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Fundamental insight into the effect of carbodiimide crosslinking on cellular recognition of collagen-based scaffolds

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ABSTRACT

Research on the development of collagen constructs is extremely important in the field of tissue engineering. Collagen scaffolds for numerous tissue engineering applications are frequently crosslinked with 1-ethyl-3-(3-dimethylaminopropyl-carbodiimide hydrochloride (EDC) in the presence of N-hydroxysuccinimide (NHS). Despite producing scaffolds with good biocompatibility and low cellular toxicity the influence of EDC/NHS crosslinking on the cell interactive properties of collagen has been overlooked. Here we have extensively studied the interaction of model cell lines with collagen I-based materials after crosslinking with different ratios of EDC in relation to the number of carboxylic acid residues on collagen. Divalent cation-dependent cell adhesion, via integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$, were sensitive to EDC crosslinking. With increasing EDC concentration, this was replaced with cation-independent adhesion. These results were replicated using purified recombinant I domains derived from integrin α_1 and α_2 subunits. Integrin $\alpha_2\beta_1$-mediated cell spreading, apoptosis and proliferation were all heavily influenced by EDC crosslinking of collagen. Data from this rigorous study provides an exciting new insight that EDC/NHS crosslinking is utilising the same carboxylic side chain chemistry that is vital for native-like integrin-mediated cell interactions. Due to the ubiquitous usage of EDC/NHS crosslinked collagen for biomaterials fabrication this data is essential to have a full understanding in order to ensure optimized collagen-based material performance.

Statement of Significance

Carbodiimide stabilised collagen is employed extensively for the fabrication of biologically active materials. Despite this common usage, the effect of carbodiimide crosslinking on cell-collagen interactions is unclear. Here we have found that carbodiimide crosslinking of collagen inhibits native-like, whilst increasing non-native like, cellular interactions. We propose a mechanistic model in which carbodiimide modifies the carboxylic acid groups on collagen that are essential for cell binding. As such we feel that this research provides a crucial, long awaited, insight into the bioactivity of carbodiimide crosslinked collagen. Through the ubiquitous use of collagen as a cellular substrate we feel that this is fundamental to a wide range of research activity with high impact across a broad range of disciplines.

1. Introduction

The extracellular matrix (ECM) of tissues is composed of a complex network of proteins, glycoproteins and glycosaminoglycans that surround cells. Purified components of the ECM have been widely employed in the design of tissue engineering scaffolds where they provide a similar cell niche to the native tissue [1]. Fibrillar collagen I is the most abundant ECM protein component, fulfilling both structural and cell adhesive roles in a wide range of

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tissue types [2]. Collagen I is a triple-helical protein which possesses the vital physical properties of strength and stiffness required for generating complex 3D structures [3]. Alongside its physical attributes, collagen I contains a series of cellular recognition motifs that interact with a diverse lineage of cells [4]. Therefore collagen I can provide appropriate physical support for a tissue replacement whilst simultaneously directing cell attachment, proliferation and differentiation. In addition to these physical and biological roles, collagen I can be isolated to high purity and is relatively inexpensive. As such there has been an explosion of interest in this area in recent years as evidenced by both recent publications and efficacious use in clinical products.

Collagen I interacts with cells via a number of cell surface receptors including integrins and discoidin domain receptors (DDRs) [4]. Integrins are a class of cell surface receptors that bind to a wide range of ECM proteins where each integrin contains a single α and β subunit. To date at least 18 α- and 8 β-subunits have been identified that dimerise to form at least 24 different integrin heterodimers [5]. Collagen I binds to four of these integrin heterodimers, all of which contain the β_1 subunit. These are integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$ [6,7]. Cell binding studies to libraries of collagen derived triple-helical sequences have identified numerous integrin-binding motifs within collagen [8]. This lead to the delineation of a Gxx′GEx″ consensus binding sequence for cell surface integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ [4]. Although x may be phenylalanine (F), leucine (L), arginine (R), or methionine (M), of these combinations, the sequence GFOGER has been demonstrated as a high affinity integrin-binding site [8,9]. Integrins bind to collagen I via an inserted A domain (I domain) contained within the α subunit of the integrin. The crystal structure of the integrin α_2 I domain when interacting with a triple-helical GFOGER motif has been resolved [10]. This shows that the carboxylate side chain on the glutamic acid (E) residues of GFOG*E*R is critical for coordination with a Mg^{2+} ion within the metal ion-dependent adhesion site (MIDAS) on the I domain. As such, integrin-collagen interactions are highly dependent upon the presence of the divalent cation Mg^{2+}, making EDTA (ethylenediaminetetra-acetic) chelation of Mg^{2+} a convenient indicator for native-like integrin-mediated cell interaction.

In vivo collagen fibres are stabilised by enzymatic modification that leads to crosslinking. For biomaterials fabrication, similar physical integrity is obtained by carbodiimide chemical crosslinking using 1-ethyl-3-(3-dimethylaminopropyl-carbodiimide hydrochloride (EDC) in the presence of N-hydroxy-succinimide (NHS) [11]. By systematically modifying the EDC/NHS crosslinking regimes the physical properties of scaffolds can be modulated. This allows discrete control over the mechanical strength and degradation kinetics of the resultant scaffold [12]. During carbodiimide-mediated crosslinking the carboxylate moiety on one amino acid side chain (Asp, Glu) reacts through a condensation reaction with a primary amine on an adjacent amino acid (Lys). Standard crosslinking conditions of 11.5 mg/mL EDC (equivalent to 5xEDC: 2xNHS: 1xCOO^- group on collagen; 60 mM EDC) are defined as 100% throughout this paper, representing a 5-fold molar excess of the crosslinker compared to the molarity of carboxylate-containing side chains on collagen. This results in a measurable, dramatic loss of primary amine, and by extension, the carboxylate side chain content of the collagen-based scaffold [11]. This is of particular importance as the carboxylate anion of glutamic acid is critical for collagen ligation with integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ and so we hypothesise that carbodiimide treatment of collagen could influence integrin-collagen ligation.

EDC/NHS crosslinking is frequently used to stabilise three-dimensional (3D) biomaterials with well-defined pore geometry [13,14] and percolation diameters [15] for optimal cell infiltration [16]. For this study we have focused on 2-dimensional films as an approximation to the pore walls of these 3-dimensional scaffolds to reduce the complexity associated with 3D systems. The influence of EDC/NHS crosslinking on the physical properties, such as morphology, dissolution and mechanics of collagen-based scaffolds is well understood [11]. Despite this there are key areas of understanding, such as cellular interactions, that appear to have been overlooked. We have recently shown that EDC/NHS crosslinking of collagen-based materials influences cation dependent platelet and HT1080 cell attachment [11]. Therefore, the goal of this study was to explore the mechanism for this carbodiimide modulation of cell adhesion and to determine its influence on the cellular response. To do this we have analysed the cellular response to fibrillar collagen I treated with incremental dilution of EDC. We defined conventional levels of 11.5 mg/mL EDC as 100% and increased EDC and NHS concentrations up to 5-fold of this or diluted the concentration of EDC and NHS down to 1% of this condition whilst maintaining a constant collagen mass. We have utilised a range of model cell lines, each of which express unique collagen binding integrins. These have enabled us to probe the availability of specific integrin binding sites as a function of carbodiimide crosslinking. Through this research we demonstrate for the first time a detailed mechanism for cell-interaction with collagen materials crosslinked with increasing EDC/NHS conditions. Carbodiimide crosslinking of collagen is widely employed for biomaterials fabrication. This work provides a comprehensive investigation of the fundamental science that underpins this technology, producing scaffolds with appropriate physical and biological activity.

2. Materials and methods

2.1. Materials

Unless stated otherwise all reagents were analytical grade and used as received from Sigma-Aldrich.

2.2. Film preparation and crosslinking

Collagen slurries were prepared by swelling a 0.5% (w/v) suspension of bovine Achilles tendon insoluble collagen in 50 mM acetic acid at 4 °C overnight then homogenising on ice for 20 min at 13500 rpm using an Ultra-Turrax VD125 (VWR International Ltd., UK) homogeniser. Air bubbles were removed from the suspension by centrifuging at 2500 rpm for 5 min (Hermle Z300, Labortechnik, Germany). Films of ~8 μm of thickness were cast by pipetting 100 μL of slurry/well into an Immulon-2HB 96-well plate (Thermo Scientific) and drying for 48 h in a laminar flow cabinet.

Films were chemically crosslinked using EDC/NHS at molar ratios of EDC/NHS/COO^-(Col) = 5/2/1 in 75% (v/v) Ethanol. This was defined as standard (100%) crosslinking conditions. The crosslinking solution was varied from this standard 100% condition by dissolving the appropriate amount of EDC/NHS into 75% Ethanol or by diluting from the 100% solution with 75% Ethanol. Non-crosslinked (0% EDC/NHS) films were incubated in 75% Ethanol only. After incubation for 2 h at room temperature the films were washed extensively with deionised water and dried in a laminar flow cabinet.

2.3. Platelet adhesion analysis

Platelets were obtained from human platelet rich plasma provided by the National Health Service Blood and Transplants (NHSBT) authority in accordance with the Declaration of Helsinki. Platelets were prepared by centrifugation for 15 min, 240*g*, the pellet discarded and 1 μL of Prostaglandin E_1 (100 μg/mL in ethanol)