行政院國家科學委員會補助專題研究計畫成果報告

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計畫類別：☐個別型計畫　☐整合型計畫

計畫編號：NSC89－2320－B038－063－　－

執行期間：89年8月1日至90年7月23日

計畫主持人：何秀娥

共同主持人：葉健全

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執行單位：台北醫學大學藥學系

中　華　民　國　90年7月1日

行政院國家科學委員會專題研究計畫成果報告

可供細胞生長的膠原蛋白間質之特性研究

計畫編號：NSC 89-2320-B-038-063
執行期限：89 年 8 月 1 日至 90 年 7 月 31 日
主持人：何秀娥　台北醫學大學藥學系

一、中文摘要

本計畫利用 glutraldehyde 當做交鏈劑，探討膠原蛋白應用於細胞培養上，增加膠原蛋白纖維之間強度的影響性。• 1% 膠原蛋白溶液以不同濃度 glutaraldehyde 處理 24 小時。最後膠原蛋白膠體溶液的黏彈性將利用動態機械分析儀(DMA)測量。Voigt-Kelvin 的 Creep compliance model 用來描述這些膠原蛋白膠體溶液的黏彈性。這些經交鏈的膠原蛋白膠體溶液，經過冷凍乾燥法形成厚度在 0.2-0.3 mm 的膠原蛋白海棉組織。以 DMA 測膠原蛋白海棉組織的 break modulus 結果顯示交鏈程度愈高，剛性愈強。由裸鼠皮取出培養的纖維母細胞，分別注入 $3x10^5$ cells/ml 纖維母細胞於不同濃度的 glutaraldehyde 膠原蛋白海棉組織，結果顯示 glutaraldehyde 的濃度高達 0.2%時，膠原蛋白海棉組織仍可與纖維母細胞有很好的相容性。

關鍵詞：膠原蛋白，黏彈性，戊二醛，動態機械分析儀

Abstract

The influence of glutaraldehyde as a crosslinking agent to increase the strength of collagen matrices for cell culture was examined in this study. Collagen solutions of 1% were treated with different concentrations (0%-0.2%) of glutaraldehyde for 24 h. The viscoelasticity of the resulting collagen gel solution was measured using Dynamic Mechanical Analysis (DMA), which demonstrated that all collagen gel solutions examined followed the same model pattern. The creep compliance model of Voigt-Kelvin satisfactorily described the change of viscoelasticity expressed by these collagen gel solutions. These crosslinked collagen gel solutions were freeze-dried to form a matrix with a thickness of about 0.2 to 0.3 mm. The break modulus of these collagen matrices measured by DMA revealed that the higher the degree of crosslinking, the higher the break modulus. The compatibility of fibroblasts isolated from nude mouse skin with these collagen matrices was found to be acceptable at a cell density of 3×10^5 cells/cm^2 with no contraction, even when using a concentration of glutaraldehyde of up to 0.2%.

KEYWORDS: Collagen， Viscoelasticity， Glutaraldehyde， Dynamic Mechanical Analysis

Introduction

The necessity of artificial skin substitutes for wound healing or model membranes for penetration studies has promoted the development of a two-layered design with a sheet of keratinocytes attached to a biocompatible collagen-based substrate within which fibroblasts grow [1]. Collagen and its derived matrices have, therefore, become a popular biomaterial for this purpose and have been suggested as bioprostheses for burn wound dressings [2],

tissue templates, etc., due to their compatibility [3]. The desired properties of these matrices which facilitate wound-healing processes are stimulation of cell migration and infiltration, support of cell proliferation, as well as the non-contractibility of the matrices [4].

Glutaraldehyde (GA) is commonly used as a crosslinking agent for collagen-based materials. A major reaction pathway involved in this crosslinking is the aldehyde groups of GA (II) with the ε-amine groups of lysine or hydroxylysine residues (I) as shown in Scheme 1 [5]. The main purpose of chemical crosslinking using GA is to minimize contraction of the collagen matrices during cell growth, but its use is associated with marked cytotoxicity [6,7]. Further incorporation of glycosaminoglycans (20% chondroitin sulfate) [8,9] or hyaluronic acid into [10] collagen gels, and subsequent crosslinking with carbodiimides and diamines or glutaraldehyde is able to enhance the strength of the collagen gels, thus inhibiting fibroblast contraction of the collagen matrices [11].

The relationship between collagen crosslinking techniques and dentinum reinforcement has been examined [12] by various methods including the modulus of elasticity, elongation at breaking point, and stiffness. The tensile behavior of fibroblasts cultured in collagen gel has also been studied with the use of a time-lapse video recording system. It was demonstrated that fibroblasts generate tension and change their orientation along the tensile direction [13]. Couette flow, shear creep, uniaxial creep, and porous bed flow all revealed that cross-linked collagen was more resistant to deformation and flow than was non-crosslinked collagen [14]. This seems to indicate that the rheological behavior of collagen-based biomaterials and their tensile strength will determine the resistance to contraction. Dynamic mechanical analysis (DMA) can provide valuable information for this purpose.

In this study, collagen was modified by crosslinking with various concentrations of glutaraldehyde. Physical properties of these crosslinked collagen gel solutions and the resulting freeze-dried collagen matrices were characterized and evaluated for compatibility with fibroblasts. Optimally, a minimal concentration of glutaraldehyde could be elucidated to produce acceptable contraction with minimal cytotoxicity

RESULTS AND DISCUSSION

The extent of crosslinking after treating the collagen gel solutions with various concentrations of glutaraldehyde was measured, and the values are listed in Table 1. Results show that the extent of crosslinking of terminal amino groups on collagen increased with increasing concentrations of glutaraldehyde. Complete crosslinking was approached when the concentration of glutaraldehyde used was higher than 0.12% at a 1% w/w concentration of collagen.

The viscoelasticity of these collagen gel solutions was measured by following the creep compliance curve, and results are shown in Tables 2 and 3. The curvature portion (A-B curve) of all creep compliance plots can be described by a Voigt-Kelvin model: Compliance = Strain (ε)/Stress (δ) = [1/Young's modulus (E)]*(1 - exp(-t/τ)), in which τ is designated as η/E. Nonlinear regression of the compliance with respect to time t, Young's modulus (E), and retardation time (τ) were calculated from the corresponding equations. Results in Table 2 indicate that both the elastic modulus and viscosity (η) of the collagen gel solutions increased with increasing concentrations of glutaraldehyde. A significant increase in viscosity ($p < 0.05$) was noticed when the concentration of glutaraldehyde used was higher than 0.12%. Results in Table 3

illustrate that retardation time increased with increasing concentration of glutaraldehyde, indicating a gradual transformation of the liquid characteristics of the collagen gel solution into solid characteristics as the extent of crosslinking by glutaraldehyde increased.

The break modulus of the collagen matrices crosslinked with various concentrations of glutaraldehyde was monitored by DMA at the break point, and the results are shown in Table 4, which clearly illustrates that the break modulus of the collagen matrices increased with increasing concentrations of glutaraldehyde. A higher break modulus means a higher stress is needed to achieve a constant strain, thus indicating a higher resistance of the collagen matrices to deformation with an increasing extent of crosslinking. Further, the break moduli of these collagen matrices become higher when they were incubated with FCM (Fibroblast Culture Medium) before measurement. This is probably a result of reinforcement of the mechanical strength by increasing hydrogen bonding between collagen fibrils. This is further demonstrated in Figure 3 by the SEM photographs of collagen matrices crosslinked with various concentrations of glutaraldehyde. Denser structures of collagen films were obtained with increasing concentrations of glutaraldehyde for the same concentration of 1% w/w collagen.

Figure 4 shows the growth of fibroblasts within the collagen matrix crosslinked with various concentrations of glutaraldehyde. The cell numbers of growth at 48 h are all comparable, and no sign of cytotoxicity to fibroblasts was shown, even within a collagen matrix crosslinked with a concentration of glutaraldehyde as high as 0.2%. Figure 5 illustrates that at the two highest concentrations (0.15% and 0.20%) of crosslinking agent, the number of fibroblast cells gradually increased with time. The compatibility of the collagen matrix with fibroblasts was demonstrated. The contraction of collagen matrices by fibroblasts was minimal as determined by comparing the measurement of length and height before and after culturing (data not shown).

CONCLUSIONS

Complete crosslinking was approached when the concentration of glutaraldehyde used exceeded 0.12% at a 1% w/w concentration of collagen. A significant increase in viscosity and a gradual transformation into solid characteristics were noticed when the concentration of glutaraldehyde used exceeded 0.12%. A higher resistance of the collagen matrices to deformation with an increasing extent of crosslinking was also demonstrated. Furthermore, the number of fibroblasts gradually increased with time within the collagen matrices crosslinked with all concentrations of glutaraldehyde examined. The compatibility of the crosslinked collagen matrix with fibroblasts was demonstrated. In conclusion, the crosslinking of 1% w/w collagen with glutaraldehyde at a concentration of 0.12% seems to be optimal in terms of contraction and cell cytotoxicity.

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