In vitro Comparison of ProColl's Recombinant Human Collagen with Bovine Collagen

Abstract

Collagen is an important component of human tissues hence it is used in many products within the medical, pharmaceutical and aesthetics industry where its structure provides mechanical robustness. Collagen is traditionally extracted from animal materials, which many applications are seeking to avoid in order to lower the risk of interspecies disease transfer, improve biocompatibility and reduce ethical concerns. In this white paper we report on the use of ProColl recombinant human collagen (RHC) to grow human cells. The growth of primary human umbilical cord endothelial cells was shown to be the same on RHC when compared with that on bovine collagen. The immune response of these cells was also investigated by studying the release of interleukins in response to different collagens. Single chain collagen (α1 and α2) stimulated IL-6 and IL-8 production but when the chains were in the form of a triple helix RHC a lower release of the interleukins was observed at the same level of triple helical bovine collagen. ProColl have developed a RHC that can be used in cell culture and applied to replace collagen derived from animal sources.

1. Introduction

Collagen is the most abundant protein within the human body and plays a central role in the maintenance and repair of all organs and tissues. Thus, it is one of the most industrially important proteins with applications in medicine, cosmetics, and food. Most collagen available is derived from animal sources such as bovine, murine and marine, however there is a growing interest in replacing these animal products [1-3]. ProColl research has now enabled the production of animal free collagen through fermentation processes harnessing the metabolism of yeast to produce the recombinant protein at scale.

There are several reasons behind the wish to use less animal products within healthcare. Firstly, the use of animal free materials within a human context removes the risk of interspecies disease transfer whether that be prions, the vectors responsible for BSE and CJD in humans, or viruses. After the BSE crisis legislation was brought in to mitigate risk and improve animal husbandry with monitoring and traceability of animals within the human food chain, this was extended to bovine materials used in the medical device industry with suppliers using raw materials from closed herds. Unfortunately, for materials derived from animals out of the human food chain there is no such monitoring. A second reason for the

move to animal free materials is the lowering of the risk of an immune response [1,3]. Finally, moral and ethical reasons with belief structures precluding the use of animals and the wish to avoid animal suffering. This moral stance has been a guiding doctrine within the research community where the wish for animal free materials is now innate with the adoption of the themes of the 3Rs; replacement, refinement, and reduction of animal research (https://nc3rs.org.uk/the-3rs).

This reasoning for animal free products within human healthcare has driven a lot of research initiatives to replace natural materials with synthetic polymers in the fabrication of scaffolds for wound healing or tissue engineering [4,5]. This has focused on aliphatic polyesters such as polyglycolic acid (PGA), polylactic acid (PLA), their copolymers (e.g. PLGA) and polycaprolactone (PCL). The advantage of using these materials are that the degradation products of these polymers (glycolic acid and lactic acid) are present in the human body and are removed by natural metabolic pathways [5,6]. However, these synthetic polymers do not participate in normal tissue function and repair as does collagen. In addition, these materials often have mechanical limitations and can illicit unwanted immune responses [6]. Their use also often require aggressive solvents and their potential contaminants that must be removed and complicate regulatory approval.

To address these concerns on the use of collagen extracted from animals, ProColl have used a synthetic biology approach. This is based on a precision fermentation processes using yeast that has been modified so that it synthesises the recombinant human collagen (RHC). Yeast cells can be grown in large numbers, as in brewery fermentations, so allowing scale of production. In this white paper we discuss research that has examined the growth of human cells on ProColl's recombinant human collagen in comparison with standard culture techniques using bovine acid soluble collagen (BASC), gelatin and uncoated tissue culture plastic . Standard cell biology analytical methods were used to assess that the cells cultured on recombinant human collagen grew and exhibited immune response equal to when they were grown on bovine acid soluble collagen.

2. Methods

2.1 Collagen Coating

Tissue culture plates were passively coated with the different collagens. This was achieved by diluting the collagen solutions to 50µg/ml and pipetting 200µl of each into different wells of 96 well culture plates in order to give a final concentration of 10 µg/cm2. The plates were then incubated overnight at 4°C, before removing the excess collagen and allowing the plates to air dry in a tissue culture hood overnight. Once dried the plates were then either used straight away or sealed and stored at 4°C before use.

2.1 Cell growth

The main cells used in this study were Primary Human Umbilical Cord Endothelial Cells (HUVECs). These are primary cells isolated from the vein of the umbilical cord. They are a model system for studying endothelial cell function, with applications including hypoxia, inflammation, oxidative stress, response to infection, and both normal and tumour-associated angiogenesis (REF). Coated and control wells on the tissue culture plate were seeded with 50,000 cells/cm2 and then cultured for 48 hours.

2.2 AlamarBlue Assay as a cell health indicator

AlamarBlue is an important redox indicator that is used to evaluate metabolic function (REF). Monitoring changes to the cellular reducing environment or metabolic activity by using such resazurin-based reagents is a well-established and reliable indicator of cellular health. The AlamarBlue assay (Invitrogen) utilizes a ready to use, non-toxic, resazurin-based solution that functions as a cell health indicator by using the reducing power of living cells to quantitatively measure viability. Following incubation of the cells with the reagent for 4 hours, absorbance was read at 570nm using a plate reader (PolarStar; BMG). The amount of absorbance is proportional to the number of living cells and corresponds to cellular metabolic activity and proliferation potential. Damaged and nonviable cells have lower innate metabolic activity and thus proportionally lower signal than healthy cells.

2.3 Lactate dehydrogenase cytotoxicity assay

Lactate dehydrogenase (LDH) is an important enzyme of a cell's metabolic pathway. It is present in all tissues and serves as an important checkpoint of gluconeogenesis and DNA metabolism. Hence its use as a signpost for cellular health. During cell death the plasma membrane is damaged resulting in the release of LDH, an enzyme that oxidizes lactate, generating NADH. The LDH cytotoxicity assay takes advantage of this process by utilizing a WST substrate mix, which interacts with NADH generating a yellow colour. Following incubation of the samples at room temperature, the absorbance of all samples and controls

at 450 nm was read using a plate reader (PolarStar; BMG). LDH activity was then normalised against medium alone to compensate for the potential medium interference.

2.4 Cytokine analysis (IL-6 and IL-8)

The release of cytokines from HUVEC and THP-1 cell lines was studied. Interleukin IL-6 (IL-6) is produced in response to tissue injuries and contributes to host defence through the stimulation of the acute phase of wound healing. Interlukin-8 (IL-8) is an important protein related to inflammation and so an integral part of the wound healing process. For cytokine analysis, the concentrations of IL-6 and IL-8 in cell culture supernatants were quantified by ELISA kits (Duoset, R&D Systems) according to the manufacturer's instructions. Following completion of the assay, absorbance was read at 450nm using a plate reader (PolarStar; BMG).

2.5 Data analysis

All data are presented as the mean ± the standard deviation (SD). All endpoints were assessed upon three independent cell cultures (n=3). Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, USA) software. A two-way analysis of variance (ANOVA) with subsequent Tukey's multiple comparisons test was performed for each endpoint.

3. Results and Discussion

3.1 Health of cells grown on recombinant collagen

There was no impairment of cellular metabolism when human cells were grown on recombinant human collagen (RHC) compared to bovine acid soluble collagen (BASC) (Figure 1). Decreases in AlamarBlue absorbance signifies an impairment of cellular metabolism [REF]. Figure 1 shows that there was no significant decrease in AlamarBlue absorbance, thus there was no difference in metabolic activity between non-coated, gelatin, RHC and BASC coatings. However, the LDH assay suggests that there was better growth in both the RHC and BASC coated wells as there were more viable cells compared to non-coated and gelatin wells. Figure 2 shows there was a significantly lower amount of LDH released by HUVECs seeded on the BASC and RHC coated wells when compared to the uncoated and gelatin-coated wells. The LDH is released from dead cells, so if a greater percentage of the cell population is viable then less LDH will be available to drive the colour change detected by the assay.

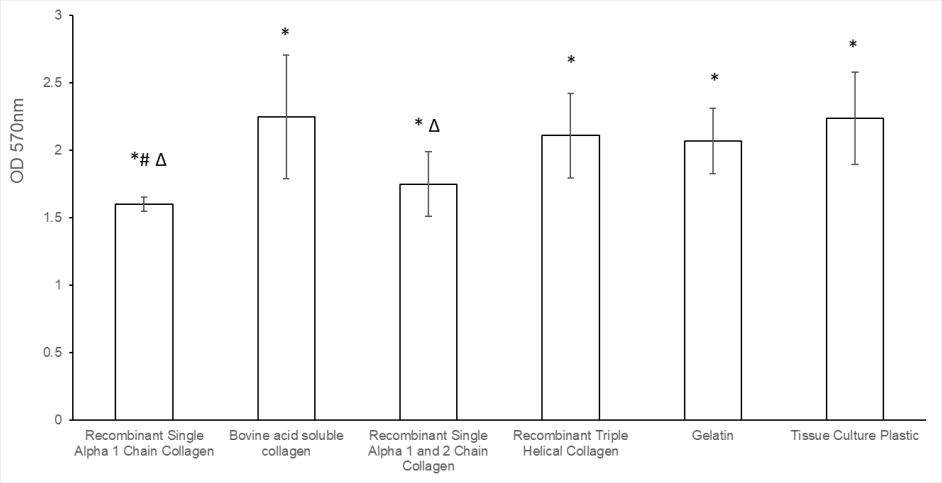

Figure 1. Comparison of collagen coating on HUVEC cellular health - AlamarBlue. *Relative fluorescence intensity of AlamarBlue, n=3 with all assays performed in triplicate. The data is presented as the mean ±Standard deviation. Significance is denoted as the following: compared to the medium control p<0.01(*); compared to Gelatin p<0.01(#); and compared to no coating p<0.01(Δ).*

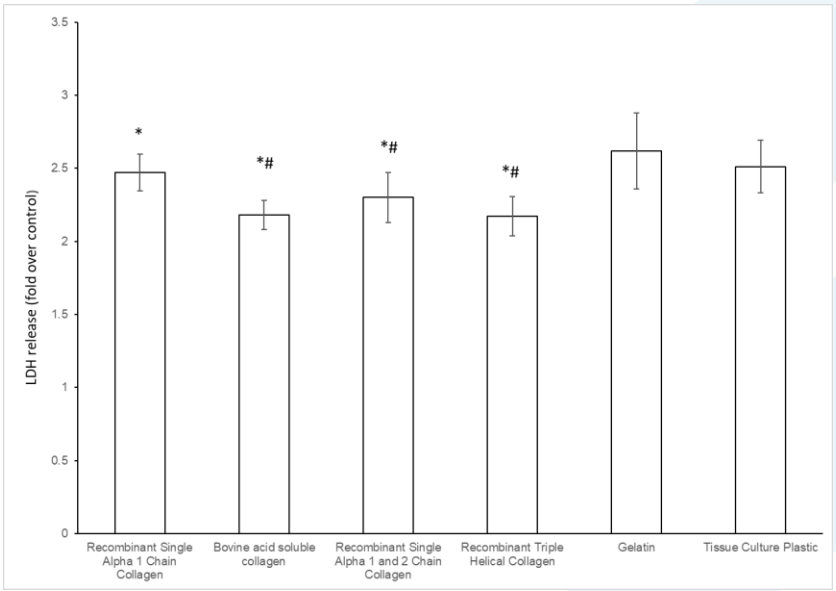

Figure 2. Comparison of collagen coating of HUVEC cellular health – LDH release. *LDH activity was normalised against medium alone to compensate for the potential medium interference and is presented as fold over control, n=3 with all assays performed in triplicate. The data is presented as the mean ±Standard deviation. Significance is denoted as the following: compared to the non-coated control p<0.01(*); and compared to Gelatin p<0.01(#).*

3.2 Immune response of cells grown on Recombinant Human Collagen.

The potential immune reaction of the collagen coatings was then investigated by examining the release of IL-6 and IL-8. HUVEC cells are capable of releasing chemokines and cytokines that can then act as a feedback loop and initiate the innate immune response if required (REF). HUVEC cells were shown to release significantly more IL-6 and IL-8 when grown in the presence of single alpha chains of collagen (Figure 3 and 4) either as single alpha 1 chains or as a mixture of alpha 1 and alpha 2 chains. Previous research has also shown that when the collagen triple helix is unwound, through partial denaturation, elements of the molecule are exposed that up regulate many pathways important in wound healing [7,8]. During the inflammation phase, the peptide fragments of collagen were shown to have a chemotactic effect in the recruitment of cells essential to wound healing, while collagen derived peptides stimulate fibroblast proliferation during the proliferation phase [9,10].

In contrast, IL-6 and IL-8 release and so immune response was lower for cells grown on bovine acid soluble collagen (BASC), recombinant human collagen (RHC), gelatin and non-coated tissue culture plastic. The HUVEC cells grown on BASC and RHC exhibited the same immune response. The immune response observed when the collagen was in the single chain alpha form was prevented after these molecules had been assembled into the triple helical form of the RHC.

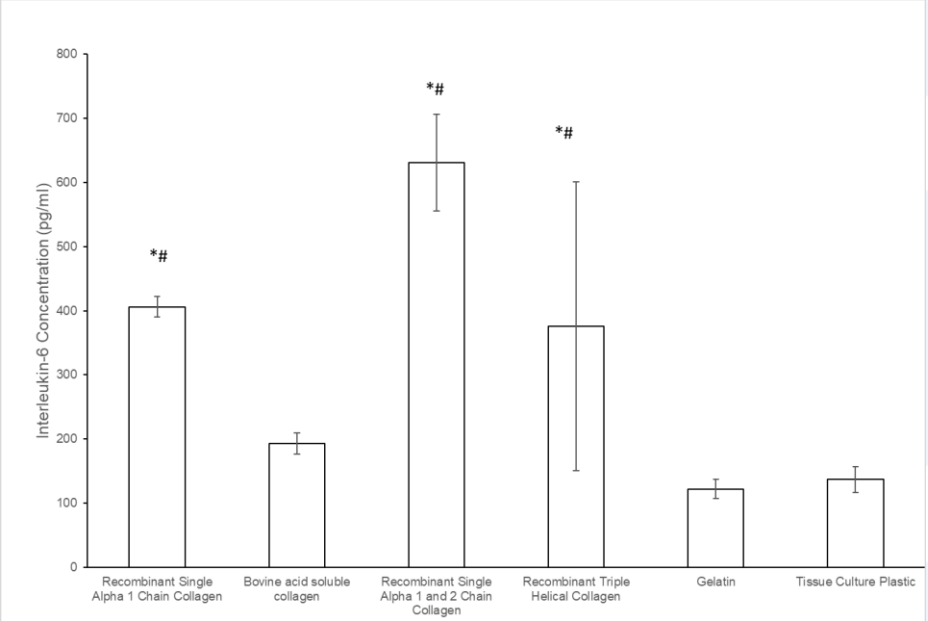

Figure 3. The influence of collagens on IL-6 expression. *The data is presented as the mean ±standard deviation. Statistical significance is denoted as the following: compared to the tissue culture plastic non-coated control p<0.01(*); and compared to gelatin p<0.01(#).*

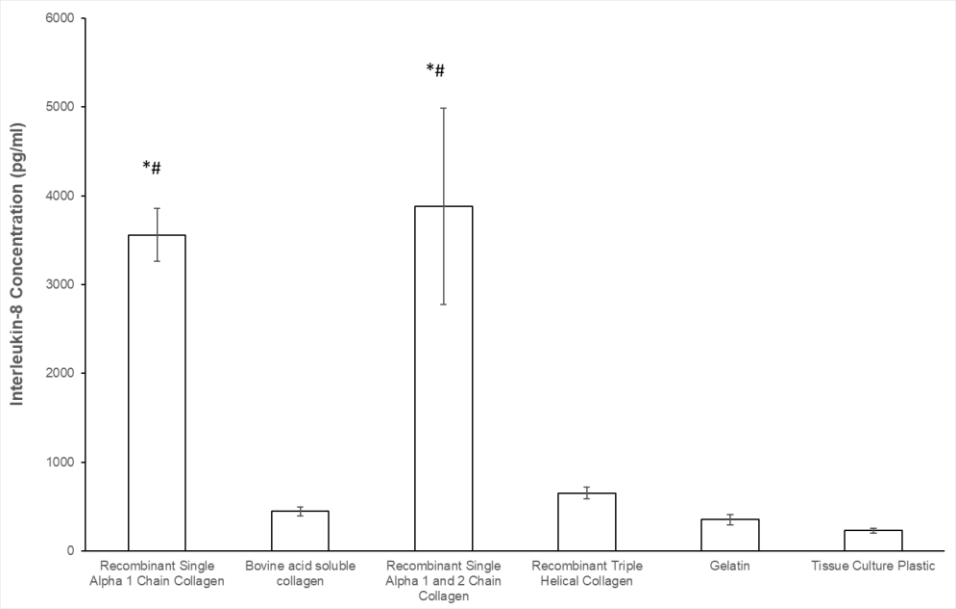

Figure 4. The influence of collagens on IL-8 expression. *The data is presented as the mean ±standard deviation. Statistical significance is denoted as the following: compared to the tissue culture plastic non-coated control p<0.01(*); and compared to gelatin p<0.01(#).*

Conclusions

The recombinant human collagen (RHC) produced by ProColl allowed the healthy growth of primary human cells. There was better growth of HUVEC cells on both the RHC and bovine collagen (BASC) when compared to that on gelatin coated and uncoated tissue culture plastic. There was no difference in immune response of the cells when they were grown on RHC or on BASC. When single alpha chains were present interleukin production was increased. This was expected as collagen fragments have previously been observed to upregulate an immune response in human cells. When the RHC is formed into a triple helix the release of IL-6 and IL-8 was lowered to a level observed for triple helical BASC. The production of animal free collagen by ProColl offers great opportunity to meet market needs within regenerative medicine, medical engineering as well as aesthetics.

Acknowledgements

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