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Effects of delayed delivery of dexamethasone-21-phosphate via subcutaneous microdialysis implants on macrophage activation in rats

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ABSTRACT

Macrophage activation is of interest in the biomaterials field since macrophages with an M(Dex) characteristic phenotype, i.e., $CD68^{+}CD163^{+}$, are believed to result in improved integration of the biomaterial as well as improved tissue remodeling and increased biomaterial longevity. To facilitate delivery of a macrophage modulator, dexamethasone-21-phosphate (Dex), microdialysis probes were subcutaneously implanted in male Sprague–Dawley rats. Dex localized delivery was delayed to the third day post implantation as a means to alter macrophage activation state at an implant site. To better elucidate the molecular mechanisms associated with M(Dex) macrophage activation, CCL2 was quantified in dialysates, gene expression ratios were determined from excised tissue surrounding the implant, histological analyses, and immunohistochemical analyses (CD68, CD163) were performed. Delayed Dex infusion resulted in the up-regulation of IL-6 at the transcript level in the tissue in contact with the microdialysis probe and decreased CCL2 concentrations collected in dialysates. Histological analyses showed increased cellular density as compared to controls in response to delayed Dex infusion. Dex delayed infusion resulted in an increased percentage of $CD68^{+}CD163^{+}$, M(Dex), macrophages in the tissue surrounding the microdialysis probe as compared to probes that served as controls.

1. Introduction

Millions of different types of biomaterials (artificial heart valves, breast implants, implanted biosensors, pacemakers, and prosthetic joints) are implanted worldwide every year. Each of these different biomaterials elicits a foreign body reaction (FBR) which is an immune response to the implanted biomaterial [1]. While normal wound healing consists of hemostasis, inflammation, proliferation and remodeling, the FBR consists of acute inflammation, chronic inflammation, and the eventual fibrotic encapsulation of the biomaterial. This encapsulation results in the biomaterial being 'walled off' from the rest of the body, residing in its own microenvironment. For many biomaterials, this encapsulation poses no significant clinical concerns, but for other materials such as sensors, this encapsulation proves to be detrimental resulting in loss of function [2]. For this reason, much effort has been put into controlling or altering the FBR to eliminate or reduce the formation of the fibrotic encapsulation.

Macrophages play a role in both innate and adaptive immunity and can phagocytize foreign materials including microbes and cellular debris [3]. Beyond being phagocytic cells, macrophages play dueling roles in both driving and resolving inflammation, antigen presentation vs. scavenging, and tissue destruction vs tissue remodeling [4]. Macrophages have been historically-identified as playing a critical role in the outcome of implanted biomaterials and their FBR [5].

Macrophages are highly plastic cells exhibiting a wide range of phenotypes [6]. Macrophage activation is a term used to describe the ability of macrophages to change phenotypes in response to biochemical signals [7,8]. Mills identified this phenotypic change as macrophage polarization and identified the extremes of these phenotypes along this plastic continuum as either M1 or M2 [9]. However, it is important to note that macrophage activation exhibits a continuum of states. M1 macrophages are classically activated by lipopolysaccharide (LPS), interferon gamma (IFN-γ), or tumor necrosis factor alpha (TNF-α). M1 macrophages secrete pro-inflammatory cytokines (IL-1β, IL-6, IL-12, and TNF-α) and high concentrations of nitric oxide [8,10]. M1 macrophages are characterized as being pro-inflammatory, highly microbicidal, and efficient antigen presenting cells expressing high amounts of major histocompatibility complex II (MHC II) [11]. M2

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macrophages consist of three subclasses, M2a,b,c, and are considered to be anti-inflammatory. M2 macrophages are induced by a variety of different modulators, IL-4 and/or IL-13 (M2a), immune complex, toll-like receptor, or IL-1 receptor ligation (M2b) and IL-10, glucocorticoids, and secosteroids (M2c) [11,12]. M2c macrophages are considered to be anti-inflammatory, pro-tissue remodeling, and pro-wound healing. Macrophage activation has become of wide interest in the fields of biomaterials and regenerative medicine [13–16]. It has been postulated that by switching macrophages to a predominantly M2c activation state at an implant site, improved wound healing and improved implant integration into the host tissue will be achieved. Recently, a nomenclature change was requested to denote macrophage polarization as macrophage activation with the macrophage phenotype being termed according to the modulator which was used to elicit the macrophage, i.e., M(LPS) rather than M1 [17]. In this paper, the M1/M2 nomenclature will be used as a broad categorization for comparisons to past literature while the modulator nomenclature, M(Dex), will be used to describe macrophages that were altered via the delivery of Dex through the implanted microdialysis probe.

Microdialysis sampling is a minimally-invasive, diffusion-based sampling technique [18]. The microdialysis probe consists of inlet and outlet tubing, inner cannula, and a semi-permeable membrane with a defined molecular weight cut-off (MWCO). Microdialysis sampling has been used *in vivo* to sample low molecular weight soluble analytes from the extracellular space (ECS) which are smaller than the MWCO of the probe membrane. This technique works by passing a fluid (perfusate), which is of physiological ionic strength and pH, through the inlet and down the inner cannula. Once the perfusate leaves the inner cannula, it passes by the membrane and exits through the outlet tubing and is collected as a dialysate. Any concentration gradient which exists between the perfusate and the ECS allows the diffusion of molecules into or out of the microdialysis probe. This feature allows microdialysis sampling to be used for the simultaneous collection of analytes from and delivery of modulators to the ECS [19]. Once collected, analytes in the dialysate can be quantified using a wide variety of chemical analysis methods.

Dexamethasone (Dex) is a synthetic glucocorticoid that is widely used as an anti-inflammatory and immunosuppressant drug [20,21]. Once inside the cell, dexamethasone binds to glucocorticoid receptors which then translocate to the nucleus [22]. In the nucleus, Dex carries out its anti-inflammatory effects via two means: transactivation and transrepression [23]. Transactivation is the increase in the expression of certain ant-inflammatory genes including lipocortin-1 and the type II IL-1 receptor [24,25]. Transrepression is the down regulation of pro-inflammatory genes through the direct binding of the glucocorticoid receptor to negative glucocorticoid receptor elements [26,27] and by interfering with activator protein-1 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [28,29]. Through these actions Dex has been shown to down-regulate pro-inflammatory cytokines such as CCL2, IL-6, and TNF-α at the transcriptional level [30] as well as reducing CCL2, IL-6, and TNF-α protein concentrations in different animal models [31].

Chemokine (C–C motif) ligand 2 (CCL2) is a 13 kDa chemokine which exists as a monomer and homodimer at physiological concentrations [32]. CCL2 was formerly known as monocyte chemoattractant protein-1 (MCP-1) due to its ability to result in monocyte migration. CCL2 has been shown to be one of the primary factors for attracting monocytes to a wound site [33]. Once at the wound site the monocytes differentiate into macrophages. The activation state of these macrophages depends on the biochemical milieu present at the wound site.

Dexamethasone has been widely used as a release agent from biomaterials studies in an effort to improve integration of the biomaterial with the host tissue [34–36]. Dex has also been identified as a modulator which produces a phenotype that has characteristics of the former M2c macrophage [8,11]. While much work has been done with incorporating Dex-release into implanted biomaterials, quantifying the molecular response, particularly *in vivo* attempts to shift macrophages to an M(Dex) phenotype, has not been reported. Most work with Dex-releasing biomaterials uses standard histological, rather than molecular assessments, of implant/tissue outcomes. Our previous work has shown that Dex delivered immediately after microdialysis probe insertion is capable of shifting macrophages to a more $CD163^+$ state, the former M2c designation [37]. This is consistent with other macrophage studies in rats with dexamethasone that have also targeted $CD163^+$ cells [38]. However, there is a body of literature suggesting that an initial inflammatory response is critical for proper wound healing [39]. Therefore, we sought to determine if a different or even more optimal response in terms of converting macrophages to an M(Dex) state can be gained if the start of Dex infusion is delayed to allow the initial inflammatory response to commence. The resulting foreign body reaction to the implanted dialysis probes was characterized not only with standard histological means, but also immunohistochemical and molecular means at the gene and protein level. Thus, this work aimed to gain a better molecular understanding of macrophage-activation modulators' effectiveness at controlling the foreign body reaction.

2. Materials and methods

2.1. Chemicals

The following chemicals were used in this study: Anti-CD68 Antibody (Santa Cruz Biotechnology, Inc., Dallas, TX); Anti-CD163 Antibody (Santa Cruz Biotechnology, Inc., Dallas, TX); Apex™ Antibody Labeling Kits (Alexa Fluor 488 and Alexa Fluor 647) (Life Technologies, Carlsbad, CA); BD OptEIA™ Rat MCP-1 ELISA Set (BD Biosciences, San Jose, CA); bovine serum albumin (BSA) (Rockland Immunochemicals, Gilbertsville, PA); chloroform (MP Biomedicals LLC, Solon, OH); dexamethasone-21-phosphate disodium salt (Dex) (Alfa Aesar, Ward Hill, MA); Dextran-500 (Sigma Aldrich, St Louis, MO); ethylene oxide (Anderson Sterilizers, Inc, Haw River, NC); Halt Protease Inhibitor (Pierce, Rockford, IL); Hoeschst 34580 (Sigma Aldrich, St Louis, MO); Horse Serum (Life Technologies, Carlsbad, CA); HPLC grade water (Fisher Scientific, Waltham, MA); isoflurane (Abbott Laboratories, North Chicago, IL); Optimal Cutting Temperature solution (Sakura®Finetek, Torrance, CA); povidone-iodine (Professional Disposables International Inc, Orangeburge, NY); Proteinase K (Qiagen, Venlo, Limburg); RNAlater (Life Technologies, Carlsbad, CA); Trizol (Life Technologies, Carlsbad, CA); Taqman® Gene Expression Assays (IL-6, Arg2, CCL2, CD163, CD206, IL-1ra, IL-10, iNOS2, Lipo-1, TGFβ-1, TNF-α, and Taf9b) (Life Technologies, Carlsbad, CA) and Vetbond™ (3M, St Paul, MN). Ringer's solution contained 147 mM NaCl, 4.6 mM KCl, 2.3 mM $CaCl_2$, pH 7.4 and was prepared in HPLC-grade water. All other chemicals were reagent-grade or higher.

2.2. Microdialysis procedure

CMA 20 microdialysis probes with a 10 mm 100 kDa molecular weight cut-off (MWCO) polyethersulfone membrane (Harvard Apparatus, Holliston, MA) were used for all microdialysis sampling procedures. All microdialysis probes were ethylene oxide sterilized (Anderson Sterilizers Inc, Haw River, NC) prior to use. A BAS Bee microdialysis pump (Bioanalytical Systems Inc, West Lafayette, IN) with 1 mL BAS syringes (Bioanalytical Systems Inc, West

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