Biomaterials 128 (2017) 160–171

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Development of fusogenic glass surfaces that impart spatiotemporal control over macrophage fusion: Direct visualization of multinucleated giant cell formation

James J. Faust [a, b], Wayne Christenson [c, d], Kyle Doudrick [e], Robert Ros [c, d], Tatiana P. Ugarova [a, b, *]

[a] School of Life Sciences, Arizona State University, Tempe, AZ 85287, USA
[b] Center for Metabolic and Vascular Biology, Mayo Clinic, Scottsdale, AZ 85259, USA
[c] Department of Physics, Arizona State University, Tempe, AZ 85287, USA
[d] Center for Biological Physics, Arizona State University, Tempe, AZ 85287, USA
[e] Department of Civil and Environmental Engineering and Earth Sciences, University of Notre Dame, Notre Dame, IN 46556, USA

ARTICLE INFO

Article history:
Received 25 October 2016
Received in revised form
21 February 2017
Accepted 26 February 2017
Available online 2 March 2017

Keywords:
Macrophage fusion
Multinucleated giant cells
Foreign body reaction
Microscopy
Live imaging
Mac-1 integrin

ABSTRACT

Implantation of synthetic material, including vascular grafts, pacemakers, etc. results in the foreign body reaction and the formation of multinucleated giant cells (MGCs) at the exterior surface of the implant. Despite the long-standing premise that fusion of mononucleated macrophages results in the formation of MGCs, to date, no published study has shown fusion in context with living specimens. This is due to the fact that optical-quality glass, which is required for the majority of live imaging techniques, does not promote macrophage fusion. Consequently, the morphological changes that macrophages undergo during fusion as well as the mechanisms that govern this process remain ill-defined. In this study, we serendipitously identified a highly fusogenic glass surface and discovered that the capacity to promote fusion was due to oleamide contamination. When adsorbed on glass, oleamide and other molecules that contain long-chain hydrocarbons promoted high levels of macrophage fusion. Adhesion, an essential step for macrophage fusion, was apparently mediated by Mac-1 integrin (CD11b/CD18, $\alpha_M\beta_2$) as determined by single cell force spectroscopy and adhesion assays. Micropatterned glass further increased fusion and enabled a remarkable degree of spatiotemporal control over MGC formation. Using these surfaces, we reveal the kinetics that govern MGC formation *in vitro*. We anticipate that the spatiotemporal control afforded by these surfaces will expedite studies designed to identify the mechanism(s) of macrophage fusion and MGC formation with implication for the design of novel biomaterials.

1. Introduction

Implantation of synthetic materials, including vascular grafts, pacemakers and other medical devices results in the foreign body reaction at the exterior surface of the implant. Chronic inflammation during the end stages of the foreign body reaction causes macrophages to fuse and form MGCs. Once formed, MGCs make destructive cellular products that clear the foreign body (e.g. MMP-9, superoxide, cathepsin K, etc.). These potent cellular products etch surfaces and promote stress fractures that can eventually lead to failure of pacemakers [1] or synthetic implantable biomaterials [2,3]. Thus, the existence of MGCs on the implant surface is thought to be detrimental to the long term function of the device [4–8].

In order for macrophages to fuse, monocytes recruited from peripheral blood to sites of inflammation differentiate into macrophages that subsequently fuse as inflammation progresses to the chronic state. The T helper 2 cytokine interleukin-4 (IL-4) promotes macrophages fusion *in vivo* [9] and when applied in cell culture can be used to study monocyte/macrophage fusion [10]. Although this *in vitro* cell system has proven invaluable to our understanding of the molecular mediators that orchestrate macrophage fusion, there is a surprising paucity of data regarding the morphological changes that macrophages undergo during fusion as well as the cellular mechanisms that govern this process. In fact, despite several long-standing predictions that purportedly account for the mechanisms

* Corresponding author. School of Life Sciences, Arizona State University, Tempe, AZ 85287, USA.
E-mail address: Tatiana.Ugarova@asu.edu (T.P. Ugarova).

http://dx.doi.org/10.1016/j.biomaterials.2017.02.031
0142-9612/

of macrophage fusion, no published study to date has shown the formation of a MGC in context with living specimens.

This deficiency is primary due to the fact that most high resolution techniques in optical microscopy require glass as substrate. However, glass surfaces are known to support very low levels of macrophage fusion in the presence of IL-4 despite robust adhesion [11]. When macrophage fusion does occur on glass, it is impossible to predict where and at what time macrophages will fuse, since increased magnification decreases the field of view. Consequently, if the goal is to observe macrophage fusion with living specimens then low magnification objectives and long imaging durations are necessary in order to capture rare fusion events. On the other hand, plastic surfaces (e.g. Permanox) are known to support macrophage fusion in the presence of IL-4 [12], and currently serve as the gold standard for *in vitro* analysis of MGC formation [13]. However, the problem with most plastic substrates is that changes in refractive index lead to chromatic aberration which is accentuated by substrate thickness. Further, birefringent properties of most plastic substrates make techniques that exploit polarity of light impossible. Finally, most plastic is not compatible with the use of high numerical aperture objectives. If plastic is used, the only technique that can be successfully employed is low-resolution phase-contrast and only when long working distance or low magnification objectives are used. These barriers have restricted studies to fixed specimens and have thwarted our ability to utilize the large number of imaging techniques that rely on optical-quality glass for image formation.

Here we describe fabrication of optical-quality glass surfaces that exploit adsorption of molecules containing long-chain hydrocarbons. Glass surfaces adsorbed with these substances promote extraordinary rates of macrophage fusion and adhesion is mediated in part by Mac-1 integrin ($\alpha_M\beta_2$, CD11b/CD18, CR3). Micropatterning glass with the aforementioned substances leads to a further increase in macrophage fusion and enables a high degree of spatiotemporal control over the formation of MGCs. For the first time, we utilize living specimens to reveal the sequence of events that result in MGC formation via macrophage fusion. We show that MGC formation is a non-linear process that requires a lag-phase and involves three types of fusion events. Moreover, macrophage fusion occurs between intercellular margins, but not through the previously proposed “cellocytosis” mechanism. We anticipate that the spatiotemporal control afforded by this surface may expedite fundamental studies related to the mechanism of macrophage fusion. Furthermore, a better understanding of this process represents a significant step towards the design of biomaterials that are resistant to inflammatory responses evoked by MGCs.

2. Materials and methods

2.1. Mice

C57BL/6J and Mac-$1^{-/-}$ (B6.129S4-*Itgam*^{tm1Myd}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). LifeAct mice [14] were a generous gift from Dr. Janice K. Burkhardt and used with permission from Dr. Roland Wedlich-Söldner. Mac-$1^{-/-}$ mice were housed in sterile cages. All mice were given *ad libitum* access to food and water and colonies were maintained at a constant temperature of 22 °C on a 12 h light/dark cycle. All procedures were performed in accordance with the animal protocols approved by the Institutional Animal Care and Use Committee at the Arizona State University and the Mayo Clinic.

2.2. Macrophage isolation and cell culture

Macrophages were isolated from the peritoneum 72 h after injection of a 0.5 mL sterile 4% solution of Brewer's thioglycollate (Sigma Aldrich, St. Louis, MO). Mice were humanely sacrificed according protocols approved by Mayo Clinic and ASU Animal Care and Use Committees. Immediately following euthanasia, 5 mL of an ice-cold solution of phosphate-buffered saline (PBS, pH 7.4) supplemented with 5 mM ethylenediaminetetraacetate was injected aseptically into the peritoneum, the lavage containing cells was collected, and the cells were counted with a Neubauer hemocytometer. Low passage number ($\leq$10) human embryonic kidney 293 cells (HEK293) stably expressing human Mac-1 integrin and wild-type HEK293 cells were previously described [15]. The cells were cultured in the complete DMEM:F12 medium and detached from the culture dish by incubation in Cellstripper™ (Cellgro, Manassas, VA). The cells were washed and then resuspended in the complete DMEM:F12 medium.

2.3. IL-4-induced macrophage fusion

Peritoneal lavage cells were applied at a concentration of 5×10^5 cells/mL and spread evenly across the glass insert (23.5 mm) of a 35 mm Fluorodish (FluoroDish; World Precision Instruments, Sarasota, FL). Where applicable, cell number was scaled linearly to account for changes in surface area. Cells were maintained in an incubator (5% CO_2 at 37 °C) for 30 min and nonadherent cells were removed and adherent macrophages were washed 3–5 times with Hank's balanced salt solution (HBSS; Cellgro, Manassas, VA) containing 0.1% bovine serum albumin (BSA). Cells were incubated in DMEM:F12 (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biological, Atlanta, GA) and 100 I.U./mL penicillin, 100 μg/mL streptomycin (Cellgro, Manassas, VA). After 2 h, the medium was removed and fresh medium supplemented with 10 ng/mL of interleukin-4 (IL-4; Genscript, Piscataway, NJ) was applied to the culture to induce fusion. In this study, the application of IL-4 is considered $t = 0$. At the respective time points, cultures were washed 2 times with PBS and incubated with ice-cold methanol for 2 min. The fixed cultures were placed in an oven at 60 °C until dried. Wright's stain (Sigma Aldrich, St. Louis, MO) was applied for 8 min and the stain was removed and substituted with fresh buffered Wright's stain (pH 6.5) for an additional 8 min. The cover glass was placed under running deionized water to remove unbound stain and dried in an oven at 60 °C. The cover glass was mounted in Permount™ (Thermo Fischer Scientific, Waltham, MA), the mountant was air dry, and the specimens were imaged with bright field optics. The extent of MGCs formation was evaluated by determining the fusion index. The fusion index is defined as the number of nuclei present in fused macrophages divided by the total number of nuclei in both fused and non-fused macrophages. Three to 5 bright field images collected at 20× objective magnification were analyzed per specimen.

2.4. Adhesion assays

Cell adhesion was determined by plating 2.5×10^5 cells in 35 mm dishes. Care was taken to ensure that cells spread evenly across the entire surface area of the dish for each experimental condition. After 25 min at 37 °C in 5% CO_2, cell images were captured before washing in order to standardize the number of cells in the field of view. Three phase contrast images (20× objective magnification) corresponding to the center of the dish, the edge of the dish and the center-edge midpoint were collected for each sample. At 30 min, the specimens were washed gently 3 times with HBSS supplemented with 0.1% BSA and 3 images were collected as described above. Adhesion was calculated by dividing the average number of adherent cells after washing by the average number of total cells before washing. For inhibition experiments, Mac-1