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Modification of Collagen Film by Certain Chemical Agents

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Abstract. Morphological changes and the content of free carboxyl groups in bovine collagen (type I) film under the influence of trypsin, hydrochloric acid (HCl) and ethylenediaminetetraacetic acid (EDTA) were studied. Incubation with trypsin and HCl was found to cause some delamination of the film and the appearance of some low-density spots. Incubation with EDTA did not cause any morphological changes. A high concentration of free carboxyl groups (10-fold higher than in control) was seen after incubation with trypsin.

Key words: collagen modification.

Introduction

Among biological materials, collagen, particulary type I, is a major component of connective tissues. Type I collagen has a helical structure composed of three polypeptide chains: two identical chains α_1(I) and one chain α_2(I). Type I collagen molecules form fibrils, strengthened by two types of covalent crosslinks: intramolecular and intermolecular. The covalent intermolecular crosslinks between collagen molecules in macromolecular fibrils are essential for the stability and various physico-chemical properties of native and performed collagen[1,9,13,22].

The application of collagen-derived products, such as wound dressings[6,19], artifical dermis[17], tendon substitutes[23] and injectable materials in plastic surgery[8], has had an increasing impact on biomedicine and in the clinic. Collagen products may be used as a support for cells in tissue culture[18] and as a scaffold for tissue repair or regeneration[7,20]. One of the advantages of using animal, particulary bovine, collagen is the possibility to obtain large quantities of pure type I or type I/III[12]. Physico-chemical changes of collagen under the influence of some agents were investigated, e.g. the study of prolonged exposure to concentrated sodium hydroxide (NaOH) solution, formic acid (FA), trifluoroacetic (TFA), tetrafluoroethanol (TFE) and hexafluoroisopropanol (HFIP)[4]. It was also proved that bovine and human collagen type I may be degraded by trypsin in experimental conditions[10]. Treated collagen was found to be a good support for growing cells[18].

The goal of this study is to describe collagen (films) treated with trypsin, HCl and EDTA and to observe morphological and physico-chemical changes in the collagen film.

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Materials and Methods

Preparation of collagen films. The collagen was prepared using native type I collagen extracted from bovine Achilles tendons of two-year old animals. The collagen was extracted and processed according to the procedures described by NISHIHARA[14], with our modification[21]. The Achilles tendons were chopped into small pieces in a mincing machine and incubated with 10% sodium chlorate. After washing and crumbing, the collagen was solubilized by 0.1% acetic acid overnight. The next steps were precipitation of the collagen with 0.1 M sodium phosphate, washing and incubation in 0.1 M HCl and then digestion by pepsin for 24 h, followed by another precipitation with 0.02 M sodium phosphate. The sediment was dissolved in 0.1 M acetic acid and homogenized, and the collagen solution was centrifuged at 4000 rpm for 20 min. The sediment was removed and the supernatant, in the form of gel collagen, was processed. Excess air was removed and the film was prepared at 37°C. The dried collagen film was neutralized (vapors NH_4 and washed with aceton and distilled water in a 1 : 1 solution). Finally, the collagen film was sterilized at 110°C for 5 h.

Collagen substrates. Disks of collagen films (thickness 0.05 mm, weight 2 mg, diameter 8 mm) were prepared. Furthermore, the disks of collagen films were treated by:

1) incubation in 0.5% EDTA for 3 × 20 min room temperature,

2) incubation in 0.25% tripsin (T) for 15 min at 37°C, pH 8.1. Inactivation of trypsin by addition 0.05% soybean trypsin inhibitor solution was done,

3) incubation in 0.1 N HCl for 3 × 20 min at room temperature and then washed in distilled water,

4) as a control, non-modified collagen was used.

In order to verify a possible convertible effect of the reagents used for the collagen film modification, some cross tests were performed according to the following schema:

1) HCl-treated collagen was incubated with trypsin solution,

2) HCl-treated and trypsin- incubated was repeatedly treated with HCl,

3) EDTA-treated collagen was incubated with trypsin,

4) EDTA-treated and trypsin-incubated was treated again with EDTA.

After these procedures, a test with crystal violet binding was performed.

Estimation of the free carboxyl groups. The free carboxyl groups in collagen were investigated by staining with crystal violet after RAPPAPORT et al.[15] using the following procedure:

1) disks of collagen film were incubated with a 0.005% solution of crystal violet in distilled water for 30 min,

2) the stain solution was decanted and the disks were rinsed 3 times with distilled water,

3) the stain bound to the collagen was removed with absolute ethanol for 5 min at room temperature,

4) the concentrations of crystal violet binding to the collagen film disks were measured with an automatic plate reader (Labsystems AUTO EIA) at 600 nm optical density,

5) the measurements were interpreted with a calibration curve of different concentrations of crystal violet.

Electron microscope. Prior to electron microscopic observation, the specimens were kept in Dulbecco's MEM medium for 24 h at 37°C and then processed after[11] as follows: the specimens were fixed with 3% glutaraldehyde in cacodylate buffer (pH 7.4), post-fixed in 1% osmium tetroxide solution at room temperature for 25 min, washed in running water for 2 h and dehydrated through a graded series of ethanol. Further, the specimens were embedded in Epon-812. The ultra-thin sections were contrasted in 2% uranyl acetate and 0.1% lead citrate and photographs were made using an electron microscope (JEM 100S Japan).

Light microscope. The dry collagen film, after being incubated with PBS solution (24 h at 37°C), underwent fixation with 4% formaldehyde-buffer for 1 h, was dehydrated and embedded in paraffin, and serial sections was cut at 8–10 μm (microtome – Leitz 513), stained with hematoxylin and observed in a light microscope.

Statistical analysis. All the reported values are expressed as means ± standard deviations. Statistical significance was assessed using the Student's *t*-test for the estimation of the free carboxyl groups in collagen. A difference was considered significant when p was less than 0.05.

Results

The influence HCl on collagen films

A low concentration of HCl (0.1 N) caused some changes in the structure of the collagen film. Collagen film treated with hydrochloric acid binds a small quantity of crystal violet. The mean value of extinction was 0.072 μM /mg of collagen, which was the lowest result compared with the others (Fig. 1).

In light microscopy, a homogenic structure with lamellar arrangements can be seen (Fig. 2A). In an elec-

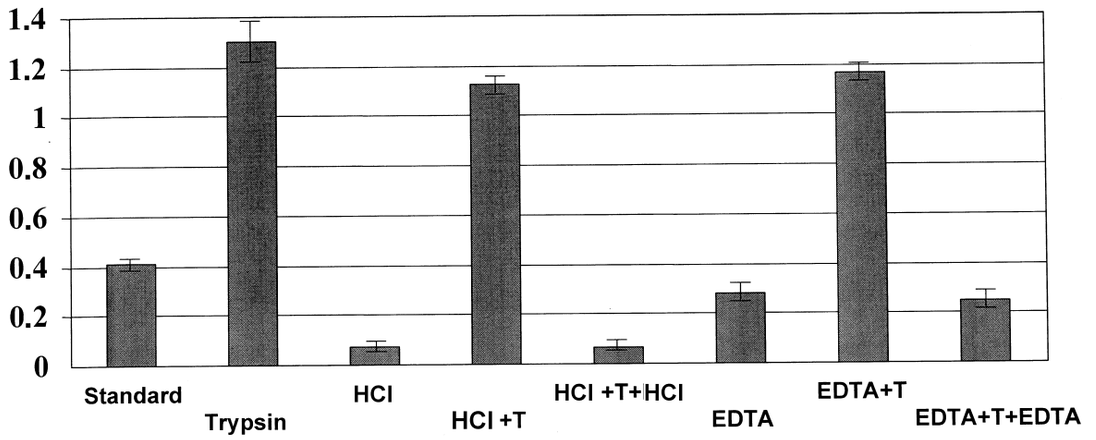

Fig. 1. Free carboxyl groups (as μM of crystal violet/mg of collagen) present in films after various experimental treatment. Significant differences from standard material (Student's *t*-test) $p<0.05$ were found in: HCl, HCl+T, HCl+T+HCl and EDTA, EDTA+T, EDTA+T+EDTA

tron microscopy of vertical slits drifted inside membrane, about a quarter of thickness were found with areas of low density (Fig. 2 B).

The influence of trypsin on collagen films

Incubation with trypsin changed the structure of the collagen film. The test with crystal violet demonstrated a high concentration of stain bound to the film of 1.3 μM/mg collagen (Fig. 1), showing that the concentration of of the free carboxyl groups is the highest in this material. The electron microscope revealed numerous stratifications parallel to the surface of the collagen film and the areas of low density (Fig. 3).

The influence of EDTA on collagen film

After incubation of collagen film with EDTA, no changes were observed in the collagen film structure. The test with crystal violet demonstrated a low concentration of free carboxyl groups of 0.28 μM/mg collagen (Fig. 1). A dense and uniform collagen structure (Fig. 4) was also seen in the electron microscope.

Non-modified collagen

The test with the use of crystal violet indicated a low concentration of free carboxyl groups in this material of 0.4 μM/mg of collagen (Fig. 1). In both the light and the electron microscope, this material was shown to be as homogeneous, with a compact structure (Fig. 5).

Discussion

This paper presents a possibility of collagen film modification by means of incubation with some chemical agents. After the applied procedures, the changes in collagen films morphology are visible in light and electron microscopes (Figs.: 2A and 3A or 2B and 3B). The concentration of the free carboxyl groups in treated collagen was affected (Fig. 1). Significant microscopic changes were detected when films were incubated with trypsin and a low concentration of HCl. No changes were found when film was incubated with EDTA. A remarkable liberation of free carboxyl groups was seen only after film digestion in trypsin, around 10 times more than in the standard material. Incubation with HCl caused a significant decrease of the free carboxyl groups in comparison with the control. After incubation with EDTA, the concentration of the free carboxyl groups was similar to that of the control. Binding to processed film was observed in the cross-tests with crystal violet. Such influence of trypsin is reversible when films preincubated with the enzyme are postincubated with HCl or EDTA. Postincubation with HCl and EDTA of trypsin-treated films confirms the dominant, effect of trypsin on the appearance of free carboxyl groups in collagen film.

Other studies[2,5,10] have shown that precipitated collagen is degraded by trypsin and HCl, especially with acid pretreatment[16]; it was also found that treatment of collagen implant with various chemicals does not diminish cellular growth on it[4]. Adhesion and proliferation of epithelial cells on modified collagen it is planned to estimate.

In conclusion: 1) film obtained from bovine collagen can be modified by incubation with trypsin or a low concentration of HCl; 2) morphological changes, such as delamination of the collagen structure and a presence of low-density spots, are seen after incubation in HCl and trypsin; 3) a high concentration of free carboxyl groups is observed after incubation of collagen film with trypsin.

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