A HISTOCHEMICAL STUDY OF THE PROTEINS OF PRECOLLAGEN FIBERS IN REGENERATING CONNECTIVE TISSUE

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There is considerable evidence in the literature that precollagen fibers are an initial phase in the formation of definitive collagen fibers [1, 2, 3, 6, 7, 8, 12, 15, 17, 20]. The precollagen fiber corresponds in its histochemical, electron-microscopic, and x-ray structural properties to collastromin, the basis of the collagen fibril. The latter is insoluble in weak solutions of alkalies, acids, and citrate buffer solution [6]. Nevertheless there is no information in the literature on the relationship between precollagen fibers and acids, alkalies, and citrate buffer solution in the course of their formation during the regeneration of connective tissue. Obviously no comparison has been made of the chemical properties of collastromin and procollagen at different periods of scar formation.

Bearing in mind that a definite time is required for the formation of the external and internal phases of the collagen fibril, we may postulate the existence of a definite series of stages in the formation of each of these phases. Reports in the literature indicate changes in the physico-chemical properties of the procollagen membranes of collagen fibrils with age and during maturation of the scar [9-11, 14].

In the present research our starting point was the suggestion that in the course of formation of precollagen fibers their physico-chemical properties are modified, and especially their solubility in acids, alkalies, and buffer solutions.

EXPERIMENTAL METHOD

The solubility of precollagen fibers was investigated at various periods of scar formation. The action of these solvents mentioned above on collagen and reticulin fibers was studied simultaneously.

The test object was skin wounds of rats inflicted 5 and 10 days before the experiments. Pieces of tissue from the healing wounds were treated: 1) with citrate buffer solution (pH 4.0) for 1, 12, and 24 h, and 5 and 10 days; 2) with 2 N acetic acid solution for 15 min; 3 with 0.5 N, 1 N, and 2 N solutions of NaOH for 1, 3, and 10 h; 4) with phosphate buffer solution (pH 7.0, 7.5, 8.0) for 10 h.

After treatment, the material was washed with distilled water and fixed in 96° alcohol for 12 h, with subsequent clearance through methylbenzoate and embedding in paraffin wax. Sections 7 μ thick were impregnated by Gomori's method, stained by van Gieson's method, and treated by Barrnett and Seligman's method to detect α-acylamidocarboxyl groups (in contrast to Gomori's impregnation method, this method gives more reliable and reproducible results when used to detect the proteins of the precollagen fiber, because it is based on a concrete chemical reaction [13].

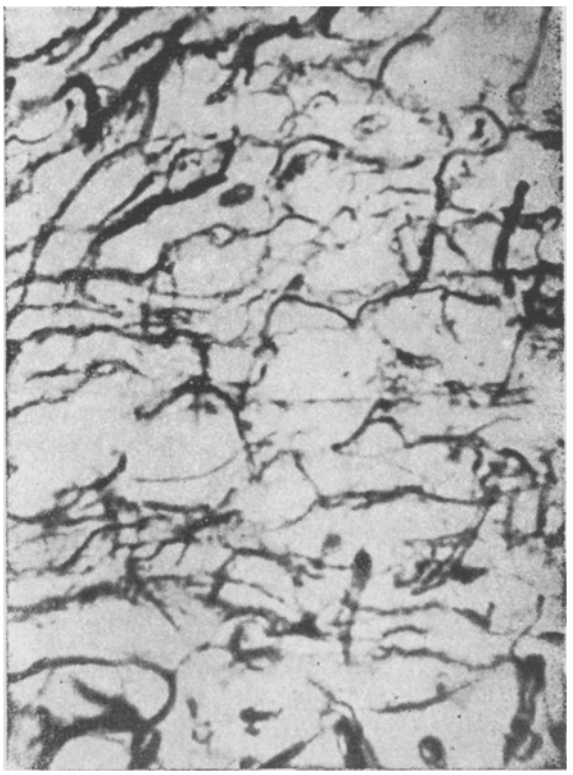
Controls to this series of experiments were 5-day and 10-day old skin wounds in rats untreated by acids, alkalies, or buffer solutions. Previously we used the following histochemical methods of detection of proteins: the tetrazonium reaction, demonstration of SH and S-S groups, determination of arginine, the reaction with DNPB, and the reaction for amino groups. The reactive groups in the proteins of the precollagen fibers either did not show up at all, or did so very weakly (SH and S-S groups).

EXPERIMENTAL RESULTS

In granulation tissue 5 days old, treated with citrate buffer solution, impregnation by Gomori's method revealed a very slight bead-shaped swelling and partial dissolving of the precollagen fibers. Mature collagen fibers in areas

of healthy skin, investigated for purposes of comparison, showed increased argentophilia as the period of treatment with citrate buffer solution lengthened. No changes were observed in the reticulin fibers of the walls of the small blood vessels.

When granulation tissue taken from the wound on the 5th day after injury was stained to show α-acylamidocarboxyl groups, the precollagen fibers took up the dye clearly but not intensively. After treatment with citrate buffer solution, this reaction showed no significant changes in these fibers (see figure). Likewise they remained unchanged after treatment with acetic acid. Mature collagen fibers treated with acetic acid showed swelling and loosening of their arrangement.



Precollagen dibers in a 5-day old skin wound in a rat after treatment for 10 days with citrate buffer solution. Reaction of Barrnett and Seligman to detect α-acylamidocarboxyl groups. Eye-piece 7, objective 90, immersion.

In solutions of phosphate buffer, slight swelling of the precollagen and collagen fibers was observed. The mature collagen fibers swelled more intensively than the precollagen. The reticulin fibers remained unchanged.

In caustic soda solutions gradual homogenization and solution of the precollagen figers took place as the concentration increased, reaching a maximum in the 2 N NaOH solution. In the mature collagen figers treated with low concentrations of caustic soda the argentophilia was increased, and in high concentrations the fibers were completely dissolved. Characteristically, complete solution of the mature collagen fibers took place more slowly than in the case of precollagen. When Gomori's method was used, thin argyrophilic fibrils remained for a long time at the site of the dissolved collagen fibers. They could also be detected by the reaction for α-acylamidocarboxyl groups. The changes in the reticulin fibers were similar but less pronounced.

In the 10-day old scar treated with citrate buffer solution, impregnation by Gomori's method and staining by van Gieson's method or to demonstrate α-acylcarboxyl groups showed gross swelling of the young collagen fibers of the scar. It began during treatment with citrate buffer solution and needed a shorter time than in the case of mature collagen fibers. In the latter, and also in the reticulin fibers, the same changes were observed as in the 5-day old scar.

Young collagen fibers swelled more intensively than old in acetic acid. In caustic soda solutions, as the concentration increased so also did the swelling and solution of the young collagen fibers, to a greater degree than the mature.

The results showed that the precollagen fibers were only slightly soluble in citrate buffer solution and acetic acid on the 5th day of regeneration. Although impregnation by Gomori's method revealed a very slight bead-like swelling of the precollagen fibers, in view of the fact that similar changes could not be detected by Barrnett and Seligman's method of staining for α-acylamidocarboxyl groups, it must be concluded that collastromin was relatively more resistant to acetic acid and to citrate buffer solution.

Treatment with phosphate buffer solution showed swelling, and treatment with alkali complete solution of the collastromin. The precollagen fibers were apparently less resistant to the action of alkali than the collastromin of the mature collagen fibers. The precollagen of the collagen fibers of the 10-day old scar dissolved readily in the citrate buffer solution, in acetic acid, and in alkali. Characteristically, as the precollagen matured (precollagen of mature collagen fibers) it became less soluble in these various solutions.

The reticulin fibers of the small blood vessels closely resembled the precollagen fibers in some of their properties, namely, in their insolubility in citrate buffer solution and acetic acid. There are reports in the literature of considerable differences also occurring between these types of fibers [5].

Hence, from the moment of its appearance in the wound, the protein of collagen type, forming precollagen fibers, possesses properties completely different from those of procollagen.

The results of our observations do not agree with those obtained by Jackson [16], according to whom precollagen fibers are soluble in saline and citrate buffer solutions. This discrepancy may be attributed to the fact that the impregnation technique is not suitable for the detection of precollagen fibers after intensive physico-chemical treatment.

Our findings confirm those of G. V. Orlovskaya and A. A. Tustanovskii, obtained in embryonic material [4]. X-ray structural analysis has demonstrated the presence of two proteins in collagen fibers—collagen 1 and collagen 2, which are structural isomers [19]. Rich and Crick suggest that collagen 1 differs from collagen 2 by the arrangement of the amino-acid side chains. They consider that in collagen 1 the hydroxy group of hydroxyproline forms a hydrogen bond within the limits of a three-chain unit, while in collagen 2 hydroxyproline takes part in the formation of bonds with neighboring three-chain units.

Earlier work by Soviet researchers furnished biochemical and electron microscopic evidence of the existence of two different proteins in the mature collagen fiber. A. A. Tustanovskii [8] claims that the insoluble protein called collastromin can be identified with the collagen 1 of Rich and Crick or the "plus" protofibril of Ramachandran [18].

The insoluble residue of the collagen fiber almost fails to give any histochemical reactions, enabling the detection of certain groups capable of reacting in the side chains of the amino acids [9]. These facts enable us to understand the results described in this paper relating to the low solubility of the precollagen fibers of the regenerating connective tissue, as the result of the inaccessibility of certain of the chemical groups in the protein molecules of these fibers. Precollagen fibers possess these properties in the early stages of their existence. We have shown that, in contrast to the proteins of newly formed, precollagen fibers, the procollagen present in the young fibers is more readily soluble than the procollagen of the old fibers at the margins of the skin wound. In the old scar [11] the procollagen has become still less soluble (in acetic acid and citrate buffer solution). Hence, whereas in the evolution of procollagen phases of high and low solubility are clearly distinguishable, in the evolution of the proteins of precollagen fibers these two phases are not observed. The solubility of collastromin (in the composition of mature fibers) is probably somewhat different from the solubility of the proteins of the precollagen fibers, although the experimental conditions were not absolutely identical (different thickness of the fibers). It may also be suggested that the proteins of the precollagen fiber pass through a stage of high solubility in the cell protoplasm. This problem requires special analysis.

SUMMARY

An inquiry was made into the action of weak solutions of acetic acid, alkali, and citrate buffer on precollagen and collagen fibers of regenerating connective tissue. Precollagen and collagen fibers were studied with the aid of Gomori's method, the method for acylamidcarboxyl groups, and van Gieson's method. Precollagen fibers in the regenerating connective tissue are hardly soluble in the citrate buffer solution and acetic acid but they are readily soluble in alkali. From the moment of its appearance in the regenerating connective tissue, procollagen is well soluble in the citrate buffer solution, alkali, and acetic acid. Precollagen solubility falls with the maturation of connective tissue.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.
