Biomaterials 74 (2016) 167–177

Contents lists available at ScienceDirect

Biomaterials

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

Effects of methoxypoly (Ethylene glycol) mediated immunocamouflage on leukocyte surface marker detection, cell conjugation, activation and alloproliferation

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ARTICLE INFO

Article history:
Received 9 July 2015
Received in revised form
25 September 2015
Accepted 29 September 2015

Keywords:
Leukocyte
PEGylation
Allorecognition
Polymer
Grafting
Immunomodulation

ABSTRACT

Tissue rejection occurs subsequent to the recognition of foreign antigens via receptor-ligand contacts between APC (antigen presenting cells) and T cells, resulting in initialization of signaling cascades and T cell proliferation. Bioengineering of donor cells by the covalent attachment of methoxypolyethylene glycol (mPEG) to membrane proteins (PEGylation) provides a novel means to attenuate these interactions consequent to mPEG-induced charge and steric camouflage. While previous studies demonstrated that polymer-mediated immunocamouflage decreased immune recognition both *in vitro* and *in vivo*, these studies monitored late events in immune recognition and activation such as T cell proliferation. Consequently little information has been provided concerning the early cellular events governing this response. Therefore, the effect of PEGylation was assessed by examining initial cell–cell interactions, changes to activation pathways, and apoptosis to understand the role that each may play in the decreased proliferative response observed in modified cells during the course of a mixed lymphocyte reaction (MLR). The mPEG-modified T cells resulted in significant immunocamouflage of lymphocyte surface proteins and decreased interactions with APC. Furthermore, mPEG-MLR exhibited decreased NFκB pathway activation, while exhibiting no significant differences in degree of cell death compared to the control MLR. These results suggest that PEGylation may prevent the direct recognition of foreign alloantigens by decreasing the stability and duration of initial cell–cell interactions.

1. Introduction

Despite tissue matching to major blood groups and HLA antigens, rejection of foreign tissues remains a major problem in transfusion and transplantation medicine [1–3]. This is due to both the antigenic diversity of human cells, which impedes tissue matching, and the complex nature of the immune response following allorecognition of foreign tissues. Together, these factors negatively impact the acute and long-term viability and function of the transplanted tissues as well as the quality of life of the recipient. To overcome these obstacles, successful tissue and organ transplantation are largely dependent on the use of broadly acting and toxic immunosuppressant drugs.

Immunosuppressant drugs typically exhibit broad inhibition, suppressing helpful as well as harmful responses, resulting in severe side effects such as cancer, infection, and direct allograft damage due to drug toxicity [4,5]. Moreover, many immunosuppressant drugs work by inhibiting downstream effector responses, after allorecognition has occurred. While some therapeutics inhibit initial activation events by targeting adhesion and costimulatory receptors, the redundant nature of receptors may decrease the efficacy of these drugs for preventing graft rejection [6]. As a consequence, receptor specific therapies are often used in combination with other costimulatory blocking agents or non-specific broadly acting immunosuppressant drugs [6]. However, preventing allorecognition and inducing immune quiescence would be preferable to the cytotoxic drugs currently used to prevent tissue rejection. As such, novel methods to prevent the recognition and rejection of foreign tissues would be beneficial.

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http://dx.doi.org/10.1016/j.biomaterials.2015.09.047

Bioengineering of cells via the covalent grafting of polymers to membrane proteins may provide a means of achieving this goal. Approved PEGylated proteins have been widely used for drug delivery to enhance bioavailability and decrease immune responses [7,8]. More recently, the direct PEGylation of intact cells (e.g., RBC, WBC) [9–14] and tissues (e.g., pancreatic islets) [15–18] have been examined. As demonstrated in these studies, PEGylated red blood cells (RBC) resulted in efficient camouflage of non-ABO RBC antigens with no significant effect on morphology, lysis, or murine *in vivo* survival, while PEGylated leukocytes (WBC) prevented allorecognition and T cell activation both *in vitro* and *in vivo*. Of particular interest, the leukocyte findings strongly suggest that PEGylated cells and tissues may have significant utility in transplantation research, where prevention of allorecognition is critical. PEGylation, unlike current immunosuppressive drugs, would be a novel and non-toxic means to prevent initial allorecognition events via the global camouflage of allogeneic cells.

While recent studies suggest that PEGylated allogeneic leukocytes induced an immunosuppressive state by altering the differentiation, proliferation, and environmental milieu both *in vitro* and in a murine model [13], they did not fully defined the initial molecular mechanisms governing these events. Indeed, studies to date have monitored late events in T cell activation such as proliferation and cytokine secretion. Although valuable, these studies do not provide the dynamic information on early T cell allorecognition and activation events. Therefore, the effect of PEGylation was assessed by examining initial cell–cell interactions, changes to activation pathways, and apoptosis to understand the role that each may play in the decreased proliferative response observed in modified cells during the course of a MLR. Understanding the mechanism of the induced immune quiescent state by immunocamouflage is important for producing improved and/or novel cellular blood products.

2. Methods and materials

2.1. Cell collection

All experiments using human blood cells were done with the approval of the University of British Columbia Clinical Research Ethics Board and in accordance with the Declaration of Helsinki. Following informed consent, donor whole blood was collected in heparinized Vacutainer® blood tubes (BD, Franklin Lakes, NJ). Peripheral blood mononuclear cells (PBMC) were isolated using Histopaque-1077 (Sigma–Aldrich, St. Louis, MO, USA) according to manufacturer's protocol.

2.2. Generation of monocyte derived dendritic cells (moDC)

moDC were prepared as previously described [14]. Briefly, freshly prepared PBMC were seeded at 5×10^6 cells/ml in AIM V medium (Gibco, Invitrogen, Grand Island, NY, USA). After 3 h incubation (37 °C, 5% CO_2), non-adherent cells were removed by washing. The remaining monocyte rich adherent cells were incubated in AIM V medium supplemented with IL-4 (100 ng/ml) and GM-CSF (50 ng/ml) (R&D Systems, Minneapolis, MN, USA). IL-4 and GM-CSF was added again on day 3. On day 6, cells were washed twice and suspended at 5×10^5 cells/ml in AIM V medium supplemented with IL-4, GM-CSF and maturation/activating cytokines TNF-α (5 ng/ml), IL-1β (5 ng/ml), IL-6 (150 ng/ml) (R&D systems Inc., Minneapolis, MN, USA) and prostaglandin E2 (1 μg/ml) (Sigma–Aldrich, St. Louis, MO, USA). Mature moDC were harvested on day 7 and assessed by flow cytometry for maturation markers CD80 and CD86 (BD Biosciences, San Jose, CA, USA) prior to downstream assays.

2.3. Polymer species and cell derivatization

Polymer modification of donor leukocytes was done using succinimidyl valerate activated mPEG (mPEG; 5, 20 and 30 kDa; Laysan Bio Incorporated, Arab, AL, USA) as previously described [13,19]. Isolated PBMC were suspended to 4×10^6 cells/ml in mPEG buffer (50 mM K_2HPO_4, 105 mM NaCl, pH 8.0) with desired amount of mPEG (5, 20, 30 kDa) to reach appropriate grafting concentration (0–4 mM). The reaction was performed for 1 h at room temperature. PBMC were stained with trypan blue (Invitrogen, Grand Island, NY, USA) and enumerated using a hemocytometer.

2.4. Surface marker analysis

Freshly isolated control or mPEG-modified PBMC were assessed for immunocamouflage efficacy by exclusion of fluorescently labeled antibodies to leukocyte cluster of differentiation (CD) markers. Surface marker analysis was examined via flow cytometry as previously described [11,13,19–21]. PBMC cell suspensions were stained with anti-CD11a FITC, anti-CD25 PE, anti-CD62L PE or anti-CD71 FITC according to manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Unstained cells and isotype control antibodies were utilized for each fluorophore to determine the degree of non-specific binding and background fluorescence. Analysis of the efficacy of immunocamouflage was based on percent positive cell (PPC) values. For all studies, a minimum of 20,000 events per sample was collected.

2.5. Mixed lymphocyte reactions

To assess the effects of polymer modified leukocytes during allorecognition, one of the two disparate PBMC population was modified with polymer (20 and 30 kDa mPEG; 0–2 mM) as previously described [13,19]. An equal number of cells from two disparate PBMC populations were suspended in AIM V medium (2×10^6 cells/ml) and incubated in a humidified, 5% CO_2 incubator at 37 °C. Cells were harvested for downstream assays at various timepoints as indicated. For proliferation and conjugation assays, cells were stained with amine reactive fluorescent probes prior to cell modification with polymer.

2.6. CFSE proliferation assay

The proliferative response of PBMC was used to assess the effects of cell modification on allorecognition (MLR) as well as the response to mitogen challenge (PHA) as previously described [13,19]. Freshly isolated human PBMCs were stained with carboxyfluorescein diacetate succinimidylester (CFSE; Cell Trace- Molecular Probes, Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Following CFSE staining, PBMC were derivatized with mPEG as described above. For mitogen stimulation, PHA was added at 2 μg/ml. Proliferation of the $CD3^+CD4^+$ population was assessed by flow cytometry on days 7, 10 and 14 for the MLR and day 5 for PHA stimulation. The $CD3^+CD4^+$ population was determined using anti-CD3 PE and anti-CD4 APC antibodies (BD biosciences, San Jose, CA, USA) according to manufacturer's instructions. Data acquisition and analysis was performed using Cell Quest Software as previously described.

2.7. Time-lapse microscopy

To investigate the effects of PEGylation on the cell–cell interaction necessary for allopresentation and alloproliferation, matured moDC and allogeneic control or mPEG-modified PBMC (20 kDa mPEG; 2.0 mM) were co-cultured in RPMI supplemented