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豬皮膠原蛋白免疫生成性之探討

Assays of the immunogenicity of the porcine dermal collagen membrane

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一、中英文摘要

本研究之目的在檢測臺北醫學院自製的豬皮膠原蛋白膜(porcine dermal collagen membrane; PDCM)是否具免疫生成性;以及戊二醛之作用是否會改變其免疫反應。30隻雄性、重350克的白鼠，分為五組，其中四組為實驗組，一組為對照組，每組6隻，將經三種不同濃度(3.00%、0.05%、0.01%)戊二醛修飾的PDCM以及未經戊二醛修飾的PDCM種入實驗組的下顎骨區;對照組之手術方式同實驗組，惟不植入PDCM。於術後3;6;9週進行心臟穿刺採集血清，並以酵素結合免疫分析法(ELISA)檢測;結果顯示未經戊二醛修飾的PDCM之免疫生成性遠高於經戊二醛修飾之PDCM，可見戊二醛可降低PDCM之免疫生成性。而未經戊二醛修飾的PDCM(抗原)與經0.01%、0.05%、3.00%三種不同濃度戊二醛修飾的PDCM刺激產生之抗體亦會產生交叉免疫反應，顯示經戊二醛修飾的PDCM產生之抗體仍能辨視未經戊二醛修飾的PDCM抗原，意即戊二醛雖能降低免疫生成性，卻不致使抗原決定部位產生明顯改變;由本實驗可知PDCM雖具免疫生成性，但可以適宜濃度戊二醛修飾降低之。

關鍵詞：
豬皮膠原蛋白，戊二醛，免疫生成性，交叉免疫反應，酵素結合免疫分析法.

Abstract

Previous studies showed that crosslinking of porcine dermal collagen membrane (PDCM) with glutaraldehyde (GA) could retard its resorption rate and still preserve its biocompatibility. The purpose of this study was to assay the relevant humoral immune response induced by PDCM and the possible effect of GA on PDCM's immunogenicity. Thirty Sprague-Dawley rats were selected and divided into five groups (n=6). PDCM reconstituted with 3 different concentrations (0.01%, 0.05%, 3%) of GA and the non-GA crosslinked (non-GAX) PDCM were implanted in the submandibular region of rats. The sham procedure was done on the control without grafting. Three, six, and nine weeks after implantation, sera were collected by cardiac puncture and assayed for anti-collagen antibodies by ELISA. The architecture of PDCMs was also observed under SEM. The results revealed that anti-collagen antibodies induced by non-GAX PDCM were statistically significant higher than GAX PDCM. The sera in rats implanted with non-GAX PDCM cross-reacted with the GAX PDCM and vice versa. It indicated that the immunogenicity of non-GAX PDCM was stronger than GAX PDCMs, and that cross-reactivity existed between antibodies induced by GAX antigen and non-GAX antigen. Under SEM, there were four different types of superficial structures: fibrillar structures, open pores, channels, and sheet-like structures. The greater the concentration of GA, the surface architecture of PDCM looks denser. It is concluded that crosslinking of GA could change the superficial stereo architecture of PDCM and reduce its immunogenicity.

Keywords: porcine dermal collagen membrane (PDCM); glutaraldehyde; immunogenicity; cross-reactivity.; enzyme-linked immunosorbent assay (ELISA).

二、緣由與目的

Collagens are the major component of mammalian connective tissues, and serve as the primary structural skeleton of tissues. Basically, they have phylogenetically conserved primary sequence and helical structure. The chemical structure and biological properties of collagen molecules make them useful in the production of suitable biomaterials.[1] It is unsurprising that collagen-based biomaterials have been used since the mid-nineteenth century, when catgut was used as suture material.[2] In the past two decades, numerous collagen-based biomaterials have been used clinically; for example, injectable soluble collagen has been used as a subcutaneous implant to correct dermatological defects.[3,4] However, questions concerning the immunogenicity were

raised soon after their clinical applications. Although conservation exists among the collagen molecules of different species, there are structural and sequence differences that can induce the host to mount an immune response to heterogenic collagens. The strength of immunogenicity is inversely associated with the degree of similarity of component and structure between host and the foreign protein. Consequently, any foreign protein that shares high degree of similarity with host protein is less likely to induce immune response and reveals high biocompatibility.

A number of immunological studies have been performed on clinically used collagen graft.[5,6] Cooperman[7] reported that about 3% of patients after receiving bovine collagen implant for therapeutic purpose produced antibody against bovine collagen. In addition, Siegle[8], Ellingsworth[9], pointed out that after intradermal injection of bovine collagen, anticollagen antibody was observed in the serum. These results document that heterologenic collagens do have immunogenicity.

DeLustro[10] compared the immunogenicity of several collagen products prepared by different methods and found that glutaraldehyde (GA) modification resulted in decrease of immunogenicity. Also, the experiments conducted by Oliver, Grant[11], Griffiths[12] and McPherson[13,14] proved that GA modified collagen, when implanted in human or animal, exhibited a great degree of fibroblast infiltration, vascularization, with only temporary inflammatory reaction, and no obvious immune response. These results indicate that chemical modification of collagen might reduce the immunogenicity of collagen.

Moreover, another problem occurred in Europe beginning in 1991 when it was found that cattle dying from bovine spongiform encephalopathy (BSE). As this infectious agent could infect the human being, concerns about the safety of the use of medical devices derived from bovine species were aroused[15].

We have been interested in developing alternatives of biomaterial from porcine collagen. Since 1992, we have prepared a biomedical device from porcine collagen[16] and named it porcine dermal collagen membrane (PDCM). Collagen was extracted from porcine dermis and treated with pepsin to remove the telopeptide, an immunogenic end, and finally GA was added to induce crosslinking reaction. Primary achievements, including studies on its biocompatibility and biodegradability[17], the healing of bony defects in guided bone regeneration technique[18], and its physical properties[19], have been made from the tests on the characteristics of PDCM after GA crosslinkage. From the findings thus obtained, the following characteristics of PDCM after GA modification have been verified: biocompatibility and biodegradability, effect in guided bone regeneration, ideal elastic modulus, controllable resoluble rate, proper cellular permeability, and controlled membrane expansion rate.

We attempted here to examine the possible effects of GA modification on the immunogenicity and surface structure of porcine dermal collagen membrane. PDCM was implanted into submandibular region of rats, and serum anti-collagen antibodies were measured by ELISA (Enzyme-Linked Immunosorbent Assay). In assessing cross-reactivity, the serum was allowed to react with both its homologous and heterologous antigens.

三、結果與討論

Results

Anti-collagen Antibody

The antibody levels in rats implanted with PDCM were shown in Fig.1. It was observed that the anti-collagen antibody levels in rats implanted with non-GAX PDCM were statistically significant higher than 3 other groups implanted with GAX PDCM ($p < 0.01$). Sera from rats implanted with the GAX PDCM demonstrated low antibody response against their respective implant materials, and the intensities of immune response among the 3 GAX test groups showed no statistically significant difference ($p > 0.05$). The amount of antibody in the non-GAX PDCM group increased gradually in accordance with the length of time after implantation. While no such tendency were observed in rats receiving GAX PDCM implants.

Cross-Reactivity Test

Sera from rats 9 weeks post-implantation with either non-GAX or GAX PDCMs were tested for cross-reactivity by ELISA, and the results were shown in Table 1. The antibody against non-GAX PDCM cross-reacted with GAX PDCM and vice versa. The antibody against non-GAX PDCM reacted stronger to its homologous antigen, the

implanted PDCM, than the heterologous antigens. It is surprising that antibodies against GAX PDCM reacted stronger to its heterologous antigen (the non-GAX PDCM), than to its homologous antigen (the GAX PDCM) significantly ($p<0.01$). And the antibodies to various different concentrations of GAX PDCM cross-reacted with each other in similar low activity with no statistically significant difference were noted ($p>0.05$).

SEM Observation

Under SEM examination, the structure of PDCM conditioned with different levels of GA could be divided into four categories:[20] (1) fibrillar structure: collagen fibers composed of aggregated collagen fibrils dispersed on the surface; (2) open pores: characterized by two aspects, surface pores formed from a series of open semiellipsoids, or hemispheres with walls that frequently had sheet-like structure; (3) channels: formed from open surface pores that stretched into the deeper layer of the sponge; and (4) sheet-like structures: formed a flat smooth surface.

In Fig.2, we can see that PDCMs crosslinked by different levels of GA displayed obvious differences in their structural patterns. For the non-GAX PDCM (Fig. 2a), loosely arranged fibrillar structure, different sizes of open pores, and channels connected with deep structure in between collagen fibers could be seen. For PDCM conditioned by 0.01% of GA (Fig. 2b), crosslinking effect among collagen fibers could be observed, while less fibrillar structure was seen than that observed in non-GAX PDCM (Fig. 2a). The distribution of pores and channels was still evident. However, its size was obviously smaller than that in Fig. 2a, mild crosslinking effect was noted for the little shallow and flat sheet-like structures. For PDCM conditioned by 0.05% of GA (Fig. 2c), crosslinking effect among collagen fibers was quite obvious, few fibrillar structure could be seen, with small size and amount of pores scattering around occasionally. Nearly complete sheet-like structure was seen on the surface. For the PDCM conditioned by 3.00% of GA (Fig. 2d), crosslinking effect among collagen fibers was quite ideal. Almost all collagen fibers were polymerized into complete sheet-like structure with folds formed. Neither pore nor channel was seen and the structure was tightened and complete.

Discussion

This study allows a comparison of the immunogenicity of GA crosslinked (GAX) and non-GA crosslinked (non-GAX) porcine dermal collagen. PDCMs were implanted into the submandibular region of rats, the original design for this animal model is to simulate the clinical condition under which the collagen membrane is used to repair jaw bone defects. Sera from rats were used to measure anti-collagen antibody by ELISA. Antibody responses to collagen were noted 3 weeks post-implantation. But anti-collagen antibody levels in sera from rats implanted with non-GAX PDCM were higher, and persisted longer, than that of rats implanted with GAX PDCM, as shown in Fig. 1. At any time during the course of immune response, the antibody levels in rats implanted with non-GAX PDCM is higher, and this discrepancy became more obvious 9 weeks post-implantation, when the antibody level is about 7-fold higher ($p<0.01$). Although the kenetics of antibody responses in all tested rats were similar, i.e., a lag period followed by a logarithmic phase, the antibody levels in rats implanted with GAX PDCMs reached peak at the 6th week post-implantation and then declined. The results suggest that GA crosslinking affect both the strength and duration of antibody response to porcine collagen.

The mechanism responsible for the decrease of immunogenicity of GAX collagen is not yet clearly understood. There might be two possible explanations, at least, as suggested in the present study. First, it is due to decrease in degradability. The development of humoral immune response to protein antigen requires the participation of Th cells. For activation of Th cells, antigen must be taken up, processed, and presented in association with major histocompatibility complex (MHC) molecule by antigen presenting cells. Any foreign protein that cannot be degraded and is inaccessible to Th cells, will as a consequence be poorly immunogenic or nonimmunogenic. As we pointed out in our previous study[17], PDCM crosslinked with 3% GA was apparently intact at the 9th week post-operation, while the non-GA crosslinked PDCM was almost completely degraded within 3 weeks. The results suggest that GA crosslinking of collagen render it resistant to degradation, resulting in longer persistence in the body and reduced immunogenicity. And secondly, the conformation of collagen molecule is changed or masked. The antibody specificity is species-

dependent and genetically regulated[21]; it is known that rats recognize the conformational determinants on collagen molecule. Due to conformation changes or stereo hindrance, GA-crosslinked collagen molecule is not recognized by rat lymphocytes any more. As can be seen in Fig. 2, the surface structure of GA-treated PDCM became denser. Recent study[22] performed by Bowers et al. showed that the response of cells or tissue interfaces could be affected by surface topography of implants.

As shown in table 1, all sera from rats implanted with either non-GAX or GAX PDCM produced antibody against the implants. The sera in rats implanted with non-GAX PDCM cross-reacted with the GAX PDCM and vice versa. The serum from rats implanted with non-GAX PDCM reacts stronger to its homologous antigen (the non-GAX PDCM) than does the heterologous antigen (the GAX PDCM). Of particular interest is one point, we found that the antibody against GAX porcine collagen reacts more strongly to the heterologous antigen than does its homologous antigen. These results suggest that the GA treatment does not change the antigenic determinants of collagen molecule. The possible explanation is that treatment with GA results in intermolecular crosslinking and lattice formation in such a way as to mask the antigenic determinants on the original collagen molecule, making them unavailable to antibody molecule. This speculation is verified by the SEM observation in the present study.

The results as shown in Fig.1 seem to suggest that the immunogenicity of non-GAX PDCM was higher than that of GAX PDCM. However, since the serum samples used in this study are undiluted, this may imply that antibody levels are not high, i.e. the immunogenicity is weak or low. It has been known that the telopeptide domain of collagen molecule contains the greatest species variability.[23] In the present study the telopeptide domain was removed by pepsin, and this minimized the immunogenicity.

四、計畫成果自評

研究內容基本上與原計畫相符，雖增加豬皮膠原蛋白膜在電子顯微鏡下之研究觀察，惟實驗動物數量有所減少，但所得實驗結果仍頗具學術應用價值。在預期目標達成上，除免疫墨點結果不如預期理想將繼續進行測試外，其餘部分皆已達成預計目標。

本研究成果足以證實臺北醫學院自製之豬皮膠原蛋白膜經蛋白胨消化酵素處理後已不具強烈的免疫生成性，而經由戊二醛之修飾作用，更可使其免疫生成性降低。

本一年期之研究成果應具相當之學術應用價值，因此已計畫將之發表於國際性學術研究期刊。

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Figure legends

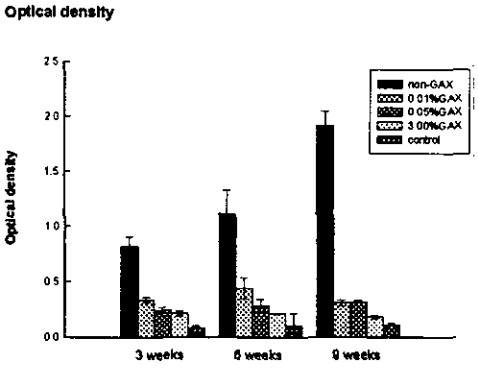

Fig.1. Antibody responses to PDCM treated or untreated with different concentrations of GA measured by ELISA at scheduled time intervals. The results were expressed in OD as mean ± S.D. for each group. Undiluted serum samples were used.

Fig. 2. Structural patterns of PDCMs treated by different levels of GA examined under SEM (× 100). (The SEM photographs can't be enclosed in the text)

Table 1. Cross-reactivity* between GAX and non-GAX PDCM

Ag / Serum Ab	non-GAX PDCM Ag	0.01%GAX PDCM Ag	0.05%GAX PDCM Ag	3.00%GAX PDCM Ag
Non-GAX PDCM Ab.	1.158±0.026	0.277±0.008'	0.290±0.012'	0.317±0.008'
0.01%GAX PDCM Ab.	1.189±0.032	0.375±0.013'	0.367±0.015'	0.390±0.011'
0.05%GAX PDCM Ab.	1.293±0.030	0.270±0.006'	0.251±0.008'	0.268±0.009'
3.00%GAX PDCM Ab.	0.821±0.024	0.259±0.011'	0.377±0.009'	0.322±0.014'
Control	0.149±0.007'	0.097±0.005'	0.090±0.006'	0.050±0.007'

* expressed in OD value (mean±S.D.)
' Significantly difference ($p<0.01$)