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In vitro response of macrophage polarization to a keratin biomaterial

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ABSTRACT

Macrophage response to biomaterials is emerging as a major focus in tissue repair and wound healing. Macrophages are able to differentiate into two distinct states, eliciting divergent effects. The M1 phenotype is considered pro-inflammatory and up-regulates activity related to tissue destruction, whereas the M2 phenotype is considered anti-inflammatory and supports tissue remodeling. Both are necessary but a fine balance must be maintained as dysregulation of naïve macrophages to M1 or M2 polarization has been implicated in several disease and injury models, and has been suggested as a potential cause for poor outcomes. Keratin biomaterials have been shown using different animal models to promote regeneration in several tissues. A potential common mechanism may be the general capability for keratin biomaterials to elicit beneficial inflammatory responses during the early stages of regeneration. In the present study, a keratin biomaterial was utilized in vitro to examine its effects on polarization toward one of these two macrophage phenotypes, and thus its role in inflammation. Exposure of a monocytic cell line to keratin biomaterial substrates was shown to bias macrophages toward an M2 phenotype, while a collagen control surface produced both M1 and M2 macrophages. Furthermore, keratin treatment was similar to the M2 positive control and was similarly effective at down-regulating the M1 response. Keratin biomaterial influenced greater production of anti-inflammatory cytokines and decreased amounts of pro-inflammatory cytokines. The use of a keratin biomaterial in regenerative medicine may therefore provide additional benefit by regulating a positive remodeling response.

1. Introduction

Biomaterials have many current and potential applications, including those in tissue engineering and regenerative medicine (TE/RM). Regardless of their intended use, all biomaterials elicit a reaction from their host, the foreign body response, which exerts a great influence over the degree of success or failure in TE/RM applications. Prominent participants in the response of the body to an implanted biomaterial are macrophages. Exposure to implanted materials generally causes macrophages to fuse into multinucleated giant cells, which ultimately leads to fibrous encapsulation and scar tissue formation around the implant [1,2]. Multinucleated giant cells are generally associated with chronic inflammation and, depending on the signals encountered within the environment, can arise from both ends of the macrophage phenotype continuum [2–4]. Within the context of TE/RM, it has been demonstrated that adherent macrophages on biomaterials (precursors to foreign body giant cells) revealed a profile that was neither M1- nor M2-polarized but somewhere in the middle [2]. While many strategies aim to avoid this process and the host immune response completely, macrophages have recently emerged in a different light as an important component of the innate immune system that can modulate and attenuate tissue remodeling following injury [5–8]. More recently it has been suggested that the key to tissue regeneration approaches may be the concept of regulating the balance between two distinctly different sub-types of macrophages.

The general utility of keratin biomaterials has been described by several investigators for applications such as drug delivery, tissue regeneration, hemostasis and wound healing [9–24]. Three general findings have been reported: excellent biocompatibility, cell adhesion and improved tissue healing. As early as 1982, scientists reported work on the general biocompatibility of wool-based keratin biomaterials [25]. This Japanese-language publication describes the preparation of both oxidized and reduced, solubilized keratins that were used to coat polyester meshes with a glutaraldehyde-crosslinked film of keratin biomaterial prior to implantation into the dorsal muscle of dogs and rabbits. After 2, 4 and 6 weeks, the implants were scored for degree of foreign body reaction by examining histological sections. The investigators found that the degree of foreign body reaction was low in all cases, with no

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apparent distinction between the different forms of keratin biomaterials used in the study. More recently, several authors have expanded on this initial finding of good biocompatibility in papers utilizing a variety of in vitro and in vivo model systems, sometimes by blending keratin with other biomaterials [13,15,26–32]. Cell adhesion to keratin substrates has also been demonstrated by several authors [33–37], and tissue healing (i.e. regeneration) studies have included skin, bone, nerve, cornea and heart, with consistent findings of improved tissue repair and little notable scar formation reported [12,13,16,17,23].

Based on our group's earlier experience in peripheral nerve regeneration [11,14,22,23], we undertook a pilot study to investigate the potential for a keratin biomaterial hydrogel to facilitate neuronal regeneration in the spinal cord [38]. Along with several observations demonstrating improved functional recovery, the data from this study suggested that downstream tissue damage normally seen due to the inflammatory cascade was mitigated by keratin biomaterial treatment. Interestingly, Kigerl et al. demonstrated that these secondary injury mechanisms in the spinal cord are dominated by a pro-inflammatory M1 macrophage phenotype, a response that overpowers the relatively smaller and transient anti-inflammatory M2 macrophage phenotype [39]. Limited staining of the spinal cord tissue from the aforementioned pilot study revealed a strong M2 presence and a notably smaller M1 population. Other studies have shown that a keratin-based implant such as a hydrogel quickly becomes infiltrated with resident cells, but that a classical foreign body reaction does not ensue, overall cell population decreases, and the relatively small, initial inflammatory response resolves itself quickly [30,31]. These observations suggest that keratin biomaterials may be influencing the cellular response to tissue injury, particularly inflammation. Based on this previous research, we postulated that keratin biomaterials may be capable of inducing macrophage polarization at sites of injury, and that this may represent a common mechanism that is partly responsible for the beneficial tissue regeneration reported by different investigators around the world, including our group.

The purpose of the current study was to investigate the role of macrophage response in keratin's capacity as a regenerative biomaterial. We hypothesized that keratin can contribute to macrophage polarization, and ultimately tissue regeneration, by favoring the growth- and regeneration-promoting M2 phenotype. To examine this, an in vitro culture system employing a human monocytic cell line was used to determine the relative ratio of M1 and M2 macrophage phenotypes that arise at different time points following growth on a keratin biomaterial substrate, as well as cytokines secreted by these cells, compared to cells grown on tissue culture plastic (TCP) and collagen substrates.

2. Materials and methods

2.1. Preparation of keratin biomaterial and coatings

The keratin biomaterial was extracted and prepared as previously described [11,31,38]. Briefly, a 2% peracetic acid solution was used to oxidize human hair fibers. Following washing with deionized (DI) water to remove residual oxidant from the hair fibers, tris(hydroxymethyl)-aminomethane (Tris) base and DI water was used to extract the soluble keratin proteins. The solution was then dialyzed against DI water, neutralized to pH 7.4 with NaOH, lyophilized and ground into a powder. The keratin powder was sterilized via exposure to a 25 kGy dose of γ-irradiation and aseptically reconstituted in phosphate-buffered saline (PBS). Keratin and type-I rat tail collagen ($\geqslant$90% purity; BD Biosciences) were diluted to a final concentration of 200 $\mu g\ ml^{-1}$ and 1 ml of these respective solutions was added to the wells of glass chamber slides (Nunc, Thermo Fisher Scientific) and incubated for 24 h at 37 °C to form coatings. After incubation, excess solution was removed and the coated surface rinsed with PBS prior to cell seeding.

2.2. Human macrophage cell culture

The THP-1 human monocytic cell line was obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 (Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 0.05 mM 2-mercaptoethanol (Sigma). To generate adherent THP-1-derived macrophages (TDM), 1×10^6 cells were added to wells in an untreated TCP six-well plate (Becton Dickinson) and treated with 5 $ng\ ml^{-1}$ of phorbol myristate acetate (PMA; Sigma) dissolved in media for 48 h at 37 °C, 5% CO_2. Macrophage phenotype ($CD14^+$) was confirmed using flow cytometry (data not shown), and for the purposes of this study, will be considered as having an M0 phenotype. TDMs were then washed with PBS, detached using 0.25% trypsin/0.1% EDTA (HyClone), pelleted and resuspended in complete media. 1×10^6 TDMs were then plated and reattached on corresponding substrates of the glass chamber slides (Table 1). For control treatments, TDMs were induced to a polarized phenotype by culturing cells with either lipopolysaccharide (LPS, 100 $ng\ ml^{-1}$; Sigma) and human recombinant (hr) interferon gamma (IFNγ, 20 $ng\ ml^{-1}$; Sigma) to produce M1 macrophages, or hr interleukin 4 (IL-4, 20 $ng\ ml^{-1}$; Sigma) to produce M2 macrophages in glass chamber slides [40]. Media, including that of the control treatments that contained cytokines, were changed every 3 days. TDM M0 macrophages were produced by incubating to their respective time points in the presence of complete media only (Table 1, no coating treatment group).

2.3. Immunocytochemistry

All stains were performed at room temperature (RT), manually, using an optimized double-immunofluorescence technique. Briefly, macrophages cultured in glass chamber slides were washed with PBS, fixed in 4% paraformaldehyde for 20 min at RT and washed with a buffer containing 0.1% bovine serum albumin (BSA) in 1$\times$ PBS. After blocking non-specific staining for 45 min (10% BSA), the first primary antibody was added (CD86 for M1 specificity, 10 $\mu g\ ml^{-1}$; R&D Systems) and incubated for 1 h at RT. After washing, the secondary antibody was added (NL-557, 1:200; R&D Systems) and incubated in the dark for 1 h. After rinsing with the wash buffer, the second primary antibody was added to the wells (CD206 for M2 specificity, 15 $\mu g\ ml^{-1}$; R&D Systems) and incubated for 1 h. Cells were incubated with the final secondary antibody (NL-493, 1:50; R&D Systems) for an additional hour, washed and the gasket removed from the slide. Slides were mounted with ProLong® Gold Antifade (Life Technologies) mounting media and visualized using a Zeiss LSM510 inverted confocal microscope.

2.4. Macrophage quantitative analysis

Quantitative analysis of CD86+(M1), CD206+(M2), CD86+/CD206+(M1/M2; co-expressing phenotype) and CD86−/CD206−(M0) cells for each treatment group at each time point

Table 1
Time points and treatment culture conditions for TDMs. $n = 6$ was analyzed for each condition at each time point.

Time points	Treatment conditions
24 h, 3 days, 7 days, 14 days	Keratin coating Collagen coating No coating LPS/IFNγ IL-4