D) presentation. The cell nuclei are stained with PI, and can be visualized. **Fig. 4** shows such a fluorescence microscopy image of bovine endothelial cells seeded and grown on FMB for 1 wk. **Fig. 5 A–D** shows composite confocal images of FMB loaded with fibroblasts at an estimated cell density of 100 million cells per 1 mL packed FMB. The rate of cell proliferation on the beads is clearly manifested.

3.7. Modified MTS Assay for Cell Number on FMB

Evaluate cell number on FMB by CellTitre 96Aqeous colorimetric assay (MTS assay) as previously described ***(8)***. For use with FMB, the assay must be modified as follows:

1. Place 200-µL samples of suspended FMB + cells in 24-well flat bottom plates (in triplicate) and add 200 µL of fresh mixture of MTS/PMS (CellTitre 96 AQueous Assay by Promega, Madison, WI) to each well.
2. After 2–6 h of incubation at 37°C, add 50 µL of dimethyl sulfoxide (DMSO) for 1 h with periodic shaking and transfer 0.1–0.3 mL of the supernatant to a 96-well plate.
3. Measure the optical density (OD) of the supernatant in a computerized automatic microwell-plate spectrophotometer (Anthos HT-II, Salzburg, Austria, or any equivalent) at 492 nm.
4. In a calibration of the procedure, various known amount of cells are seeded in plates, and when they attach they are incubated with the MTS reagents for 2, 4, and 6 h. The OD readings of the MTS should correlate well ($r > 0.95–0.99$) with the number of seeded cells.
5. Choose the incubation time at which the OD readings are within the optimal range for the assay of cell number on FMB. Depending on the cell types tested, the assay can be used to monitor cell number until a dense extracellular matrix (ECM) is deposited and masks the cells, typically after 4–5 d (*see* **Note 3**).
6. To monitor the proliferation of the cells, vortex for up to 3 s to disperse clumps, remove 100-µL samples of suspended FMB with cells at regular intervals, allow the particulate FMB to settle (1 min), and assay the cell number. **Fig. 5E** shows the proliferation of fibroblasts on FMB. **Fig. 6** shows that the highly populated FMB can be used to transfer seeded cells onto a plastic culture dish.

3.8. Pig Skin Wound-Healing Model

1. Make full-thickness excisional wounds using an 8-mm circular punch into the paravertebral skin of pigs as previously described ***(9)***.
2. To each wound space, add a mixture of 150 µL of 3 mg/mL fibrinogen and 2 U/mL human α-thrombin (*see* **Note 4**). However, in some cases, prior to the addition of the fibrin, 2 million of the cultured syngeneic fibroblasts in suspension or on FMB should first be added to the wound (*see* **Note 5**).

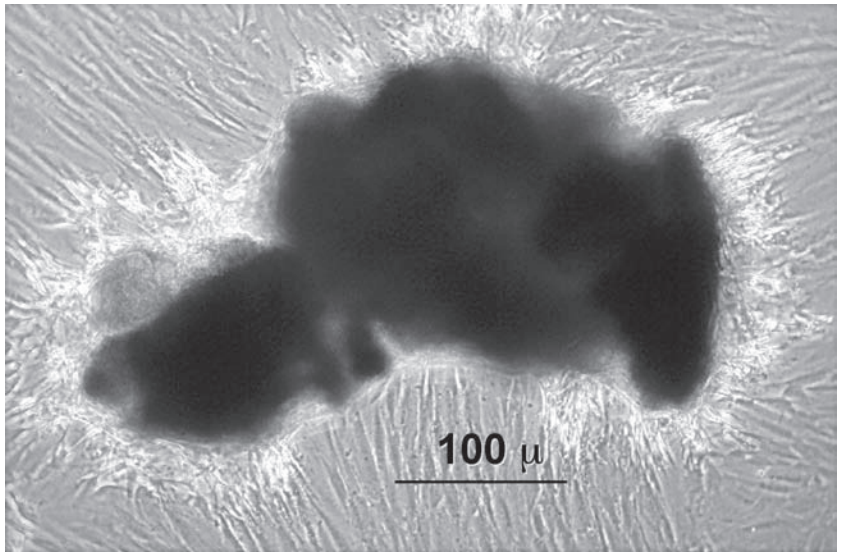

Fig. 6. Downloading of HF grown on FMB. Cells were grown on FMB for 14 d to reach saturation and downloaded from the FMB to plastic culture dish.

3. Dress the wound sites with an occlusive dressing, and harvest after 4 d. **Fig. 7** shows a comparison of wounds filled with fibrin alone (A), fibrin + naked FMB (B), and fibrin with FMB + cells (C), each tested in duplicate. In the control wounds, no granulation can be observed at this time-point; FMB alone seems to initiate vascularization and formation of granulation at the wound bed; FMB + cells fill the wound bed with newly formed granulation tissue (*see* **Note 6**).

4. Notes

1. The ideal size of FMB for cell culturing appears to be between 50 and 300 microns. Below 20 microns, the cells appear to engulf the particles rather than just adhere to them.
2. Cell growth on FMB appears to be optimal under conditions of low shear. Thus, we employ roller bottles or test-tubes made of non-cell-adherent polymers, rather than stirred suspensions in spinner flasks to grow cells on FMB. Experiments not described here demonstrate that the FMB are biodegradable, in vitro as well as in various animal models.
3. It is worth noting that the previously-described modified MTS assay provides good evaluation of the number of cells on FMB for only a few days after seeding. When the cells generate a significant amount of ECM, this tends to clump the FMB, and the cells become embedded within the whole aggregate. Thus, the

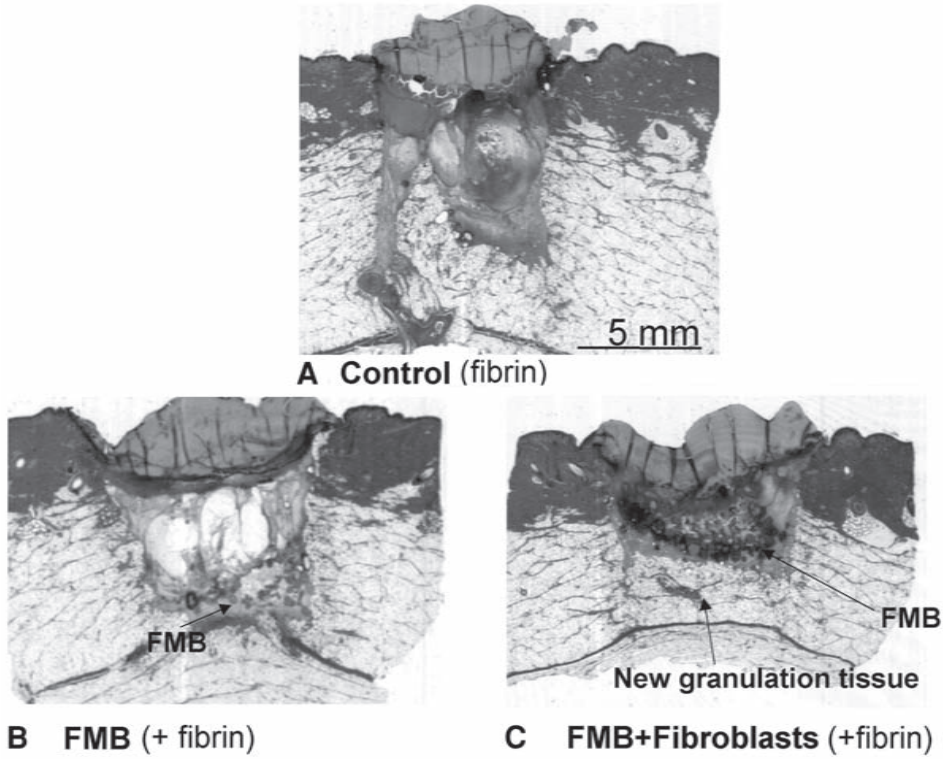

Fig. 7. Pigskin wound healing (d 4): histology of cutaneous wounds implanted with 3 mg/mL fibrin and combinations of FMB, human skin fibroblasts (HF), PDGF-BB, and controls: **(A)** Control wound (no fibrin or cells) shows no evidence of granulation tissue. **(B)** Addition of human fibrin and trypsinized HF shows no evidence of granulation tissue. **(C)** Wound to which syngeneic PF loaded FMB were added in fibrin. We observe FMB along the base of the wound and robust granulation tissue and neovascularization between the FMB and the underlying subcutaneous tissue. Additional PDGF did not further augment granulation tissue formation (not shown). (See color plate 1 appearing in the insert following p. 112.)

penetration of the MTS reagent into the cells is reduced, thereby underestimating cell number.

4. A major consideration for delivering cells-on-FMB in dilute fibrin glue to a wound site is the use of the appropriate applicator. Thus, for skin repair, a spray-type device may be adequate ***(22)***. For internal use, an endoscopic delivery system can be developed. Currently available fibrin glue applicators are not adequate for such delivery of cells on FMB because of internal clogging, clotting, and shear force. We are currently designing applicators to allow the delivery of cells-on-FMB that are convenient for tissue-engineering purposes.

5. The general approach is to implant viable cells-on-FMB to a wound site and fix them in place with a concomitantly formed low concentration of fibrin. Thus, the cells-on-FMB are suspended in fibrinogen (~3–5 mg/mL) and delivered simultaneously with thrombin, equivalent to the use of fibrin glue to seal surgical wounds, but in much lower concentrations ***(18,19)***.
6. The issue of immunogenicity is also relevant to materials employed for tissue engineering. Because FMB are composed of human fibrin(ogen) and thrombin, they are not expected to induce immune reactions to these materials in humans.

Acknowledgments

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