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Enzymatic Remodeling of Connective Tissue as a Novel Approach to Rejuvenation in Mammals

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Abstract

Background: The principal timing mechanism in a mammal's ageing remains unrevealed despite more than one century of gerontologic investigations. We hypothesized that modifications of connective tissue composition could reverse the physiological processes associated with aging.

Objective: To describe a novel method for rejuvenating aging mammals by means of enzymatic remodeling of connective tissue.

Methods: Collagenase was used as the collagenolytic agent (CLA) for selective destruction of mature and old collagen in a rodent model. CLA was injected in the rat's tail vein once every two weeks for 12 weeks. The monitored parameters were: urine hydroxyproline excretion (as an indicator of mature and old collagen destruction), the content of mature and old collagen and elastin in the dermis of the skin biopsy sections, and oxygen consumption (as an indicator of whole-body metabolic oxygen activity).

Results: Following CLA injections, a significant increase in hydroxyprolinuria (average 1.7-fold; $p<0.05$) was observed. Decreases were observed after the injection in the mean percentage of dermal area involved by mature and old collagen. The rats' oxygen consumption rate increased 1.25-fold after the course of CLA injections ($p<0.001$ vs. the pre-injection value).

Conclusion: This study provides evidence for the breakdown of mature and old collagen, the formation of a new collagen matrix, and a significant increase in elastic fiber formation, following CLA injections. All-body oxygen consumption also increased. These data can be interpreted as indications of systemic CLA anti-aging activity.

Keywords: Ageing; Colloidal systems; Connective tissue; Extracellular proteins; Proteoglycans; Rejuvenation

Introduction

The rapid progress of theoretical and practical medicine throughout the last century resulted in a considerable increase in life expectancy. Humans are expected to approach the 100-year mark and reach their biological limit in the 21st century. Increased life expectancy, however, is achieved mainly due to the extension of the old-age period [1,2] which is characterized more by physical limitations and chronic pathologies, rather than by the extension of the years of physical fitness and productivity. Differences in life expectancy among biological species and the similarity in the spectrum of signs of senescence within species indicate that aging is a genetically programmed process. This claim is evidenced by several studies that investigated the role of genetic mechanisms in aging [3-9].

To create a reliable and efficient method for biological rejuvenation in mammals, including humans, it is necessary to understand the mechanism of ageing and how the "Biological clock" works. Current theories of ageing are based mainly on the influence of random environmental factors (e.g., free radicals), and they fail to explain the common physiological pathways of the aging process. All attempts to create a method of rejuvenation,

or at least a slowing down of the aging process, on the basis of existing theories, have thus far been unsuccessful.

A biological system that most probably drives the "Biological clock" is connective tissue. The role of connective tissue in the process of ageing has been investigated in a number of gerontological schools. Sixty-five years ago, F. Verzar [10], the well-known Swiss gerontologist, demonstrated in rats, the age-dependent increase of the tensile strength developed by progressively heated collagen fibers. This finding led to a new realm of aging biology, which entails the aging of the extracellular matrix, independent of the aging of cells [3]. In higher animals, body cells are protected from direct contact with the external environment by skin and mucous membranes. Cells exist within an internal habitat, i.e., within the connective tissue that permeates all the organs and tissues of the body. Blood and interstitial fluids circulate through the connective tissue, bringing nutrients to body cells and removing the end-products of their metabolism. Blood and tissue fluids are also involved in the regulation of cell activity, supplying information in the form of various hormones [11-14]. Connective tissue is vital for protection of cells from the external environment. Decreasing permeability of connective tissue over time, however, causes aging and eventually death of the whole organism. The important role played by connective tissue in the process of aging is evidenced by the fact that unicellular and primitive multicellular organisms, which have no connective tissue, do not display any signs of aging. Under laboratory conditions and with regular refreshing of their habitat, they can live indefinitely, so that for all practical purposes they are virtually immortal. The cells of these organisms both feed off and release the waste products of their metabolism directly into their surroundings [11-16].

Connective tissue is comprised of cells and intercellular spaces that are filled with fibrillar structures of proteins, such as collagen and elastin [17-20]. An interfibrillar space is filled with basic substances, specifically, colloidal solutions of proteoglycans (including hyaluronate) and glycoproteins [17-20]. The permeability of connective tissue depends on the colloidal state of these basic substances [17-20]. Colloidal solutions differ from true solutions in that the particle content of dissolved substances agglutinate over time, become larger, and eventually form a sediment of continuously increasing density [21]. The human body fights against this phenomenon by enzymes that break down the "Aging" protein lumps. Extracellular proteins, however, are not easily accessible to enzymes, which is the reason they are destroyed and replaced by new and "Young" proteins, though only at a very slow pace [22-24]. The extent of activity of these enzymes progressively and significantly decreases over time [22-24]. Thus, old proteins accumulate and form cross-links between their polypeptide chains [25-29]. This process increases the density of connective tissue and decreases its permeability, which, in turn, slows the circulation of tissue fluid. As a result, cell nutrition and the removal of metabolic waste are disrupted, and the hormonal regulation of cell functioning becomes deficient. These disruptions lead to a decline in the functional capacity of the organism, which is the essence of the aging process. The accumulation of old, dehydrated, and dense extracellular proteins of connective tissue in the skin of aging people leads to a change in appearance. Investigations have revealed that characteristic features of skin connective tissue aging are largely due to aberrant collagen homeostasis [30,31].

Our approach to systemic biological rejuvenation in mammals is based on a connective tissue concept of ageing. We are unable to modify a genetically determined structure of connective tissue proteins and enzymes that are involved in its natural remodeling, but we can attempt to artificially change connective tissue remodeling. Therefore, we propose a new method for systemic rejuvenation by replacing old dehydrated extracellular proteins with young ones that are able to form hydrophilic colloid systems with high permeability. This involves active destruction of the old extracellular proteins and simultaneous synthesis of young proteins, as a reaction of the fibroblasts, to the oligopeptides and glycosaminoglycans produced by the destruction of the old proteins [3,22]. We reason that the resultant changes in the environment of the internal body cells will contribute to slowing and even reversing of the aging process in the entire body.

Materials and Methods

Materials

Systemic destruction of mature and old collagen in animal tissue was performed by intravenous injection of a collagenolytic agent, CLA (collagenase from clostridium histolyticum, Type VII, 1000-3000 CDU in mg, high purity, purchased from Sigma-Aldrich Company). The animals included 18- to 20-month-old wild-type white laboratory rats (10 males and 9 females) purchased from Jerusalem University and housed in its vivarium. Testing was performed in the Biotechnological Laboratory of the Jerusalem Technological College (Israel) and at the Warren Alpert Medical School of Brown University (USA) and of the Jerusalem University Skin Aging Laboratory (Israel).

The ability of CLA to destroy the mature and old collagen was estimated by measuring the rodents' daily hydroxyproline urinary excretion. Collagen is the only source of hydroxyproline. Therefore, increased urinary excretion of this amino acid is a reliable measure of the intensification of collagen destruction. Histochemical and histological examination of the rat's skin biopsy was also used to detect changes in the extracellular space of connective tissue after a CLA injection. Biopsies were obtained by means of bronchoscopic forceps from the skin of the animal's flank. Change in metabolic rate was assessed by measuring the animals' oxygen consumption. Since this index decreases with

age, [32] any increase after a CLA injection may be considered a sign of a youthful-like change in the metabolic process.

Measurement of urine hydroxyproline excretion

The animals were fed with a standard diet low in hydroxyproline for one week, and each was kept in a metabolic cage (Labotal, Israel) in order to collect daily urine output before the start of the experiment. The concentration of hydroxyproline was measured in the daily urine collection by the colorimetric method [32]. The urine hydroxyproline excretion measures were taken one day before, one day after and 10 days after a single injection of CLA. Five ml of urine collected during a single day were transferred to a pyrex glass tube with a screw cap, and a series of tubes was prepared for calibration using Stock standard hydroxyproline solution 5. Five mmol/l of concentrated hydrochloride were added to each of the tubes, which were closed and placed in a hot air oven overnight at 95°C. The tubes were cooled on the following day, the contents were filtered through Whatman #1 filter paper, diluted to 10 ml with water, and 7.5 ml of this mixture were loaded onto a column containing 20 g Biorad AG 11 A8 ion-retardation resin (obtained from Bio-Rad Laboratories) for removal of HCl**.** The column was eluted with water, after which the first 10 ml of eluate were discarded, and the next 15 ml were collected in a 25-ml cylinder. Then, 0.5 ml of eluate was transferred to a pyrex tube containing 1.0 propan-2-ol; 0.5 ml oxidant solution was added, and the contents of the tube were mixed on a Rotamixer for 10 seconds. The oxidation was allowed to proceed for 4-6 minutes at room temperature. One ml of Erlich's reagent was then added and, after another 10 seconds of mixing on the Rotamixer, the tube was capped and heated at 80°C for four minutes. The tube was cooled for five minutes and the color of the solution was read in 1-cm cells at 560 nm on a spectrophotometer (Spekol1300A, Analytik Jena AG, Thuringia, Germany).

All reagents that were used to perform the calorimetric method for determining the amount of hydroxyproline were purchased from Sigma Company. The 24-hour hydroxyproline elimination through urine was calculated per one kg of animal body weight.

Quantitative measurement of the collagen and elastic fibers distributed in the dermis of the skin biopsies (Figure 1)

The quantity of the mature and old collagen, and of the elastic fibers distributed in the dermis of the skin sections, was evaluated in histological sections prepared from paraffin blocks. Each slide contained a single or a series of skin tissue sections stained by the following histochemical stains: Masson trichrome (to delineate mature and old collagen fibers, stained blue) and Van Gieson stain (to delineate elastin fibers, stained black). The histological slides were optically scanned at a maximal magnification of x400 using an automatic slide scanner system (Aperio Technologies, Inc., Vista, CA). The results were collected in a database containing a virtual (digital) pool of all scanned slides. Multiple microscopic fields from each histological section that covered the entire dermal area were analyzed to obtain quantitative measurements. We used the Image-Pro Plus version 6.2 image analysis software for the computerized segmentation and measurement of the collagen and the elastic fibers. Only the slides stained by Masson trichrome (for mature and old collagen) and the Van Gieson (for elastic fibers) were quantifiable. We were not able to stain the young (newly synthesized) collagen fibers at this stage of the experiment.

The morphometric variables that were evaluated in this analysis included the percentage of the dermal area populated by both collagen and elastic fibers, the percent of the dermal depth of the collagen fibers, the degree of collagen fragmentation (assessed by the total parameter of the areas of the holes), and the length and width of the fibers.

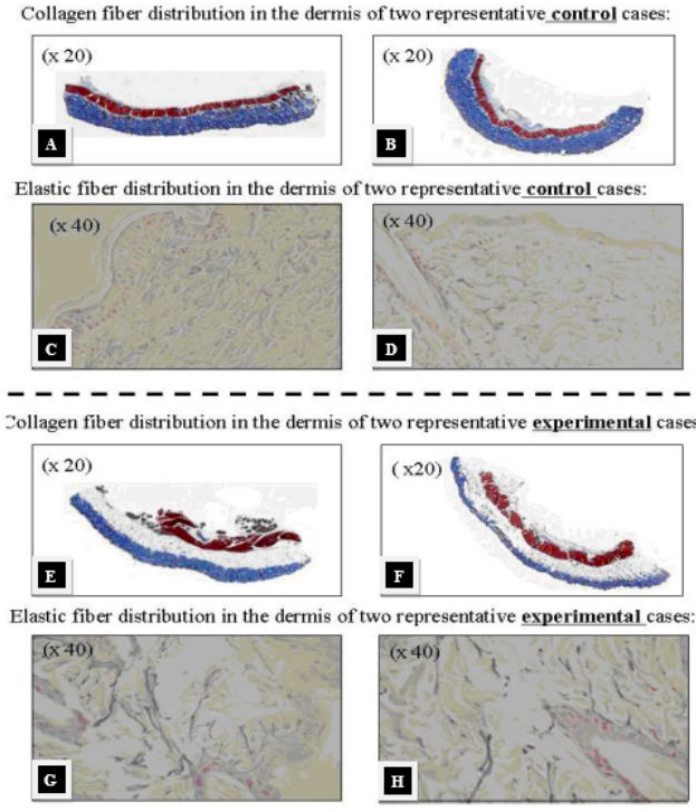

Figure 1(A-H): Characteristics and distribution of collagen and elastin fibers in rat dermis before (control) and after the course of collagenase injections. A, B. The skin sections taken before CLA injection stained Masson Trichrome. The thick layer of mature and old collagen (blue) can be seen. C, D. The skin sections taken before CLA injection stained by Van Gieson demonstrates the distribution of elastic fibers, stained black. E, F. Skin sections from the same animals taken after CLA injection. Masson Trichrome stain demonstrates an unambiguously thinner blue

layer of mature and old collagen. The unstained "transparent" layer is presumed to contain the newly synthesized collagen. G, H. Skin sections from the same animals taken after CLA injection and stained by Van Gieson. Elastic framework looks denser, and the fibrils are longer, thicker and more densely distributed in the dermis.

Measurement of oxygen consumption

The rats' oxygen consumption was measured in a hermetic cylindrical glass chamber (metabolic cage). Once sealed in the chamber, the rats consumed oxygen and exhaled carbon dioxide, which was absorbed by soda lime placed in the bottom of the chamber. Therefore, as oxygen was consumed, the pressure inside the chamber dropped and the change was measured by a manometer connected to the chamber. A pre-calibrated manometer calculated the pressure drop in the chamber relative to the amount of oxygen absorbed by the rat, expressed in ml/1 min/kg [19,33].

Experimental design

Baseline values of all monitored parameters were determined during the two weeks prior to the experimental injections (self-control study). The CLA was injected into the rats' tail vein once every two weeks for 12 weeks (a total of six injections per course). An individual CLA dose for single injection was calculated as the quantity required to dissolve 1% of the total collagen of the rat body [34]. To determine the total amount of collagen, the dry weight of the animal (25% of the total body weight) was calculated, the amount of protein in the dry weight (80%) [35] was measured, and the amount of collagen in the protein (20%) [36,37] extrapolated. The calculated amount of the CLA was dissolved in one ml of normal saline and injected into the animal's tail vein. The hydroxyproline excretion was measured in the urine before, one day after and 10 days after the CLA injection. Oxygen consumption was measured, and skin biopsies were performed just before the first injection and again after the course of injections.

Statistical analysis

Statistical analysis was performed by Student's t parametric method and Wilcoxon's non-parametric test. A p value <0.05 was considered a significant difference. Calculations were performed using SAS 8 software (SAS Institute, Cary, NC).

Results

Table 1 shows rates of urine hydroxyproline excretion before and after a single injection of CLA. One day after the injection, a significant increase in mean excretion was observed (by 1.7 times; $p<0.05$). At the end of the second week, the mean level returned to baseline. The histochemical distribution and the density of the mature and old collagen and elastic fibers were significantly different in the biopsies that were obtained before and after the course of CLA injections (Table 2). Despite the changes that transpired in the mature, old and young collagen distribution, the total thickness of the dermis remained the same (Table 2, Figure 1).

	Before injection	After injection	
		One day	10-14 Days
Rats (number)	19	19	19
Mean±Standard deviation	4.2±2.90	7.0±3.54	4.0±2.54
Range	0.9–10.0	1.7–10.0	0.8–8.1
P value was < 0.05 for the difference between hydroxyproline excretion before and one day after CLA injection. The P value for the difference between hydroxyproline excretion 10-14 days after injection and baseline was > 0.05.			

Table 1: Urinary Hydroxyproline Excretion before and after Collagenase (CLA).

Indexes	Rats (number)	Before course of CLA injections	Range	After course of CLA injections	Range	P value
Dermis diameter(μm)	19	2,297±703.8	1,233-4,306	2,405±495.8	1,786-4,067	p> 0.05
Deepest collagen diameter (μm)	19	2,108±681.4	942-4,306	1,429±343.6	828-1,927	p< 0.001
Deepest collagen dermal penetration (%)	19	91±8.2	70-98	61±4.2	32-92	p< 0.001
Elastic fiber area (μm^2)	19	5,931±2,122.9	418-28,237	9,493± 2,277.8	716-23,951	p< 0.001
Maximal elastic fiber diameter (μm)	19	58±12.0	11-200	76±18.1	24-325	p< 0.001

Table 2: Mature and Old Collagen and Elastic Fiber Distribution in the Dermis of Rats before and after the Course of Collagenase Intravenous Injections. (M±SD).

The dermal framework of the mature and old collagen was more abundant, and characterized by greater amount of collagen fibers before the course of CLA injection than after it. The layer of mature and old collagen in the dermis was significantly thinner after completion of the course of CLA injections than at baseline (by 1.5-fold, $p<0.001$; Table 2). The collagen fibers were less dense (i.e., more sparsely and more superficially distributed). In the elastic fibers that had shown an inverse pattern: the elastic framework was denser, and the fibrils were longer, thicker and more densely distributed in the dermis. This resulted in the appearance of an "Empty" space between the layers of mature and old collagen, and the muscle cells. We assumed that this space contained the young, newly formed collagen that comprised a substance that was invisible in the Masson trichrome-stained slides.

Figure 1 illustrates the histological and histochemical changes in the pattern of biopsy specimens after administration of the CLA. It provides examples of two representative slides from the two initial biopsies and the two biopsies that were taken after the end of the experiment from the same animals. Table 2 shows some of the morphometric variables that were obtained from this analysis. The following parameters declined significantly after the course of CLA injections: the mean percentage of dermal area involved by mature and old collagen (by 1.5-fold, $p<0.001$), the extent of fiber fragmentation, and the deepest dermal penetration in the skin (each by 1.5-fold, $p<0.001$). Quantitative indicators for the presence of elastic fibers increased after the course of CLA injections. Elastic fiber areas increased by 1.6-fold ($p<0.001$; Table 2), and the maximal elastic fiber diameters increased by more than 1.3-fold ($p<0.001$; Table 2). Oxygen consumption increased by 1.25-fold following the CLA injections ($p<0.001$; Table 3).

	Before injection	After injection	p*
Rats (number)	19	19	
Mean ± Standard deviation	1.2±0.29	1.5±0.18	
Range	0.84-1.30	1.20-1.72	
		<0.0005	<0.001
*The Wilcoxon signed rank test.			

Table 3: Oxygen Consumption of Mature and Old Rats Before and after the Course of CLA Injections (ml/1min/Kg).

Discussion

This study describes the implementation of a new method for systemic rejuvenation, which involves replacing old dehydrated extracellular proteins by young ones that are able to form hydrophilic colloid systems with high permeability. We demonstrated signs of such connective tissue remodeling following intravenous injections of collagenase in rats, specifically: (1) Destruction of the mature and old collagen after the injection of collagenase, as evidenced by increased excretion of hydroxyproline in the urine; (2) Decrease of the area containing mature and old collagen, as confirmed by histochemical staining: (3) Formation of new young extracellular connective tissue proteins in rats after treatment with collagenase, as evidenced by a 1.6-fold increase in the amount of elastin and the appearance of white spots at the sites of the mature and old collagen. We obtained evidence of energy metabolism stimulation after treatment with collagenase, as demonstrated by increased total body oxygen consumption. Niedermüller and Petersen [23] also demonstrated biochemical and rheological changes in the dermis of rats aged 14 to 32 months, which corresponded to the dermis of young rats, following stimulation by bovine plasma factor of endogenous collagenase activity.

It seems reasonable that the process of aging in the extracellular proteins of connective tissue is actively influenced by the genetic program of aging. The rate of aging of protein colloidal solutions is widely believed to depend on the chemical structure of polypeptide chains, which is programmed genetically. This assumption is supported by correlations between collagen melting points and different life expectancies in animals of different species. The melting point of collagen depends on its chemical composition [3,18]. The determinant role of the connective tissue structure in the lifespan of mammalian species is illustrated by the naked mole rat (*Heterocephalus glaber*), which has a maximum lifespan exceeding 30 years [8]. This is the longest lifespan reported for a rodent species and is especially striking considering its small body mass. In comparison, a similarly sized house mouse has a maximum lifespan of 3 years. Naked mole-rat fibroblasts were found to secrete extremely high molecular mass hyaluronan, which is over five times larger than human or mouse hyaluronan. This high molecular mass hyaluronan accumulates abundantly in naked mole-rat tissues due to the decreased activity of hyaluronan-degrading enzymes and a unique sequence of hyaluronan synthase 2 (HAS2) [7]

Another possible application of the genetic program of aging entails the system of the proteins (mainly enzymes) that are involved in remodeling of the extracellular component of connective tissue. This remodeling process appears to become less and less effective with time, starting from infancy [22-24]. The current study attempted to achieve youth-like changes in the aged rat organism by artificial destruction and remodeling of old collagen structures throughout the animal's body. We showed systemic disintegration of mature and old collagen, following injections of CLA, with subsequent appearance of young collagen and younger-like structured elastin, as well as increased oxygen uptake. These changes appear to represent signs of a rejuvenating effect of CLA. Therefore, the data presented herein support the major role of colloidal connective tissue in the aging process. We demonstrated that intravenous injections of collagenase in aging

rats may serve as replacement therapy for insufficient activity of endogenous collagenase due to old age. This finding may be implemented in the search for new ways to rejuvenate human as well as other higher animal (mammalian) organisms.

Limitations

Several limitations of this study bear mention. First, we did not measure the natural lifespan of the rodents after the course of CLA injections. Second, we were unable to stain the newly synthesized collagen fibers (the "Young collagen") and had to evaluate the quantity of the young collagen in dermal tissue indirectly (i.e., by the thickness of the bright layer in derma between the layer of old collagen and the muscle tissue). This is thus a very preliminary investigation and first attempt to perform artificial remodeling of connective tissue at the level of whole organisms. Several questions remain to be answered in future investigations, such as possible systemic CLA non-target side effects on other tissues/organs (lung, blood vessels, immune system etc.) and the long-term effect of CLA on survival and lifespan.

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