Biomaterials 33 (2012) 7497–7507

Contents lists available at SciVerse ScienceDirect

Biomaterials

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

Senescence and quiescence induced compromised function in cultured macrophages

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ARTICLE INFO

Article history:
Received 22 June 2012
Accepted 30 June 2012
Available online 17 July 2012

Keywords:
Macrophage
Foreign body response
Phagocytosis
Senescence
Quiescence
In vitro culture

ABSTRACT

Implants are predisposed to infection even years after implantation, despite ostensibly being surrounded by innumerable macrophages as part of the host foreign body response. The local implant environment could adversely influence the implant-associated macrophage phenotype, proliferative capacity, activation states, and ability to neutralize pathogens. This study monitored cultured macrophage proliferative states and phagocytotic competence on tissue culture plastic to address the hypothesis that extended contact with foreign materials alters macrophage phenotype. That such macrophage alterations might also occur around implants has significance to the foreign body response, infection, cancer, autoimmune and other diseases. Specifically, multiple indicators of macrophage proliferation in various culture conditions, including cell confluence, long-term culture (21 days), lipopolysaccharide (LPS) stimulation, passaging, and mitogenic stimulation are reported. Importantly, primary murine macrophages became quiescent at high confluence and senescent during long-term culture. Senescent macrophages significantly reduced their ability to phagocytose particles, while quiescent macrophages did not. Cell senescence and quiescence were not observed with repeated passaging. Primary macrophage stimulation with LPS delayed senescence but did not eliminate it. These results prompt the conclusion that both cell quiescence and senescence are observed under common macrophage culture conditions and could alter macrophage behavior and phenotypes in extended in vitro culture, such as the ability to phagocytose. Such macrophage transitions around foreign bodies in vivo are not documented: quiescence and senescence reported here in macrophage culture could be relevant to macrophage behavior both in vitro in bioassays and in vivo in the foreign body response and implant-centered infection.

1. Introduction

Macrophages play a primary role in modulating the foreign body response, immediately localizing to surfaces of every implanted material [1]. At the implant site, they are responsible for removing cell debris, foreign bodies and pathogens. After acute phase inflammation subsides, macrophages may reside at implant surfaces throughout the duration of the implantation, possibly for decades [2,3], in some case producing multi-cellular macrophage layers around monolithic implants [1,4], completely infiltrating porous implants [5], and fusing to form foreign body giant cells at these surfaces [1,4,6–8]. That any of these commonly observed chronic responses result from macrophage in situ proliferation versus continual new cell recruitment is not clear. However, a recent study found that during T helper 2 (Th2) inflammation, macrophages were capable of undergoing rapid proliferation in vivo [9]. Importantly, changes in their resident phenotypes, functional competence and capabilities to address infection risk over this implant duration, prompted by or correlated with their prolonged exposure and reaction to a foreign body (e.g., implant) are largely unknown.

Despite macrophage persistence at surfaces of implanted materials, implants retain substantial infection risk even years after implantation [10,11]. This may be due to the fact that unlike host tissue that is continuously renewed, thereby limiting opportunities for bacterial colonization, tissue surrounding implanted materials remains relatively unchanged, encapsulated in fibrous scar tissue [1,12–14]. This suggests that while abundant macrophages are present, they may be transformed by their chronic reactions to implants into states of relative inactivity, incapable of addressing

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http://dx.doi.org/10.1016/j.biomaterials.2012.06.099

microbial presence as effectively as during initial implant site recruitment.

Many cells in normal tissue are quiescent, a reversible, viable, non-dividing state-of-rest. Importantly, quiescent cells can be stimulated to divide [15,16]. Cells can also become senescent, a viable but irreversible non-dividing state that cannot be overcome even with mitogenic stimuli [17]. Senescent and quiescent cells are distinguished by altered patterns of gene expression [18,19]. Senescent and quiescent transitions in macrophages at implant surfaces could explain their inability to adequately address bacterial infection in vivo in this context.

Previous studies have demonstrated a decreased phagocytic ability in aged macrophages [20] and a susceptibility of cells under oxidative stress to senesce [20,21]. That macrophages demonstrate increased intracellular reactive oxygen species with age [22] and reside in high oxidative stress environments surrounding foreign bodies [13] could indicate their propensity to senesce and their subsequent incompetence to phagocytose pathogens at implant surfaces over time. Interestingly, foreign body giant cells, the chronic multinucleated macrophage-derived phenotypic hallmark surrounding implanted materials, also display decreased phagocytic ability [23], and increased lysosomal activity [23,24], consistent with senescent cells [25] also known to multinucleate [26]. Macrophages have also been purported to undergo frustrated phagocytosis, an exhausting metabolic phenomenon that could compel macrophages to senesce around implants [1,4,7,8]. However, macrophage senescence and phagocytosis around chronically implanted foreign bodies or in long-term cultures on materials remains unaddressed in current literature.

Cultured macrophages are commonly employed in assays seeking information on aspects of their involvement in pathologies such as cancer, autoimmune diseases, and the foreign body response [27–32]. As an immunomodulatory cell, macrophages are highly susceptible to telomere attrition [22], increasing their potential to senesce. However, they are not commonly assayed for this phenotype. As both quiescence and senescence alter cell genetic profiles [18,19], macrophage transitions to these states during in vitro culture likely influence assay outcomes, potentially leading to false conclusions, irreproducible results, and inconsistencies, especially when compared to in vivo phenotypes they intend to mimic. Maintenance of consistent macrophage phenotypes and activation states between in vivo and in vitro conditions is likely critical to ensuring proper in vitro model fidelity. Therefore, understanding the possible consequences of macrophage senescent and quiescent transitions has important implications both in vitro and in vivo.

This study identified proliferation states for both primary and secondary macrophages in several experimental culture conditions, including cell confluence, culture time, passage number, and biochemical stimulation. Cultured macrophage capacity to phagocytose in quiescent and senescent states raises important questions about macrophage phenotypic competence in extended contact with materials. Should this behavior also be observed in vivo, it has important implications for implanted biomaterials in the context of the foreign body response.

2. Methods and materials

2.1. Cell culture

2.1.1. Immortalized RAW cell culture

Macrophage-like transformed murine cell line RAW 264.7 was purchased from the American Type Culture Collection (TIB-71, ATCC, Manassas, USA) and cultured in 96-well tissue culture-treated polystyrene plates (BD Falcon, San Jose, USA), unless otherwise specified, at 37 °C with 5% supplemental CO_2 according to the experiments detailed below. All RAW cells were used below passage 10 after purchase, unless passage number was explicitly specified. RAW cells were passaged by scraping with a rubber scraper (Starstedt, Newton, USA). Cells were always cultured in complete media (Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), and 1% antibiotic/antimycotic, Invitrogen, Carlsbad, USA). Full media exchanges were performed every other day.

2.1.2. Murine primary cell sourcing

Specific pathogen-free, 2–3 month-old male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, USA). Animals were kept in the University of Utah animal facility, and provided water, mouse chow, bedding, and modes of enrichment ad libitum throughout this study. For primary macrophage harvests, animals were euthanized via CO_2.

2.1.3. Primary macrophage cell culture

Bone marrow cells were collected from the femurs and tibias of 4–5 month-old euthanized male C57BL/6 mice and differentiated into bone marrow macrophages (BMMΦs) using a previously described method [33,34]. On day 7, cells were removed from surfaces by rinsing cells 3X and incubating them in Ca^{+2}/Mg^{+2}-free phosphate buffered saline (PBS, Invitrogen) for 30 min at 37 °C, and then rinsed from the surface using a 1 ml pipette tip, and collected. Cells were counted using a hemocytometer and cultured in 96-well tissue culture-treated polystyrene plates (BD Falcon, San Jose, USA), unless otherwise specified, at 37 °C with 5% supplemental CO_2 according to the experiments outlined below. At least an equal volume of complete BMMΦ media (DMEM with 10% heat-inactivated FBS, 10% L929-conditioned media, 1% antibiotic/antimycotic, 1% MEM nonessential amino acids, 1% HEPES, and 1% sodium pyruvate, Invitrogen) was added to the suspended cells after plating, and fresh media was replaced every 2–3 days. Unless otherwise specified, all BMMΦs were passaged once after their differentiation for experimental use.

2.2. In vitro culture conditions for senescence examination

2.2.1. Cell confluence

Immortalized RAW macrophages were plated at densities of 5×10^3, 1×10^4, 2×10^4, 4×10^4, and 8×10^4 cells/well, and primary macrophages were plated at densities of 5×10^3, 1×10^4, 2×10^4, 4×10^4, 8×10^4, and 1.6×10^5 cells/well and cultured for 24 h prior to fixing. To determine cell senescence versus quiescence, an equivalent cell density of 1.60×10^5 cells/well was cultured in a 30-mm Petri dish (BD Falcon) in parallel and passaged and plated at low confluence. These cells were then cultured for 7 days further (seen previously to be the time for maximum proliferation). Both control media and media with 50% serum (to encourage growth) were utilized to confirm macrophage proliferative capacity.

2.2.2. Comparisons of long-term macrophage cultures

Primary and immortalized macrophages were plated at 5×10^3 cells/well and cultured for 1, 2, 3, 5 and 7 days for secondary RAW 264.7 macrophages and 1, 2, 3, 5, 7, 10, 12, 14, 17, 19, and 21 days for primary BMMΦs prior to fixing. To determine senescence or quiescence in primary macrophages, a 30 mm Petri dish (BD Falcon) with the same cell seeding density as the long-term experiment was passaged on Day 21 and plated at 5×10^3 cells/well and analyzed for proliferation 5, 7, and 10 days later (i.e., the time at which the greatest proliferation during the initial 21 days was seen). Both control media and media with 50% serum (to encourage proliferation) were utilized to confirm macrophage proliferative capacity.

2.2.3. Lipopolysaccharide (LPS) treatment of cell cultures

For LPS-treated conditions, primary and immortalized macrophage cultures were treated with their respective media supplemented with 1 μg/ml LPS replaced every 2–3 days until the end of the experiment (i.e., 7 days for RAWs and 21 days for BMMΦs). This concentration was selected because it has been shown to effectively activate macrophages [35,36]. RAW cells were also stimulated with LPS during studies of increasing confluence (details listed above).

2.2.4. Cell passaging

RAW 264.7 macrophages were cultured in 30-mm tissue culture-treated polystyrene Petri dishes (BD Falcon, San Jose, USA) and passaged 30 times and subsequently plated into 96-well plates and assayed for senescence and proliferation markers. Primary BMMΦs were cultured in 100-mm tissue culture-treated polystyrene Petri dishes (BD Falcon), passaged and plated into 96-well plates for subsequent characterization. This was repeated up to 10 passages, where passage 1 was the first passage after differentiation. Passages were fixed after 24 h of culture for characterization. Both primary and secondary macrophage culture passages were performed every other day, to allow stock cultures sufficient time to properly adhere before serial passaging.

2.2.5. Cell culture biochemical stimulation

Primary BMMΦs were plated at 5×10^3 cells/well and cultured for 21 days and then treated for 48 h with cytokines IFN-gamma, IL-6, MCP-1, TNF, GM-CSF, MIP-1β, MIP-1α, IL-4, RANTES, and IL-10, and mitogens TGF-β, IL-1β, MCP-1, and also 50% and 100% serum prior to fixing. This 48-h incubation period was selected as the time reported for quiescent cells to reactivate [37].