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ULTRASTRUCTURAL INVESTIGATION ON FIBROBLAST INTERACTION WITH COLLAGEN SCAFFOLD

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Abstract:

Collagen based scaffolds are widely used as temporary or permanent coverings to help dermal wound healing. Under natural conditions, wound healing is affected by many factors, including different cell types, growth factors and several components of the extracellular matrix. Due to the complexity of the cell-to-matrix interaction, many cell based mechanisms are not yet properly known. To improve our understanding the entire wound healing process can be partly simulated *in vitro* by allowing stabilized cell lines to interact with 3D collagen scaffolds. Under these conditions, such parameters as cell adhesion and migration to the collagen matrix can be more easily assessed. In this study we aimed at providing a detailed analysis of how NIH3T3 fibroblasts migrate and proliferate *in vitro* when seeded in co-culture with a collagen scaffold. To this end, samples were collected at regular time intervals between 4 hours and 14 days and analyzed by Light Microscopy (LM), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). Through this experimental approach we demonstrate that fibroblasts migrate through the collagen matrix by embracing collagen fibers with long filopodia and fold them back onto the cell surface to form large intracellular vacuoles. A number of microvesicles are also released from the filopodia extremities and dispersed inside the collagen matrix. We interpret these observations as indicating the occurrence of an extracellular collagen processing followed by cell uptake and intracellular degradation.

Key Words

Fibroblasts, collagen matrix, electron microscopy, *in vitro* culture, microvesicles.

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INTRODUCTION

Wound healing is a physiological process naturally addressed to repairing skin injury. It consists of a complex cascade of molecular events, from removal of damaged tissues to restoration of normal morphogenesis.[1,2] To this end, the extracellular matrix provides a structural scaffold for cells to migrate through the wounded area and differentiate into specific functional types.[3] As a major constituent of the extracellular matrix, the collagen plays a key role in the regenerative process of the wound by acting as an anchoring support for the migrating cells.[4,5] Because of its relative abundance in bodily proteins, low immunogenicity and high stability, the collagen is *par excellence* the best biomaterial employed as a prosthetic medical device to treat dermal injury.[6,7] However, to satisfy these requirements, the collagen scaffold should be mechanically integral, structurally uniform and have a matrix porosity suitable for cells to migrate and be retained up to the completion of tissue remodelling.[8]

Prosthetic collagen matrices may differ in type and concentration as in the chemical nature of the substances added to modulate matrix absorption and/or stability.[9-11] Therefore, a wide variety of collagen-based dressing differing in structural and biochemical properties have been developed with the intent to simulate the overall function of the extracellular matrix and improve the regenerative process of the injured area in normal or chronic situations.[12,13] Amongst the physiological variables that characterize the collagen matrix, its overall three-dimensional architecture is most relevant since it conditions such cell behaviours as proliferation, migration and ultimately signalling and differential gene expression.[14-16] Matrices with high porosity values provide healthier healing environments, especially in case of chronic wounds, due to their capacity to absorb more efficiently blood and lymphatic fluids from the wounded area.[8,17]

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Wound healing entails proliferation of a variety of cell types including fibroblasts, endothelial cells and epithelial cells, besides restoration of the extracellular matrix. Since it is highly improbable that such a complexity could be mimicked in monolayer cell cultures, cell-to-matrix interactions have been explored in 3D collagen matrices with the expectation to create a more realistic *in vivo*-like environment. [8,18] Under these conditions, any structural alteration the collagen scaffold may undergo or any eventual absorption by the host tissue can be experimentally monitored with relative ease. Because of these unique structural features of the collagen matrices,[19-21] we have recently embarked in a project with the intent of studying the collagen matrix infrastructure in the presence or absence of specific cell types. We thus aim at studying how collagen based matrices interact with cultured fibroblasts for over a period of two weeks by using transmission electron microscopy (TEM) and scanning microscopy (SEM). In this study we provide evidence of how the collagen matrix is structurally modified under various conditions of *in vitro* culture, how migrating cells interact with the 3D matrix by degrading and incorporating disassembled collagen fibrils. We show also how these cells anticipate their migratory behaviour by releasing a number of microvesicles into the surrounding medium.

MATERIALS AND METHODS

Collagen Scaffold Preparation

The collagen scaffold used in this study is a heterologous Type I collagen (BIOPAD) obtained from equine Achilles tendon. The extraction and purification procedures are such as to maintain the native structure of the collagen fibres.

Cell Culturing

Stabilized murine fibroblasts (NIH 3T3) were used throughout this study. To collect enough cells for plating on collagen matrices, NIH 3T3 fibroblasts were first cultured in 75 cm^2 flasks

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containing DMEM (Dulbecco's Modified Eagle's Medium) culture medium, supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin, and maintained in an incubator at 37 °C under continuous 5% CO_2 flow and controlled humidity. Upon reaching confluence, cells were passed into new culture vessels in a 1:5 ratio and the medium was changed every three days.

3D collagen scaffolds were cut with a sterile razor blade into blocks of approximately 1 cm^2 of surface area each and inserted into 24-well plates. Aliquots of $2x10^5$ cells were seeded on the upper surface of every collagen block and incubated at 37 °C in 1.5 ml of culture medium under a continuous flow of 5% CO_2 for time intervals ranging from 4 h to 14 days. Medium was then renewed every three days.

For 2D control experiments, NIH 3T3 fibroblasts were seeded on 24 well plates and incubated in 1 ml of culture medium at the same conditions as the 3D cell-cultures and assayed at regular time intervals for cell viability and microvesicle release.

Cell Viability

Cell viability was monitored by measuring the activity of the mitochondrial enzyme succinate dehydrogenase by using the MTT test. Since reduction of MTT is correlated with cell metabolic activity, cells metabolically active are expected to yield high MTT values. At each incubation time, 1 ml aliquots of culture medium, containing both collagen scaffolds and cells, were withdrawn from each well. Subsequently 0.1 ml MTT were added to every aliquot and left to incubate for 4h in the dark at 37 °C under a 5% CO_2 flow. By the end of this incubation time, the resulting formazan crystals were completely dissolved in DMSO glycine buffer at pH 10.5 and the relative absorbance measured at a wavelength of 570 nm. Absorbance values for NIH 3T3

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cells were measured at each time point of the entire culture period in both 2D plates and 3D matrix.

Cell Retention

The initial number of cells trapped onto the collagen scaffolding at 4 h of incubation time (Ci) was considered equivalent to the number of cells present in the original cell suspension (Cs) subtracted of the cells adhering to the well (Ca). Cs was determined by manual cell count using a Neubauer chamber, while Ca was determined by counting cells stripped from each well following trypsin treatment.

Collagen matrix structure

The scaffold architecture of the collagen matrix was structurally characterized by Scanning Electron Microscopy (SEM). To this end, collagen scaffolds were cut with a sterile razor blade into a number of small blocks and attached to aluminum stubs with a carbon tape. Blocks were oriented in such a way as to expose either the upper or the lower surfaces, while others were glued on their longitudinal cut profile. They were sputter-coated with gold in a Balzers MED 010 unit and observed in a JEOL JSM 6010LA electron microscope. The native structure of collagen was also checked by Transmission Electron Microscopy as specified below.

Fibroblasts and collagen matrix co-culturing

Collagen scaffolds inclusive of their cellular loads were cut into a number of small blocks with a sterile razor blade and fixed overnight at 4 °C with 3% glutaraldehyde in phosphate buffer (PB) at pH 7.2. Following extensive rinsing with the same buffer at 4 °C, they were immersed for 1h in 0,5% tannic acid in PB at 4 °C. They were then rinsed again four times in the same buffer for 15 min at 4 °C, and eventually post-fixed for 1h with 1% osmium tetroxide in PB at 4 °C. Specimens were finally washed in distilled water, block-stained with 1% uranyl acetate in

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distilled water and then dehydrated in a graded series of ethanols. For Scanning Electron Microscopy (SEM), samples were first dried by the critical point method in a Balzers Union CPD 020, sputter-coated with gold in a Balzers MED 010 unit and then observed in a JEOL JSM 6010LA electron microscope. For Transmission Electron Microscopy (TEM) samples were fixed and dehydrated as described above and then infiltrated for two days with graded mixtures of LRWhite resin/ethanol. By the end of this procedure, samples were embedded in fresh LRWhite resin and cut with Reichert Ultracut ultramicrotome equipped with a diamond knife. Ultrathin sections (60-80 nm thick) were collected on copper grids, stained with uranyl acetate and lead citrate, and observed with a JEOL 1200 EX II electron microscope. Micrographs were acquired by the Olympus SIS VELETA CCD camera equipped the iTEM software.

For Light Microscopy (LM), 1 μm thick sections were collected on glass slides, stained with toluidine blue and observed with an optical Zeiss Axioskop2 plus microscope. Images were collected with a video camera AxioCam MRC and elaborated with the software Axiovision4.

Matrix Porosity

SEM images were subjected to thresholding to convert matrix pores and collagen fibrils into black and white images. This procedure allows to adjust pixel density and to eliminate collagen fibrils from the image background. The 2D black and white matrix representations that are so obtained are then evaluated as particle sizes and their distribution analyzed with an image program (Image J).

Statistical analysis

The data from 8 independent experiments on cell migration in 3D collagen scaffolds were used and expressed as means ± SD. Statistical analysis was preformed using one-way ANOVA test with a Stat-Plus software (AnalystSoft ©2009) with the threshold of significance set at $P<0.05$.

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RESULTS

Scaffold characterization

To assess the overall stability of the collagen scaffold, 0.5 x 0.5 mm blocks were cultured *in vitro* for periods ranging from a minimum of 4h for up to 14 days, either in the presence or absence of cells. Figure 1 shows how the collagen scaffold changes during this culturing period. From a compact structure with highly intertwined fibers (Figs. 1a-c), the scaffold is gradually transformed into a rather loose assembly of dispersed collagen fibers (Figs. 1d-f). These changes occur regardless of whether cells are present or not. However, they are somehow accelerated and collagen fibers become more dispersed if the scaffold is cultured in the presence of cells (compare Figs. 1e and 1f).

Cell/collagen matrix interaction

NIH 3T3 fibroblasts were cultured on collagen scaffold matrices as specified in Materials and Methods, and their distribution assessed at regular time intervals by LM and SEM. Figure 2 shows that at 4 h after seeding, numerous fibroblasts were retained on the upper scaffold surface and some cells had already migrated into deeper regions of the collagen matrix (Fig. 2a). On entering the matrix, fibroblasts became highly intertwined with the collagen fibers, having most of their cell surface in close contact with the collagen fibers and occasionally forming specific adhesion plaques (Figs. 2b and 2c). In the presence of the collagen scaffold, fibroblasts apparently assumed different shapes, ranging from very roundish cells (Fig. 2d) to highly polarized ones (Fig. 2e). As culturing time was prolonged to 14 days, fibroblasts became even more entangled with the collagen fibers, eventually forming thick bundles of weaved filaments (Fig. 2f).

Cell/collagen matrix interaction: incorporation

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Following 1 day of culture, fibroblasts were already anchored onto the collagen laminas, but on average they maintained a low cell surface roughness with very few filopodia extending outwardly (Figs. 3a and 3b). However, with the continuation of the co-culture period for up to 14 days, the fibroblast plasma membrane weaved intimately with the collagen matrix by extending with an increasing number of filopodia (Fig. 3c). Figure 3d shows how some of these filopodia can actually embrace entire collagen fibers. A number of small vesicles appeared to be shed from the fibroblast cell surface in the proximity of the embraced fibril (Fig. 3e). Additional disaggregation of the collagen scaffold could be observed when fibers started to be gradually frayed into thinner fibrils (Fig. 3f). As these changes occurred, fibrils were seen gradually reduced in mean diameter, deprived of their staggered banding patterns and eventually taken up into large vacuoles by fibroblasts (Fig. 3g). Fibroblast filopodia are likely to play an active role in this engulfment process by first forming hairpin-like protrusions and then folding them back onto the cell surface to trap collagen fibers in a sort of vacuolar enclosure (Fig. 3d).

An extensive ultrastructural analysis indicated that filopodia are dynamic structures, highly variables both in mean diameter and overall length (Fig. 4a). Besides taking direct contact with the collagen fibers (Fig. 4b), filopodia are also involved in releasing a number of microvesicles onto the collagen matrix. In all likelihood, these microvesicles emerge from the fibroblast surface through an active process of budding from the filopodial tips. Instances of actual continuities of filopodia with emerging microvesicles have been recorded several times (Fig. 4c). As microvesicles bud off from the fibroblast cell surface they recruit inside part of the cortical cytoplasm, including a number of smaller vesicles (Fig. 4d). From this cell site they may migrate at a certain distance from the cell surface and disperse into the collagen matrix (Fig. 4e). With the continuation of the co-culture for up to 14 days, microvesicles increase in number, appearing

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also at a far distance from the fibroblast cell surface and highly intermingled with frayed collagen fibrils (Fig. 4f).

With the continuation of the cell culture, both fibroblasts and collagen matrix undergo additional changes. In particular, the fibroblast cytoplasm gradually filled with vacuoles containing material of varying consistency and density. Occasionally, some of the material in these vacuoles was seen in actual continuity with collagen fibrils (Fig. 5a). These vacuoles are likely to be formed by phagocytosis of collagen fibers through embracement and folding of the filopodia followed by invagination of the enclosed plasma membrane (Fig. 5b). However, these collagen vacuoles are complex organelles containing not only exogenous material but also material conveyed by other vesicles. These latter are likely to be Golgi-associated vesicles delivering their load of hydrolytic enzymes to the collagen containing vacuoles for additional processing (Figs. 5b and 5c).

***In vitro* cultured 3T3 fibroblasts**

As a control of the cell-to-collagen interaction experiments, fibroblasts were also cultured *in vitro* and allowed to grow for up to 14 days. Under these conditions, fibroblasts grew and migrated as flat elongated cells, while remaining firmly attached onto the substrate (Figs. 6a and 6b). Although variable both in shape and extension in relation to such conditions as cell crowding and incubation time, the number of filopodia present on the fibroblast cell surface appeared to increase steadily during the entire culture period (Figs. 6c and 6d). Numerous microvesicles were also present along the cell contours of many fibroblasts, even though they could not be discerned in regions far from the cell surface, indicating that they had to remain in the cell proximity for lack of any additional anchoring site. Fibroblasts appeared to release microvesicles *in vitro* in much the same way as in the presence of collagen scaffolds, i.e., as buds

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from the filopodial tip, the only difference being their number and position relative to the cell body (Figs. 6e and 6f) that appeared to be smaller than in the presence of the collagen scaffold.

Cell Kinetics

A rough estimate of the number of fibroblasts migrating in collagen scaffolds as a function of culturing time indicated that a progressively higher number of fibroblasts could be found in matrix regions further away from the seeded side of the scaffold (data not shown).

However, taking the MTT test as a reliable tool to measure cell viability and cell proliferation, the fibroblast/collagen interaction seems to be more complex than simple cell counting could suggest. Figure 7 indicates that cells increased their MTT activity quite rapidly within a day and then stayed constant for the rest of the incubation period. This is probably caused by fibroblasts starting to proliferate soon after entering the collagen matrix, and declining upon becoming involved in collagen breakdown and absorption. By comparison, cells cultured *in vitro* with no collagen scaffold grew according to a symmetrical kinetics inclusive of slow rise followed by an equally slow decline. This is probably due to the fact that confluence in vitro is attained after 3 days of cell culture. Afterward cells may stop growing and presumably start to be removed by apoptosis (Figure 7).

Discussion

A variety of collagen scaffolds are available today to help wound healing and skin regeneration. To accomplish these goals, scaffolds ought to satisfy several requirements. First, the pore size has to be maintained within a restricted size range for cells to adhere to the collagen matrix and migrate inside the scaffold. Second, the scaffold itself should be sufficiently stable to allow new granulation tissue to mature while the injured tissue is replaced by new collagen deposition. Third, the collagen matrix should not persist indefinitely, but be degradable within the time

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periods compatible with the healing process. All of these requirements were actually monitored in this study by simulating wound healing in the *in vitro* co-culture system of the Biopad collagen scaffold with 3T3 fibroblasts.

A careful series of observations and measurements allowed us to establish that the collagen scaffold used in this study offers a rather uniform matrix structure for cells to migrate through, with pore sizes falling within the expected range of about 100 ± 150 μm.[8] This value is in line with earlier observations that explored the effects of upper and lower limits of this range[22] and tested cell migration in scaffolds of different collagen compositions.[13]

Would healing is a rather complex phenomenon requiring the contribution of several cell types and the activation of a variety of growth factors and cytokines[23], primarily for sustaining extensive syntheses and remodelling of the dermal tissues. Fibroblasts appear to play a major role in the process by providing collagen for the extracellular matrix to be regenerated.[24] In this study, we followed the fate of fibroblasts as they migrate and proliferate in a collagen scaffold for up to 14 days of *in vitro* culture. The observed effects included adhesion to the collagen matrix, changes in the overall fibroblast morphology, disaggregation and fraying of the collagen fibers and eventually phagocytosis and intracellular digestion of the engulfed collagen fragments. That fibroblasts may actually migrate inside the collagen scaffold is clearly indicated both by cell counting and by their recurrent interaction with collagen fibers by means of focal adhesion plaques (see Fig. 2c). Through these type of cell junctions fibroblasts appear to be highly involved in complex mechanical interactions with the collagen matrix, presumably as a precondition for their attachment onto fibrils far distant from the seeded side. Small adhesion plaques are in fact formed close to the leading edge of the migrating fibroblasts where contractile forces act to propel migration.[25] The extent by which cells migrate into the collagen scaffold is

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an important parameter to determine how quickly collagen matrices are processed *in vivo* and how long it takes for a new extracellular matrix to be fully deposited. Understanding of these parameters should in principle help to design better collagen substrates for wound healing.[26]

Micrographs shown in this study are highly suggestive of the occurrence of collagen remodeling as sustained by both extracellular and intracellular degradation. Clear indications of collagenolysis stem from the observation that collagen fragmentation increases as culture time is prolonged from 1 to 14 days. During this time period, collagen fragments accumulate in the culture medium and gradually fray into bundles of woven thin fibrils. Numerous instances of intracellular collagen degradation have also been recorded as more and more material, ranging from banded collagen fragments to bundles of highly disorganized filaments, accumulate in the fibroblast vacuoles. These observations are in line with the notion that intracellular collagen degradation in fibroblasts requires the presence of specific collagen receptors and that collagen may accumulate in the culture medium if co-cultured with receptor deficient fibroblasts incapable of undergoing endocytosis.[27] This condition may actually simulate what have been previously described *in vivo* as fibrotic lesions, where fibroblasts with marked deficiencies in phagocytic activity affect the process of collagen degradation and eventually lead to an excessive deposition of extracellular matrix in the impaired organ.[28] Additional evidence that collagen remodelling entails intracellular degradation, comes from the observation that the extent of collagen degradation can be changed *in vitro* by modulating the pH-dependent activity of lysosomal enzymes in the presence of acidotropic agents.[29,30] However, for collagen to be taken up by fibroblasts it has first to be partially degraded by specific collagenases. Collagen phagocytosis and degradation are in fact inhibited whenever the expression of these enzymes is blocked by treatment with specific RNA interference.[31] Taken together, this evidence makes it

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highly probable that collagen remodelling *in vivo* requires both the action of secreted collagenases in the extracellular milieu and the activity of some hydrolytic enzymes in the intracellular space.[32]

As clearly documented in this study, filopodia are dynamic structures involved in a number of different cell functions. They are endowed with the capacity to explore the external milieu through transient adherence to the substrate and spreading along the collagen laminae. This behaviour is typical of cells capable of altering their actin cytoskeleton in response to variations in the substrate roughness.[33,34] However, filopodia have also the additional function of acting as cellular tentacles to engulf some partially degraded collagen fragments and retreat them into vacuole-like enclosures. By doing so they are likely to predispose the fibroblast plasma membrane for phagocytosis. Indeed, the appearance of new filopodia in myoblasts coincides with the activation of metalloproteinase precursors involved in collagen degradation.[35,36] Interestingly, this type of correlation is not restricted only to connective tissues, since metalloproteinases are also controlling the distribution of postsynaptic receptors in dendritic spins through a similar mechanism.[37,38]

However, besides providing a substrate attachment for the migrating fibroblasts and protruding tentacles for engulfing and degrading collagen fibers, filopodia are also involved in the release of microvesicles. This study has clearly documented the occurrence of extensive budding and fission of microvesicles from the filopodia tips and the inclusion of numerous smaller vesicles inside them. The role played by this shedding process remains to be proved experimentally, although microvesicles are likely to serve as vehicles for the horizontal transfer of enzymes required for collagen degradation. Microvesicles are in fact known to be a universal type of organelle that cells use to release factors involved in many metabolic and developmental

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functions.[39] Shedding from filopodial protrusions has recently been demonstrated to occur in endothelial cells where microvesicles appear to regulate vessel sprouting during human brain vascularization.[40] In conclusion, the study of fibroblast interaction in collagen scaffolds has allowed us to show that such basic functions as cell migration and adhesion, along with shedding of microvesicles are preconditions for the degradation of the prosthetic collagen and presumably for the deposition of a new extracellular matrix in would healing.

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FIGURES LEGENDS

FIGURE 1. LM and TEM images showing laminas of the collagen scaffold following 4h (a and b) and 14d (c and d) of *in vitro* culture in the presence of cells (a and c) or without cells (b and d); (a) and (d), Bars 50 µm; (b) and (c), Bars 1 µm; (e), Bar 2,5 µm. (f), Bar 3 µm.

FIGURE 2. (a), LM image showing numerous fibroblasts on the upper surface of the collagen scaffold. Note that some cells have already migrated inward, Bar 20 µm. (b), TEM micrograph showing fibroblasts closely adhering to a collagen fiber (see arrowhead), Bar 5 µm. (c), enlargement of the adhesion point, Bar 200 nm. (d), SEM micrograph showing a number of fibroblasts on the top surface of the collagen scaffold, Bar 20 µm. (e), SEM micrograph showing a fibroblast amidst collagen fibers 4h after seeding, Bar 5 µm. (f), SEM micrograph showing fibroblasts on the collagen scaffold 7 days after seeding, Bar 5 µm.

FIGURE 3. 3T3 fibroblasts cultured on collagen scaffold for up to 7 days. SEM (a) and TEM (b) micrographs showing fibroblast anchored onto collagen fibers after 1 day of culture. (a), Bar 2 µm. (b), Bar 5 µm. SEM (c) and TEM (d) micrographs showing a fibroblast highly intertwined with a collagen fiber after 4 days of culture. (c) and (d), Bars 2 µm. (e), enlargement of Fig. 3d showing a number of microvesicles, Bar 500 nm. SEM (f) and TEM (g) micrographs showing an enlargement of a fibroblast surface with several collagen fibers entering the cell interior, as seen in cross sections. (f) and (g), Bars 1 µm.

FIGURE 4. (a), SEM micrograph showing the rough surface of a fibroblast, Bar 1 µm. (b), TEM micrograph showing a fibroblast surface with numerous filopodia and microvesicles closely adjoined to collagen fibers, Bar 1 µm. (c), TEM micrograph of fibroblast showing numerous filopodia and microvesicles protruding from the cell surface, Bar 500 nm. (d), TEM micrograph showing a microvesicle budding from a filopodium tip, Bar 500 nm. (e), TEM micrograph of a 7

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day fibroblast highly entangled with collagen fibers and microvesicles, Bar 2 μm. (f), TEM micrograph of collagen fibers and microvesicles in regions of the collagen matrix far way form the fibroblast cell body, Bar 5 μm.

FIGURE 5. (a), TEM micrograph showing a large vacuole in the cortical cytoplasm of a fibroblast containing a collagen fiber, Bar 1 μm. (b), TEM micrograph showing some collagen-containing vacuoles merging with smaller vesicles deriving from the Golgi apparatus, Bar 500 nm. (c), TEM micrograph showing several vesicles with some electron dense material therein, close to a Golgi apparatus, Bar 500 nm.

FIGURE 6. (a), SEM micrograph of 1 day *in vitro* cultured fibroblasts, Bar 5 μm. (b), SEM micrograph showing a number of filopodia on the fibroblast cell surface, Bar 2 μm. (c), SEM micrograph of 7 day *in vitro* cultured fibroblasts showing a number of cells highly enriched with filopodia and microvesicles, Bar 5 μm. (d), enlargement of the previous figure showing the extent of microvesicle budding from the cell surface, Bar 1 μm. (e), TEM micrograph of an *in vitro* cultured fibroblast showing a number of filopodia and microvesicles protruding from the cell surface, Bar 2 μm. (f), TEM micrograph showing several microvesicles amidst some tortuous filopodia, Bar 2 μm.

FIGURE 7. Histogram showing cell viability of 3T3 fibroblasts cultured for over a period of 14 days (grey columns) or co-cultured for the same length of time in the presence of a Biopad collagen scaffold (black columns). Asterisks (*) for $P<0.05$.

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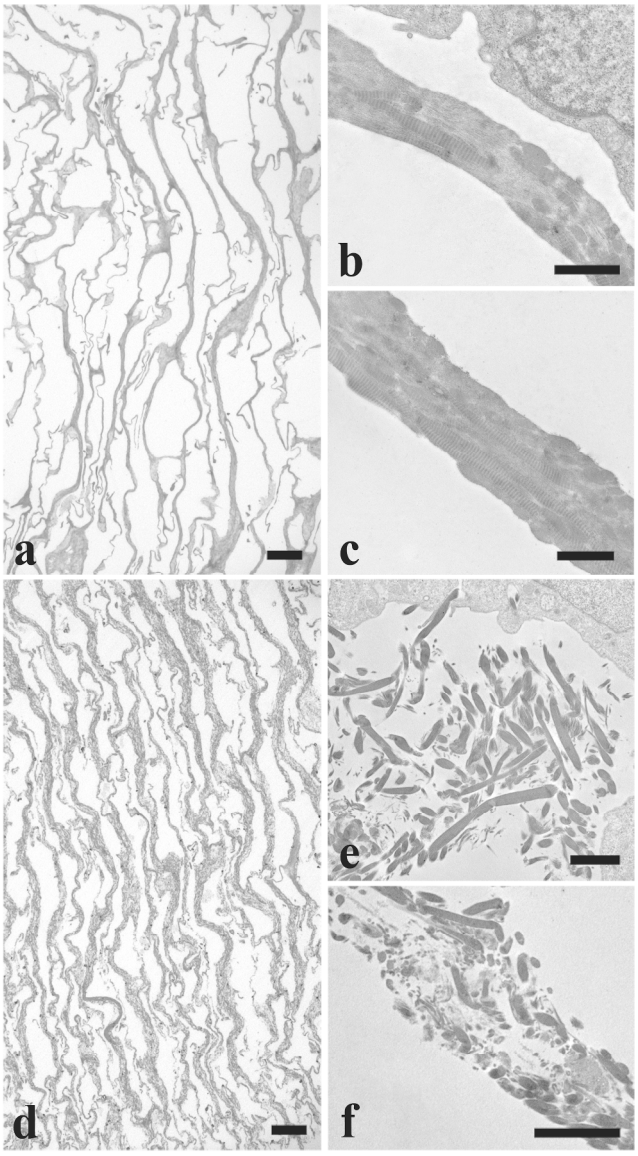

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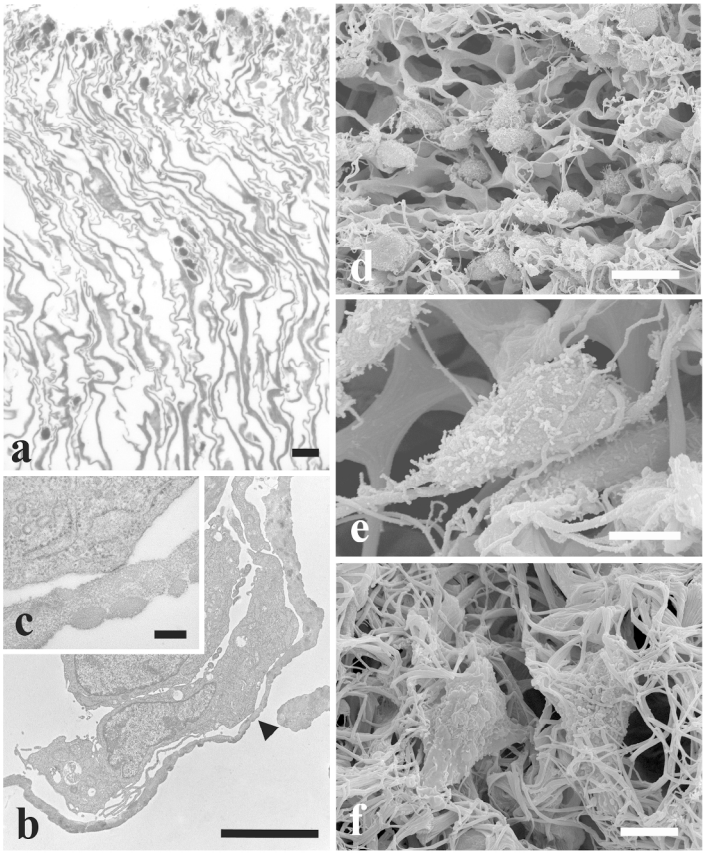

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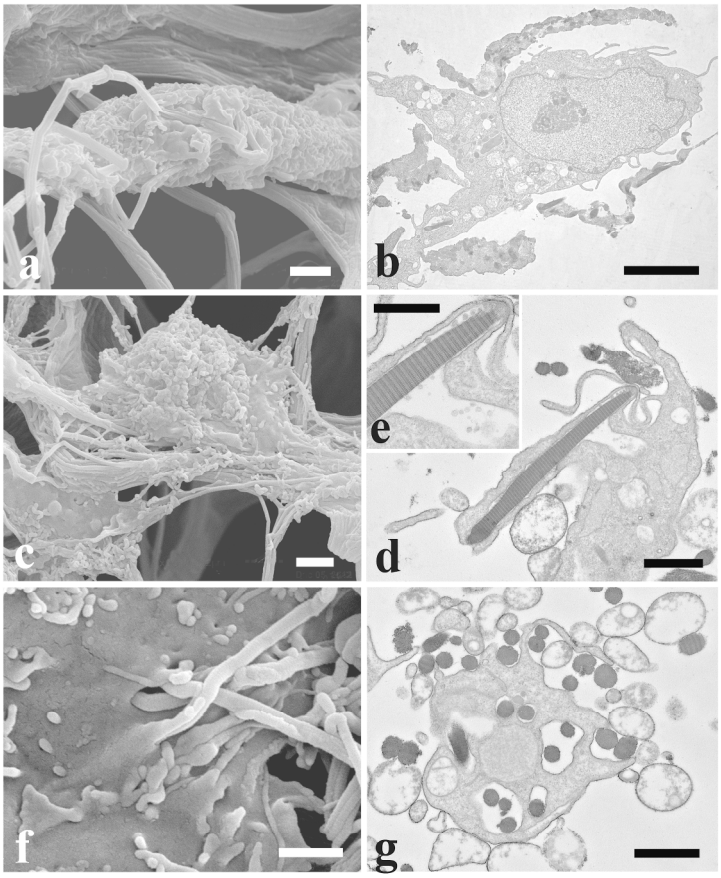

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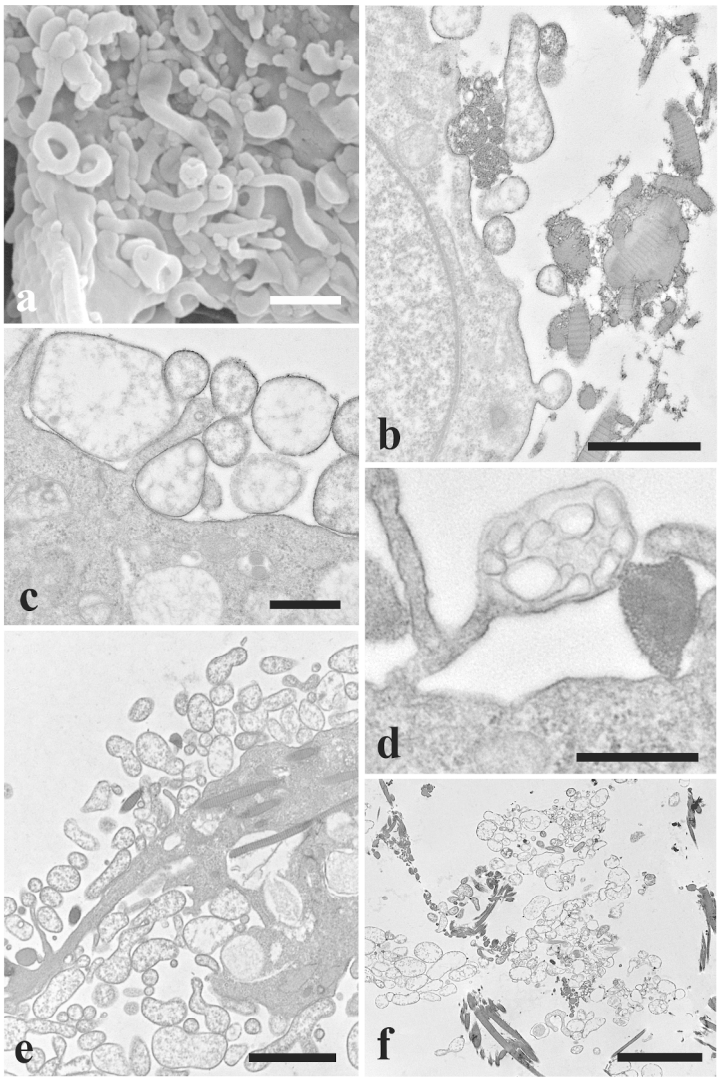

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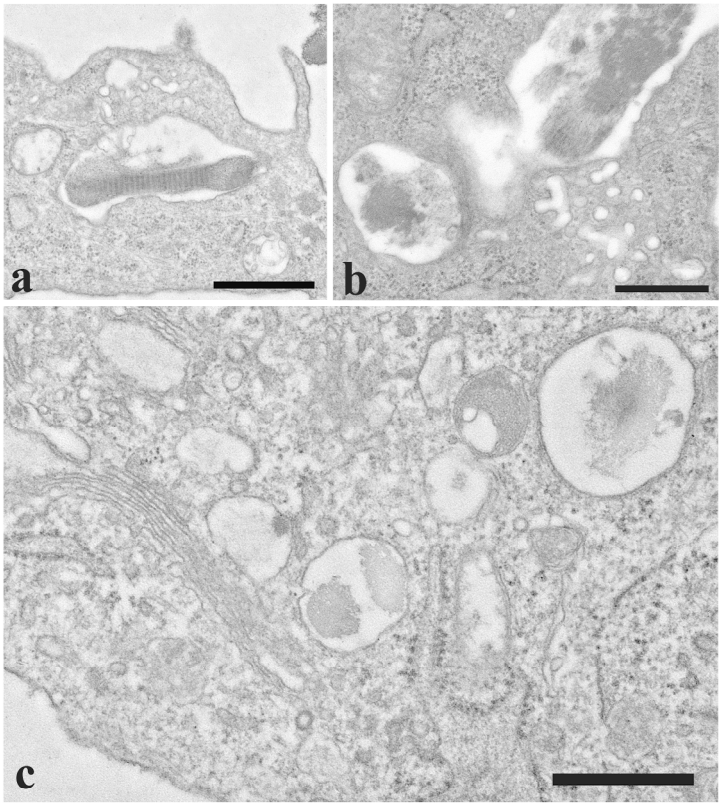

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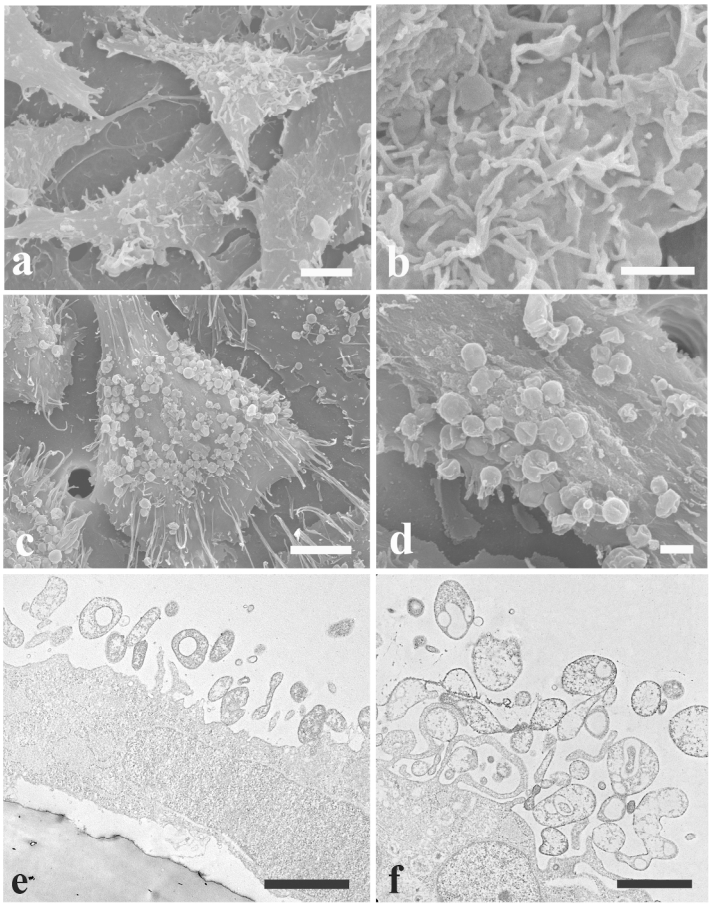

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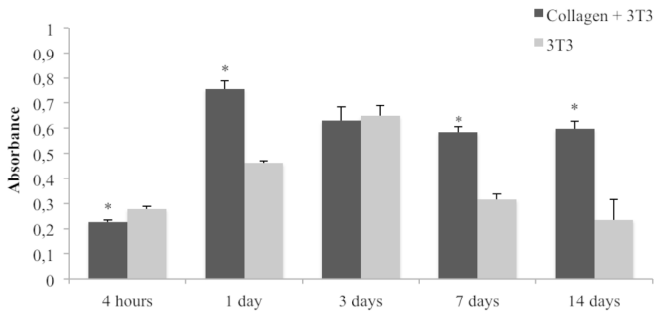

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