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Extended interaction of β1 integrin subunit-deficient cells (GD25) with surfaces modified with fibronectin-derived peptides: Culture optimization, adhesion and cytokine panel studies

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Abstract

The modification of biomaterials with extracellular matrix-mimicking factors is a common technique used to influence the cellular response through integrin-mediated signaling. The inherent limitations of antibody-inhibition studies necessitate the use of complementary methods to block integrin function to confirm cell–surface interaction. In this study, we employed a β1 integrin-deficient cell line, GD25, to investigate the role of β1 subunit in cell adhesion and subsequent cytokine (granulocyte macrophage colony stimulating factor; interleukin (IL)-1α; IL-1β; IL-6; monocyte chemoattractant protein-1; regulated upon activation, normal T-cell expressed, and secreted; tumor necrosis factor-α) release kinetics in the presence of tissue culture polystyrene (TCPS) and semi-interpenetrating polymer networks (sIPN) modified with fibronectin (FN)-mimic peptides (RGD, PHSRN). Culture conditions (i.e. seeding density, medium, serum supplementation) were optimized for long-term observation. Differences in cell adhesion, cell viability and cytokine release behavior were dependent on the presence of the β1 integrin subunit, FN, sIPN cast method and peptide identity. By comparing two complementary techniques for assaying integrin function, we observed both similarities (i.e. decreased adhesion to FN-absorbed TCPS and increased IL-1β release at 96 h) and differences (i.e. no difference in adhesion or IL-1β release in the presence of different sIPN surfaces) when the function of the β1 subunit was blocked in cell adhesion and signaling in the presence of biomaterials.

Keywords: Biological response; Polymeric biomaterial; Tissue engineering; Cellular growth; Surface structure

1. Introduction

One of the main emphases of biomaterial research is to influence cell–substrate interactions. A frequently employed technique involves modifying the biomaterial with extracellular matrix (ECM) components or ECM protein-mimics to imitate the normal cell microenvironment [1,2]. Integrin receptors are primary mediators of cell–ECM interactions. RGD, a common integrin-binding motif, is found in many ECM proteins, including collagen, vitronectin, laminin and fibronectin [3]. Unique to fibronectin is the synergy sequence, PHSRN, which is specific to the α5β1 integrin and can lead to an increase in cell adhesion and mobility as well as enhance fibronectin matrix formation [4–6]. Several other integrin pairs are able to bind to fibronectin, including α4β1, α3β1, αvβ6 and αvβ3, the latter binding the RGD motif without employing a synergy sequence [5–7]. Depending on the integrin pair that complexes with fibronectin, a variety of downstream effects can occur within the cell through differential activation of outside-in signaling pathways.

Antibodies against individual integrin subunits are commonly used to elucidate the specific role of integrins in cell adhesion, signaling and other subsequent cell behaviors modulated by ECM-containing biomaterials. The inherent

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limitations of antibody inhibition studies, including the kinetic antibody binding, non-specific antibody binding, steric interference of normal cell adherence, and altered intracellular signaling events, call for the use of complementary methods to neutralize integrin function. Such alternative methods include the use of siRNA or cell lines with knocked-out genes of interest. In this study, we employed a β1 integrin subunit-deficient fibroblastic cell line (GD25) derived from the embryonic stem cell line ES-D3 [8] to study the role of this subunit in regulating cell adhesion to a poly(ethylene glycol) (PEG)- and gelatin-based semi-interpenetrating polymer network (sIPN) and the subsequent release of common inflammatory cytokines. Through our extensive literature surveys and database searches, we found no other readily available β1 integrin subunit knock-out human cell line. The binary in situ photopolymerizable sIPN system contains PEGylated immobilized peptides derived from fibronectin (FN) and previous work has demonstrated its use as a tissue scaffold and drug delivery vehicle [9–12].

Previous studies examining the behavior of human-derived primary monocytes pretreated with anti-β1 or anti-β3 integrin subunit antibodies in the presence of the aforementioned biomaterial as well as tissue culture polystyrene (TCPS) found variations in protease and cytokine release patterns depending on antibody inhibition [13]. On sIPN surfaces, matrix metalloprotease-2 release was reduced by a greater amount when cells were treated with anti-β1 as compared to anti-β3 integrin subunit antibodies. This trend was reversed, however, in the case of interleukin (IL)-1β release. On fibronectin-adsorbed TCPS (TCPS-FN), IL-1β release from cells treated with anti-β1 subunit antibodies increased by 96 h compared to the release from untreated cells. To complement these findings, this study investigated the release kinetics of seven common fibroblast-derived cytokines from the β1 subunit-deficient cell line: granulocyte macrophage colony stimulating factor (GM-CSF), IL-1α, IL-1β, IL-6, monocyte chemoattractant protein 1 (MCP-1 or CCL2), RANTES (regulated upon activation, normal T-cell expressed, and secreted; CCL5) and tumor necrosis factor-α (TNF-α). IL-1, IL-6 and TNF-α are proinflammatory cytokines expressed by a variety of cell types which encourage host responses typical of early stage wound healing [14,15]. GM-CSF has been shown to play a role in the local activation, recruitment and survival of macrophage lineage cells [15,16]. MCP-1 and RANTES are potent stimulators of monocyte and T-cell migration [16–18]. Overall, this panel of key inflammatory proteins should provide a representation of the β1 subunits' role in inflammatory signaling.

The behavior of the GD25 cells in the presence of biomaterials was compared to that of GD25 cells expressing the β1A integrin (GD25β1A). The β1A integrin splice variant is ubiquitously expressed [19,20], has a broad distribution in vivo [21,22], is capable of activating focal adhesion kinase tyrosine phosphorylation and localizes to focal adhesions [23]. Previous studies employing the GD25 and GD25β1A lines have not considered the reaction of the cells past 24 h of adhesion and have not examined how the loss of the β1-integrin affects the cellular response to biomaterials beyond the use of proteins, adsorbed to TCPS [6,24–34]. In this study, cytokinerelease from cells adhered to PEGylated-RGD-modifed gelatin sIPNs (RGD-sIPN) and PEGylated-PHSRN-modified gelatin sIPNs (PHSRN-sIPNs) were compared to cells adhered to TCPS, TCPS-FN, as well as sIPN and PEG hydrogel controls. To do so, we first optimize culture conditions to allow for 7 days of cell survival while minimizing the non-specific adhesion and subsequent cell response caused by the absorption of proteins present in fetal bovine serum (FBS) in the culture medium.

2. Materials and methods

2.1. Synthesis and characterization of sIPN-containing gelatin modified with PEGylated-peptide

RGD and PHSRN were synthesized using a solid phase synthesis scheme based on Fmoc resin cleavage and deprotection (EMD Biosciences, Darmstadt, Germany) [35]. Peptides were analyzed with a reverse-phase high performance liquid chromatography (HPLC) system (Gilson, 10–100% acetonitrile, 1 ml min^{-1} flow rate for 30 min) coupled with ultraviolet/visible (UV/Vis) and evaporative light scattering detectors and MALDI or electrospray ionization in the positive ion mode. Samples analyzed by MALDI utilized dihydroxybutyric acid as the matrix. The method for the modification of gelatin with PEGylated-peptide has been previously published in detail [36]. All starting materials used were purchased from Aldrich unless otherwise specified. Briefly, the terminal alcohol groups of PEG-diol (2 kDa) were converted to ethylacetate and then to carboxylic acid to form bis-carboxylate-PEG (bis-COOH-PEG). The final product was characterized using a reverse-phase HPLC system (Gilson, 10–100% acetonitrile, 1 ml min^{-1} flow rate for 30 min) coupled with UV/Vis and evaporative light scattering detectors. *N*-Hydroxysuccinimide and *N*,*N*′-dicyclohexycarbodimiide were then reacted with the carboxylic acid groups of bis-COOH-PEG to produce bis-*N*-hydroxysuccinimide-PEG (bis-NSu-PEG). The resulting mixture containing bis-NSu-PEG was characterized using the reverse-phase HPLC method described earlier. A total of 1.5 eq. mol GGG, RGD or PHSRN in combination with 1.5 eq. mol *N*,*N*-diisopropyl ethylamine was added to 1 eq. mol bis-NSu-PEG in order to graft the ligand onto one of the two PEG terminal groups to form NSu-PEG-peptide. Next, 1 eq. mol of NSu-PEG-peptide was mixed with 1% gelatin (Type A, 300 bloom, porcine skin) in phosphate-buffered saline at pH 8.0 for 1 h in order to graft remaining terminal group of the PEG-peptide to the lysine groups in gelatin. A pressurized ultrafiltration system with a 30 kDa membrane filter was used to separate peptide-PEG-gelatin from the remaining bis-peptide-PEG and unreacted NSu-PEG-peptide. A similar