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The Functional Behavior of a Macrophage/Fibroblast Co-culture Model Derived from Normal and Diabetic Mice with a Marine Gelatin - Oxidized Alginate Hydrogel

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Abstract

Tissues/cells-mediated biodegradable material degradation is epitomized by the constantly changing tissues/cell-implant interface, implicating the constant adaptation of the tissues/cells. Macrophages and fibroblasts are multi-functional cells highly involved in the interactions; the two cell types modulates the behaviors of each other, but their combinatorial functional behavior in the presence of interactive bioactive wound dressings has not been adequately examined. The activity is further complicated by the implantation of biodegradable materials, such as hydrogels commonly utilized as wound dressings, in a pathological environment and this is exemplified by the macrophages with a diabetic pathology producing an alternative cytokine profile which is implicated in wound healing delay. In this study, an in situ gelable formable/conformable hydrogel formulated from modified alginate and marine gelatin was used as a model biodegradable interactive wound dressing to elucidate the combinatorial behavior of macrophages/fibroblasts derived from both normal and diabetic hosts. Cell proliferation, migration and distribution were first characterized; this was followed by simultaneous quantitative detection of 40 inflammatory cytokines and chemokines by a protein microarray. The results showed that the macrophages/fibroblasts co-culture promoted fibroblasts proliferation and migration in the presence of the hydrogel; moreover, the expressions of inflammatory cytokines and chemokines were altered when compared with the corresponding fibroblasts or macrophages monocultures. The inflammatory cytokines patterns between the normal and diabetic hosts were considerably different.

Keywords

Wound dressing; hydrogel; diabetes; fibroblast; macrophage; co-culture

1. Introduction

Both gelatin and alginate have been utilized as components of wound dressings; the beneficial effects of hydrogels composed of both of them for wound treatment have also been shown [1–2]. The most investigated gelatins are mammalian derived, but the thermal gelation property of mammalian gelatins has generally limited their utilities in formulating in situ formable dressings capable of fully conforming to wound beds. We have recently formulated a series of

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biodegradable in situ gelable hydrogels composed of partially oxidized alginate and marine gelatin; this approach has circumvented the limitations posed by mammalian derived gelatins, moreover, the hydrogel is amenable to cell migration, cell growth and extracellular matrix (ECM) deposition [3].

The primary value of utilizing gelatin and/or alginate as “interactive dressings” for wound healing has been commonly regarded as a matter of initiating hemostasis in conjunction with maintaining a moist environment [4]. Moreover, it has been demonstrated that an alginate-containing wound dressing (e.g., Kaltostat®) is able to activate macrophages resulting in the elevated levels of inflammatory cytokines including TNF- α and IL-6; the presence of these inflammatory signals in the wound could accelerate healing [5]. This type of “interactive dressing” is thought to be capable of modifying the physiology of the wound environment by modulating both cellular activities and releases of growth factors. To our knowledge, the underlying mechanisms of this type of dressings in promoting wound healing, particularly its collective interaction with cells/tissues, have hitherto not been fully explored.

In general, all implanted biomaterials invoke wound healing responses from the adjacent tissues. Inflammation is the initial phase of wound healing and macrophages play critical roles in both the acute and chronic inflammatory phases characterized by monocyte adhesion, macrophage differentiation, activation and fusion [6]. The second phase effect involves the attachment of macrophages to the implant surface leading to their secretion of pro-inflammatory cytokines, chemokines and growth factors; these in turn directly or indirectly recruit, activate and modulate the activities of various cell types including fibroblasts for wound repair [7]. Macrophages and fibroblasts are the two dominant cell types simultaneously interacting and coordinating their responses to the implanted materials, eventually leading to foreign body response (FBR) and fibrous encapsulation [8]. The effects of macrophages on non-biodegradable polymer implants (e.g., polyurethane, polycarbonate, etc.) have been extensively investigated and the results demonstrate that the interactions between macrophages and polymer surfaces are integral components of inflammation, foreign body reactions and other wound healing events occurring at the tissue/material interface [9].

Tissues/cells-mediated biodegradable material degradation is epitomized by the constantly changing compositions of the tissues/cell-implant interface, implicating the constant adaptation of the tissues/cells until the complete resorption of the implant and hence reaching full resolution. Two of the more imminent issues on the interaction of an implanted biodegradable material with tissues/cells are: (1) activation of macrophages in response to the implanted biomaterial, and (2) influence of macrophages on fibroblasts and their cooperative/interactive behavior on the biomaterial. A better understanding on the interactions between macrophages/fibroblasts and the biodegradable biomaterials is needed to facilitate the future development of biomaterials of improved performance characteristics and we have begun to address this issue [10–11]. The nature of the interactions between tissues/cells and implants is further complicated by implantation of biodegradable materials, such as hydrogels commonly utilized as wound dressings, in a pathological environment (e.g., diabetic host); this is exemplified by the macrophages with a diabetic pathology producing an alternative cytokine profile which is implicated in wound healing delay [12–13].

The overall aim of this investigation is to elucidate the cooperative aspects of the interaction of co-culture models, comprised of primary macrophage/fibroblast derived from diabetic and wild type mice, respectively, with a biodegradable interactive wound dressing. The hydrogel composed of marine gelatin and partially oxidized alginate was deployed as the in vitro 3-D model wound dressing to characterize cellular behavior parameters including cells viability, proliferation, migration and inflammatory response; temporal profiling of various biomediators were also performed. The information gathered will not only facilitate the

development of a more comprehensive and quantitative method of biocompatibility research, for both normal and diabetic hosts, but will also have implications on the future development of more optimal hydrogel formulations as wound dressings specifically targeting the healing of chronic diabetic wounds and other healing-impaired wounds.

2. Materials and methods

2.1. Preparation of hydrogel from marine gelatin and oxidized alginate

Hydrogels composed of marine gelatin and oxidized alginate were prepared and lyophilized to form porous scaffolds following the methodology described previously [3]. These scaffolds were cold-disinfected with 70% ethanol, followed by extensive rinsing in sterile PBS [14]. The disinfected scaffolds were equilibrated with DMEM culture medium to form hydrogels at 37 °C under a humidified atmosphere of 5% CO₂; this was followed by further equilibration with fresh DMEM every 30 min for two consecutive hours. Finally, uniform sized hydrogel pieces (10 mm diameter, 1 mm thick) were stamped out with a skin biopsy punch. Hydrogels were deposited individually in the wells of a 48-well cell culture plate.

2.2. Cell isolation and culture

Diabetic and normal control hosts derived macrophages and fibroblasts collected from 8 weeks old male Lepr^{db/db} mice with type 2 diabetes and impaired wound healing (BKS.Cg-Dock7^m +/+ Lepr^{db}/J, stock No. 000642, The Jackson Laboratory, Bar Harbor, ME) (abbreviate “db”) and age-matched, non-diabetic control Lepr^{db/m} mice (C57BLKS/J, stock No. 000662, The Jackson Laboratory, Bar Harbor, ME) (abbreviate “c57”). Three mice per group were used. Mice pelage hairs were first removed by applying a depilatory paste and their skins were obtained from the dorsal side. Primary fibroblasts were extracted by a standard technique previously described [15]. Briefly, the harvested tissue was cut into 3×3 mm pieces and deposited on a 10 cm Petri dish with the subcutaneous side down. Adhesion was achieved after partial dehydration and the tissue pieces were incubated with DMEM containing 10% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin (GIBCO-BRL, Rockville, MD) for 1 week in a humidified atmosphere of 5% CO₂ at 37°C; the medium was changed every other day. The tissues were removed once sufficient numbers of fibroblasts had migrated out of them, and the cells collected were passaged by trypsin-EDTA digestion; passages 4–6 were used and the cell culture media were changed every other day.

Macrophages were isolated and cultured as previously described [16]. Briefly, peritoneal macrophages were obtained by lavaging the peritoneal cavity with 3 ml of ice-cold PBS (repeated thrice) and isolated by adherence on plastic culture dishes for 2 hour at 37°C; thereafter, the macrophages were detached by applying a 0.02% EDTA-PBS solution (Lonza, Walkersville, MD) at 37°C for 10 minutes.

Five hundred microliters of cell suspension (1×10⁴ cells/ml, per cell type, seed alone or seed by mix, see Tab. 1) was deposited in each well of a 48-well cell culture plate where round hydrogel sections were pre-positioned. After 8 h of incubation, hydrogels with cells seeded and attached were rinsed with PBS twice and transferred to a new 48-well plate containing fresh cell culture media. Hydrogels not seeded with cells were used as negative controls. All studies were repeated twice and performed in triplicate (n = 3 per time-point per group).

2.3. Cell viability and proliferation assay

Colorimetric MTS assays (CellTiter 96® AQueous One Solution Cell Proliferation Assay kit, Promega, Madison, WI) were performed at 3, 7, 10 and 14 days after cell seeding to quantify cell viability and proliferation. The hydrogels were removed from the original cultures, rinsed with PBS followed by incubation with a 20% MTS reagent in serum-free DMEM for 1 h. Two

hundred microliters of media from each well were transferred to a 96-well plate and the absorbance (490 nm) was determined by a microplate reader (Infinite 200, Tecan, Switzerland).

2.4. Cell morphology, viability, migration and their 3D distribution in hydrogel

Cells seeded hydrogel were retrieved 3, 7, 10 and 14 days after seeding; they were rinsed with PBS, incubated in 500 μ l of “Live/Dead™” dye solution (viability/cytotoxicity staining kit for mammalian cells, Molecular Probes, Eugene, OR). The morphology, viability, migration and spatial distribution of cells were assessed by laser confocal microscopy (LSCM). Random areas on every sample were selected for scanning and each area was observed, profiled and analyzed in its entirety beginning from the surface at 2 μ m increments. Digital planar images (along the X–Y plane) were captured incrementally along the depth (Z-axis) of the sample; cell densities and the distribution patterns of live (green) and dead (red) cells were determined layer by layer. Tomographic reconstruction of 3D images and measurement of cell migration depth were conducted by Zeiss LSCM 510 META software.

An alternative staining protocol for cytoplasm and nucleic acid in live cells was utilized. Cells seeded hydrogel were incubated with 500 μ l of a dye solution containing 5 μ M of cell tracker orange CMTMR and 1 μ M of Hoechst 33342 (Carlsbad, CA 92008) for 30 min at 37°C in serum-free DMEM.

2.5. Protein microarray of inflammatory cytokines and chemokines

Cell culture supernatant collected on days 3, 7, 10 and 14 from cell-laden hydrogel were frozen at –80°C until use. One hundred microliters of cells culture supernatant (of each time-point, per group) was used for cytokine and chemokine detection and quantification by a Quantibody Mouse Inflammation Array I kit (RayBiotech, Inc. Norcross, GA; Cat. No. QAM-INF-1) according to the manufacturer’ instructions. The array was designed to quantitatively detect 40 inflammatory cytokines, chemokines, and growth factors simultaneously (Tab. 2). The signals (green fluorescence, Cy3 channel, 555nm excitation, and 565nm emission) were captured using a GenePix 4000B laser scanner (Bio-Rad Laboratories, Hercules, CA) and extraction with GenePix Pro 6.0 microarray analysis software. Quantitative data analysis was performed using RayBiotech mouse Inflammation Array 1 software (QAM-INF-1_Q-Analyzer). Sample concentrations (pg/ml) were determined from mean fluorescence intensities (median values) compared against a five-parameter linear regression standard curves generated from standards provided by the manufacturer. A series of isochronal maps reflecting the concentrations of the cytokine/chemokines detected by the microarrays were constructed by Matlab software (R2009a, MathWorks, Inc., Natick, MA), in order to transform numerical data into simplex graphical patterns.

2.6. Statistics

All data represent as mean $\pm$ standard deviation. Cell proliferation and migration depth data were compared among groups by multiple-way analysis of variance (MANOVA) with Tukey’s post-test. Microarray data were compared among groups by repeated-measures ANOVA [17–18]. SPSS statistical software v16.0 (Minitab, State College, PA) was used to conduct the analyses. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cell viability and proliferation assay

As depicted in Fig 1, there was no significant difference among all groups on day 3. By day 7, the cell numbers of the c57F group (OD: 0.925 ± 0.082) was the highest among the four groups and was significantly different from that of the c57MF group (OD: 0.765 ± 0.051 , $p < 0.05$).

The extent of cell proliferation moderated on day 10, with the cell number of the dbF group significantly lower than those of the c57F and the dbMF groups (OD: 0.549 ± 0.094 vs. 0.695 ± 0.029 & 0.675 ± 0.046 , $p < 0.05$). By day 14, the dbMF group reached the highest proliferation and its OD (1.137 ± 0.007) was significantly higher than those of the other three groups, and there was also significant difference between the c57F and the dbF group ($p < 0.01$).

3.2. Cell morphology, viability, migration and 3D distribution in hydrogel

The overwhelming majority (>95%) of the cells were alive with no visible difference in their morphology and distribution patterns (data not shown). As shown in Fig. 2a, the cell migration depth increased with the incubation time and Fig. 2b showed the cells' 3D distribution inside the hydrogel (one of the triplicates). The depth of cell migration was evidently different among the groups in the order of: dbMF group > c57MF group > c57F group > dbF group ($P < 0.05$).

3.3. Protein microarray of inflammatory cytokines and chemokines in cell-hydrogel co-culture system

The cytokines/chemokines microarray laser scanning maps were shown in Fig. 3. Among the 40 inflammatory cytokines targeted, eighteen showed up positive (Tab. 3, in bold font and underscored) and quantitative analyses were performed (Fig. 4). A series of isochronal maps constructed by Matlab software were shown in Fig. 5. These cytokines could be further sorted into four functional classes as depicted in Tab. 4 and discussed as follows.

3.3.1 Macrophages/fibroblasts co-culture compared with macrophages & fibroblasts monoculture—There were three different cytokines expression patterns in both the db and the c57 genotypes when their corresponding macrophages/fibroblasts co-culture and fibroblasts monoculture groups were compared: 1) up-regulated in the co-culture group: PF-4, MCP-5, MIP-1 α , MIP-1 γ ; 2) down-regulated in the co-culture group: IL-13, TNF RI, LIX, MCP-1, RANTES, Eotaxin, TCA-3, M-CSF and TIMP; 3) oppositely regulated in the co-culture group: IL-6, TNF RII, KC, G-CSF and GM-CSF, specifically, in the dbMF group these cytokines were up-regulated, whereas, they were down-regulated in the c57MF group.

All the inflammatory cytokines detected in the macrophages/fibroblasts co-culture were significantly greater ($p < 0.05$) than those of the macrophages monoculture in both the db and the c57 hosts (See Tab. 5).

3.3.2 db mouse primary cells groups compared with c57 mouse primary cells groups—In general, the magnitudes of cytokines and chemokines expressions among individual groups were in the order of: dbMF group > c57F, dbF and c57MF group > dbM and c57M group. In particular, among the eighteen inflammatory cytokines examined, the dbMF group showed considerable higher expression patterns of thirteen inflammatory cytokines; when compared with the c57MF group, they were, TNF RI, TNF RII, KC, LIX, PF-4, MCP-1, MCP-5, MIP-1 α , MIP-1 γ , Eotaxin, G-CSF, GM-CSF and TIMP-1 ($p < 0.05$). The levels of all the inflammatory cytokines detected in the dbM group were greater than that of the c57M group ($p < 0.05$) (Tab. 6). The dbF group has lower expressions of MCP-5, RANTES, TCA-3 and M-CSF but with higher expressions of LIX and MIP-1 α (data not shown).

4. Discussion

In this investigation, we utilized a hydrogel formulated from marine gelatin and oxidized alginate as the model wound dressing; primary macrophages and fibroblasts harvested from diabetic and wild type mice were used as model cell types. The objective was to construct an in vitro model of macrophages/fibroblasts co-culture to mimic the in vivo microenvironment for elucidating the interactions between cells and biodegradable wound dressings. Apparently,

co-culturing fibroblasts with macrophages enhanced both fibroblasts proliferation and migration into the hydrogel. In response to the presence of the hydrogel, the levels of inflammatory cytokines increased synergistically with the macrophages/fibroblasts co-culture above the basal production by the macrophages monoculture alone, in both the diabetic cells (db) and their wild type counterparts (c57). There were three different expression patterns between the dbMF group and the c57MF group when compared to their corresponding fibroblasts monocultures (see Tab. 5). We theorized that this difference in the secretion patterns in the co-culture milieu signified that macrophages were indeed interacting with the other cell type (i.e., fibroblasts), which resembled the cell interactions in vivo. Furthermore, the inflammatory cytokines patterns of the db and the c57 primary cells were considerably different in their corresponding macrophages or fibroblasts monocultures, as well as in their co-cultures (see Tab. 6). Evidently, different cytokines patterns were associated with the difference in the cellular behavior of fibroblasts such as proliferation and migration on/into the hydrogel. These results strongly suggested the importance of taking the interactions between different cell types into consideration when investigating the biological mechanisms of the response of cells to implantables in vitro; not only macrophages plus fibroblasts, but also the different pathophysiological status of cells.

Pro-inflammatory cytokines, particularly IL-1 α , IL-1 β , IL-6 and TNF- α , are strongly up-regulated during the inflammatory phase of wound healing [19]; polymorphonuclear leukocytes and macrophages are clearly the major sources of these cytokines. It is very likely that their presence influence various processes at the wound site, including stimulation of fibroblast proliferation, synthesis/degradation of ECM proteins, fibroblast chemotaxis, and modulation of immune responses. The coordinated expression of these cytokines is undoubtedly important for normal wound repair, as indicated by the dramatic reduction of the expressions of these genes after wounding of healing-impaired mice treated with glucocorticoid [20], moreover, the expressions of these cytokines are prolonged in db/db mice [21–22].

In our investigation, both IL-1 and TNF- α were not detected because the cell culture media were first collected on day 3 and the expression of these pro-inflammatory cytokines occurred considerably earlier than day 3, however, high levels of IL-6 and moderate amounts of TNF RI and TNF RII were detected. It was previously shown that the expression of IL-6 was elevated after wounding and tended to persist in chronic wounds [23]. Moreover, IL-6 knock-out mice exhibited severe deficiency in cutaneous wound repair, but this abnormality was completely reversed by the administration of the IL-6 protein before wounding [24], implicating the crucial role of IL-6 in initiating the healing response. A low level of TNF- α promotes wound healing by indirectly stimulating inflammation thus increasing the levels of macrophage-produced growth factors. TNF acts through two distinct cell surface binding receptors, TNF RI and TNF RII; the former is implicated in eliciting fibroblasts proliferation, whereas, the latter initiates signals for thymocyte and cytotoxic T cell proliferation [25]. The results obtained from our investigations showed that the expressions of IL-6 and TNF RII in the dbMF group were the highest compared to the others, we thus theorized that more extensive inflammatory and wound healing responses occurred in the dbMF group as compared to the others; leading to the downstream alterations of the chemokines, CSF and TIMP profiles, eventually mitigating fibroblasts proliferation and migration.

The chemokine superfamily contains more than 45 members and they are divided into four groups depending on the spacing of their first two cysteine residues. The CC chemokine (or β -chemokine) proteins have two adjacent cysteines, near their amino terminus. The two N-terminal cysteines of CXC chemokines (or α -chemokines) are separated by one amino acid, represented with an "X". It is known that wide varieties of chemokines are present at wound sites [26] and they are important modulators of wound healing. Recruitment of macrophages is closely regulated by chemokines [27]. With implant (hydrogel)-stimulated macrophages as

a potential paracrine source of pro-inflammatory factors, the release of chemokines from fibroblasts could lead to further recruitment of macrophages. Conversely, CC chemokines have been shown to influence the release of pro-inflammatory cytokines from macrophages in vitro [29]. Many chemokines are capable of influencing the behaviors of fibroblasts, which are both producers and targets of a variety of chemokines and cytokines upon proper stimulation [30]. It has been demonstrated that the CXC chemokine cCAF could stimulate differentiation of fibroblasts into myofibroblasts and accelerate wound closure [28]. Chemokines attract fibroblasts and are also capable of enhancing their activation, proliferation and migration [31], this is in good agreement with the results of our studies showing the presence of hydrogel in the fibroblasts monoculture producing different cytokines expression patterns comparable to those of the macrophages/fibroblasts co-culture system.

Murine KC and human GRO- α are homologous and it is believed that their expressions during acute or chronic inflammation play important roles in both neutrophil activation and their recruitment to the sites of inflammation [29]. IL-8 and GRO- α (in human) and MIP-2 and KC (in murine) play key roles during wound repair. It has been shown that topical application of either IL-8 or GRO- α to wounds on mouse skin produce favorable effects on re-epithelialization [32]. The results from our in vitro investigation showed that the expression of KC was up-regulated in the dbMF groups, but down-regulated in the c57MF groups when compared with their corresponding fibroblasts monocultures. The potential in vivo significance is that the amplified expression of KC could be beneficial to wound healing and this will be a goal of our future investigations.

The time course of MCP-1 expression has been shown to correlate well with macrophage infiltration, suggesting its role in macrophage recruitment and activation during wound healing [21]; the persistence and late infiltration of both neutrophils and macrophages in the wounds of healing-impaired db/db mice correlated with a surge and sustained induction of MCP-1. It should be pointed out that MCP-1 plays a critical role in the biological responses to biomaterials by promoting macrophage fusion to form foreign body giant cells (FBGC) [33]. The increased productions of MCP-1 and MIP-1 α from a monolayer of fibroblasts in the presence of monocytes have been reported. [34]. The use of fixed cell populations suggested that the MIP-1 α is derived from monocytes and the MCP-1 is derived from both cell populations.

MIP-1 α and MIP-1 γ are the major factors produced by macrophages, they are involved in activating human granulocytes and inducing synthesis of pro-inflammatory cytokines in both fibroblasts and macrophages. In murine, MIP-1 α is the primary stimulator of TNF secretion by macrophages [35], it also doubles as a macrophage activator and induces the production of other inflammatory mediators including MCP-1, MCP-5, etc. The role of MIP-1 α in wound repair has been shown [36], with both MIP-1 α mRNA and proteins detectable in mouse wounds between 12 h and 5 days after wounding; evidently, these time points coincided with maximum macrophage infiltration. Neutralizing MIP-1 α before injury results in a reduction of macrophages at the wound site leading to an eventual reduction in collagen production [36]. The results of our study revealed significant increase of MIP-1 α , MIP-1 γ and MCP-5 in the macrophages/fibroblasts co-culture and it exhibited higher expression of MCP-1 than the fibroblasts monoculture only on day 14; these cytokines expression profiles in the dbMF group were higher than that of the c57MF group at all time-points. This suggested a self-perpetuating event dependent on the interaction between macrophages with fibroblasts. From a pathological point of view, release of CC chemokines from fibroblasts is associated with chronic inflammation, and this is exemplified by interstitial fibroblasts derived from tissue undergoing a Th2 cell-mediated immune response exhibiting higher levels of MCP-1 production, when compared to normal fibroblasts [37]. We therefore theorized that the higher expression of chemokines (i.e., exhibiting inflammatory type reaction) observed in diabetic cells as compared to their wild-type counterpart was due to the pathological dysfunction.

The beneficial effects of GM-CSF on treating both normal and chronic wounds have been demonstrated in a series of animal and clinical studies [38]; moreover, the level of GM-CSF level is elevated in full-thickness excisional wounds [39]. Subcutaneous injections of GM-CSF in DFUs result in quicker resolution of cellulites, trending towards ulcer healing and lower incidence of amputation [40–41]. Wound healing in GM-CSF knockout mice models are significantly delayed; this is also accompanied by reduced IL-6, MCP-1 and MIP-2 productions, consequently, a moderation of recruitment of neutrophils and macrophages and impaired vascularization in the wounds [42]. Using the macrophages/fibroblasts co-culture model in our study, in the db group, we observed GM-CSF released from macrophages/fibroblasts co-culture with a similar but more moderate trend in the c57 wild type co-culture on both day 7 and day 10; we thus theorized that the presence of the hydrogel played a role in vivo by modulating the GM-CSF production leading to exerting its beneficial effects in wound healing; this will have to be investigated in animal models in the future using the hydrogel as a wound dressing.

Our results showed that TIMP-1 had similar temporal expression patterns as MCP-1 within different cell-hydrogel co-culture groups (see Fig. 5 and Fig. 6), implicating a correlation of the expressions of both factors. These results were also consistent with previous research showing MCP-1 enhancing the gene expression of TIMP-1 in human dermal fibroblasts [43].

Overall, we observed the difference in fibroblasts proliferation and migration in the macrophages/fibroblasts co-culture groups as compared to the fibroblasts monoculture group. It could be inferred that this effects was mediated, at least in part, through the chemo-attraction functions of KC, MCP-1, MCP-5, MIP-1 α and MIP-1 γ . Furthermore, the levels of these chemokines were relatively high and their expressions were persistent in the dbMF group; accordingly, both fibroblasts proliferation and migration in the dbMF group were higher than those of the c57MF group. We theorized that the hydrogel stimulated fibroblasts proliferation and migration through modulating the functions of macrophages during the early inflammatory stage. Evidently, the cytokine secretion profiles of the monocultures composed of either fibroblasts or macrophages were altered when the two cell types were co-cultured. That was very likely attributable to the “communications” between different cell types; however, the presence of receptors on these cells could also result in captivating some of these cytokines present in the culture supernatant, thereby evading detection.

5. Conclusion

We have developed an in vitro macrophages/fibroblasts co-culture model suitable for investigating the complex interactions among different cells and implanted biodegradable materials. This model system has contributed to a better understanding on the mechanisms related to the cell-mediated interactions with a biodegradable composed of gelatin and alginate. Our finding has provided quantitative evidence to support the hypothesis that the macrophages/fibroblasts co-culture is capable of modulating cellular behavior through different cytokines, chemokines and growth factors production patterns. Furthermore, this model has the potential of being utilized as a tool for elucidating the differential mechanisms of wound healing in diabetic hosts and efficacy screenings for newly engineered wound dressings designed to treat diabetic chronic wounds.

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Abbreviations

G-CSF	Granulocyte-colony Stimulating Factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
IL-6	Interleukin 6
IL-13	Interleukin 13
KC	CXCL1/keratinocyte-derived chemokine
LIX	CXCL5/LPS-induced chemokine
MCP-1	Monocyte Chemoattractant Protein 1
MCP-5	Monocyte Chemoattractant Protein 5
M-CSF	Macrophage-colony Stimulating Factor
MIP-1 α	Macrophage Inflammatory Protein 1 α
MIP-1 γ	Macrophage Inflammatory Protein 1 γ
PF4	Platelet factor 4
RANTES	Regulated upon activation, normal T-cell expressed, and presumably secreted
TCA-3	CCL1/T-cell activation gene 3
TIMP-1	Tissue inhibitor of metalloproteinases-1
TNF-RI	Tumor necrosis factor-receptor I
TNF-RII	Tumor necrosis factor-receptor II

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Fig. 1.

MTS assay and cell viability

Day 7 * $p < 0.05$, c57F vs. c57MF

Day 10 * $p < 0.05$, dbF vs. c57F and dbMF

Day 14 * $p < 0.01$, dbMF vs. the other three groups, c57F vs. dbF

The data were presented quantitatively as mean $\pm$ standard deviation ($n=3$).

Fig. 2. Cells' migration and 3D distribution in hydrogels (days 7 and 14, one of triplicate results): (A) migration depth, and (B) 3D distribution. Cells migration and spatial distribution were assessed by laser confocal microscopy (LSCM). Random areas on every sample were selected for scanning and each area was observed, profiled and analyzed in its entirety beginning from the surface at 2 μ m increments. Digital planar images (along the X–Y plane) were captured incrementally along the depth (Z-axis) of the sample. Tomographic reconstruction of 3D

images and measurement of cell migration depth were conducted by Zeiss LSCM 510 META software.

Fig. 3.

Inflammatory cytokines/chemokines microarray laser scanning map. The signals of light intensity of the array spots (green fluorescence, Cy3 channel, 555nm excitation, and 565nm emission) were captured using a GenePix 4000B laser scanner. Each cytokine/chemokine antibody was arrayed in quadruplicate (see Tab. 2 for the cytokine/chemokine map). The four spots on the top of the left side of each panel are the positive controls, and the four spots next to the positive controls are the negative controls. There are six standard curve detection panels (from 1 to 6) depicted at the top of first map.

Fig. 4.

Inflammatory cytokines/chemokines concentration (pg/ml) of different cell-hydrogel co-culture groups at different time points. The signals of laser scanning map were extracted with GenePix 6.0 microarray analysis software. Quantitative data analysis was performed using RayBiotech mouse Inflammation Array 1 software (Quantibody-INF-1Q-Analyzer) and the background fluorescence was subtracted from the experimental arrays. Sample concentrations (pg/ml) were determined from mean fluorescence intensities (median values) compared against a five-parameter linear regression standard curves generated from standards provided by the manufacturer (linear regression standard curves). The data were presented quantitatively as mean $\pm$ standard deviation (n=4).

Fig. 5. Isochronal maps of inflammatory cytokines/chemokines concentration of different cell-hydrogel co-culture groups at different time points. The colorful maps were constructed by Matlab software aiming to transform multiple numeric data into simplex graphical patterns. They are visually depicted as three color grid maps: (A) high concentration, and (B) low concentration cytokines pattern of cell culture supernatant of FBs or Ms+FBs cultured with hydrogel; and (C) cytokines pattern of cell culture supernatant of Ms cultured with hydrogel. An intensity scale bar is included at the top of each color grid to indicate the magnitude.

Tab. 1

Cell culture groups and their abbreviations

Cell-hydrogel co-culture system	Cells origin	
	db/db	Wild type
macrophages + Hydrogel	dbM	c57M
fibroblasts + Hydrogel	dbF	c57F
macrophages + fibroblasts + Hydrogel	dbMF	c57MF

Tab. 2

Quantibody® Mouse Inflammation Array 1

	1,2,3,4	5,6,7,8	9,10,11,12
a	POS	NEG	BLC
b	CD30L	Eotaxin	Eotaxin-2
c	Fas L	G-CSF	GM-CSF
d	ICAM-1	IFN- γ	IL-1 α
e	IL-1 β	IL-2	IL-3
f	IL-4	IL-5	IL-6
g	IL-7	IL-10	IL-12p70
h	IL-13	IL-15	IL-17
i	IL-21	KC	Leptin
j	LIX	MCP-1	MCP-5
k	M-CSF	MIG	MIP-1 α
l	MIP-1 γ	PF-4	RANTES
m	TARC	TCA-3	TIMP-1
n	TNF- α	TNF RI	TNF RII

Tab. 3

Positive detection of inflammatory cytokines/chemokines by Quantibody® Mouse Inflammation Array 1 (presented in bold font and underscored).

	1,2,3,4	5,6,7,8	9,10,11,12
a	POS	NEG	BLC
b	CD30L	<u>Eotaxin</u>	Eotaxin-2
c	Fas L	<u>G-CSF</u>	<u>GM-CSF</u>
d	ICAM-1	IFN- γ	IL-1 α
e	IL-1 β	IL-2	IL-3
f	IL-4	IL-5	<u>IL-6</u>
g	IL-7	IL-10	IL-12p70
h	<u>IL-13</u>	IL-15	IL-17
i	IL-21	<u>KC</u>	Leptin
j	<u>LIX</u>	<u>MCP-1</u>	<u>MCP-5</u>
k	<u>M-CSF</u>	MIG	<u>MIP-1α</u>
l	<u>MIP-1γ</u>	<u>PF-4</u>	<u>RANTES</u>
m	TARC	<u>TCA-3</u>	<u>TIMP-1</u>
n	TNF α	<u>TNF RI</u>	<u>TNF RII</u>

Tab. 4

Classification of inflammatory cytokines detected positive in the microarray

Pro-inflammatory factors		IL-6, IL-13, TNF-RI, TNF-RII
Chemokines	CXC family	KC, PF-4, LIX
	CC family	MCP-1, MCP-5, MIP-1 α , MIP-1 γ , RANTES, Eotaxin, TCA-3
Colony stimulating factors		G-CSF, GM-CSF, M-CSF
MMPs/TIMP-1		TIMP

Tab. 5

Summary of the regulatory patterns of inflammatory cytokines: comparison of MF co-culture and M & F monocultures.

		dbMF vs. dbF	c57MF vs. c57F	MF vs. M
Pro-Inflammatory factors	IL-6	↑	↓	↑
	TNF RII	↑	↓	
	TNF RI		↓	↑
	IL-13		↓	↑
CXC chemokines	KC	↑	↓	↑
	LIX		↓	
	PF-4		↑	↑
CC chemokines	MCP-5		↑	
	MIP-1 α		↑	
	MIP-1 γ		↑	↑
	MCP-1		↓	↑
	RANTES		↓	
	Eotaxin		↓	
	TCA-3		↓	
CSF	G-CSF	↑	↓	↑
	GM-CSF	↑	↓ day 3, 14 ↑ day 7, 10	↑
	M-CSF		↓	
MMP/TIMP	TIMP-1		↓	

↑: up-regulation in co-culture group, ↓: down-regulation in co-culture group, $p < 0.05$

Tab. 6

Inflammatory cytokines microarray results: comparison of db primary and c57 primary cell groups

		dbMF vs. c57MF	dbM vs. c57M
Pro-inflammatory factors	IL-6	↓3, ↑7-14	↑
	IL-13	↓3, ↑7	
	TNF RI	↑	↑
	TNF RII	↑	↑
CXC chemokines	KC	↑	↑
	LIX	↑	
	PF-4	↑	↑
CC chemokines	MCP-1	↑	
	MCP-5	↑	
	MIP-1 α	↑	↑
	MIP-1 γ	↑	↑
	RANTES	----	
	Eotaxin	↑	
	TCA-3	↓	
CSF	G-CSF	↑	↑
	GM-CSF	↑	↑
	M-CSF	↓	
MMP/TIMP-1	TIMP-1	↑	

↑: up-regulation in db group, ↓: down-regulation in db group, p<0.05