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Increases in Type III Collagen Gene Expression and Protein Synthesis in Patients with Inguinal Hernias

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Objective
The aim of this study was to determine if alterations in fibrillar collagen synthesis were associated with the development of inguinal hernias.

Summary Background Data
Previous work has suggested that alterations in connective tissue accumulation may play a functional role in the development of inguinal hernias. In particular, several investigators have suggested that alterations in collagen synthesis, causally related to connective disorders such as osteogenesis imperfecta, may also be responsible for the inguinal herniation that is markedly increased in such patients. This study was undertaken therefore to study collagen synthesis in patients with inguinal hernia in the absence of any other connective tissue disease.

Methods
Skin fibroblasts from 9 patients with hernias and 15 control individuals were radiolabeled with ^{3}H-proline. Trypsin–chymotrypsin-resistant type I and III collagens were isolated and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and recovery was quantified by laser densitometry. Steady-state levels of α1(I) and α1(III) procollagen mRNAs were also determined by northern and slot-blot hybridization analysis.

Results
The α1(I)/α1(III) collagen ratios were shown to be 6.3 ± 0.34 in fibroblasts from control individuals and 3.0 ± 0.25 in fibroblasts from patients with inguinal hernias. This statistically significant difference ($p < 0.0001$) was caused by an increase in the secretion of α1(III) procollagen from the fibroblasts of patients with hernias. A concomitant increase in the steady-state levels of α1(III) procollagen mRNA was observed in total RNA isolated from the patients' fibroblasts.

Conclusions
A constitutive and systemic increase in type III collagen synthesis may result in reduced collagen fibril assembly in the abdominal wall, eventually leading to the development of herniation. Although it is not yet clear what genetic factors are responsible for the elevation in type III collagen synthesis in patients with hernias, this study represents the first attempt to define individuals with an abnormality in collagen production that may be specifically related to herniation. A clearer

understanding of the possible genetic factors that influence the pathophysiology of this disease will be important to improve the treatment of patients in whom inguinal hernias develop.

Several factors have been implicated in the development of inguinal hernias. These include repeated elevations in intra-abdominal pressure and a weakening of muscle fascia and connective tissue.[1] Possible pathologic alterations in connective tissue associated with inguinal hernias were recognized as early as 1924.[2] More recently, a marked increase in the frequency of hernia defects has been shown to be associated with a variety of connective tissue disorders, such as the Marfan's and Ehlers-Danlos syndromes, cutis laxa, and osteogenesis imperfecta.[3] Moreover, more common disorders such as congenital hip dislocation in children were also shown to be associated with an increased frequency of inguinal herniation.[4]

Numerous authors have demonstrated thinner, lighter anterior rectus sheaths, conjoined tendons, and transversus abdominus aponeuroses in patients with inguinal hernias.[5-8] Moreover, bilateral hernias were associated with more severe atrophy of the anterior rectus sheath than were unilateral hernias.[9] Further studies revealed a diminished amount of insoluble (polymeric) collagen, and hydroxyproline, in the anterior rectus sheath of patients with inguinal hernias compared with that in controls.[5] Collagen synthesis, as measured by the incorporation of radiolabeled proline in fibroblast cultures, was also significantly lower in patients with inguinal hernias compared with that in controls.[10] Electron microscopy performed on the anterior rectus sheath of patients with inguinal hernias revealed a variable diameter and periodicity of collagen fibrils compared with that in controls.[11] Interestingly, similar changes were noted in pericardial and skin biopsies taken from a patient with a direct inguinal hernia.[10,11] Furthermore, a marked attenuation of the transversalis fascia and a significant reduction in the thickness of connective tissue was demonstrated in the area of the internal ring on the clinically normal side of patients with inguinal hernia.[12] This lends further support toward a more generalized defect that is expressed in the inguinal region, possibly as a result of local mechanical factors.[12] Hernias, for example, were induced in 95% of laboratory rats by surgical enlargement of the internal ring when coupled with the injection of β-aminopropionitrile (an inhibitor of lysyl oxidase), an enzyme that is important in the crosslinking of collagen.[13]

There is increasing evidence, therefore, that many common problems thought to be associated with a weakening of connective tissues, including hernias, may indeed be the result of a more generalized metabolic derangement of collagen. To address the possibility that such defects in collagen accumulation may be an important contributing factor in the etiology of inguinal herniation, this article describes the synthesis of type I and type III collagen (two of the major fibrillar collagens) in patients with inguinal herniation but no evidence of other connective tissue disorders.

MATERIALS AND METHODS

Patient Population

The controls ranged in age from 24 to 72 years and had no history of inguinal herniation. Male patients with inguinal hernias ranged in age from 17 to 67 years. Five patients with inguinal hernias had relatives with a history of inguinal herniation (three with first-degree relatives and two with second-degree relatives). One patient initially operated on for inguinal herniation was found to have only a lipoma of the spermatic cord and was considered a control for the purposes of this study.

Fibroblast Cultures

Skin specimens from patients undergoing various surgical procedures (controls, n = 15) and from patients undergoing inguinal herniorrhaphy (hernias, n = 9) were obtained under sterile conditions after informed consent. Skin samples were placed in sterile culture media as described subsequently, and fibroblast cultures were prepared from explanted skin samples as previously described.[14]

Labeling of Fibroblast Procollagens and Determination of Type I/III Procollagen Ratio

Confluent skin fibroblasts from passage 2 to 3 were used for the quantitative analysis of secreted types I and III procollagen. The labeling of secreted procollagens

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with ^{3}H-proline and the isolation of media procollagens by ammonium sulfate precipitation was performed as described earlier.[14,15] The quantitative analysis of secreted α1(I), α2(I), and α1(III) procollagens by nonreducing polyacrylamide gel electrophoresis was carried out in triplicate for all samples using a previously described procedure.[14]

RNA Extraction and Messenger RNA (mRNA) Quantitation

Total RNA was isolated from confluent skin fibroblast cultures using a well-described guanidine isothiocyanate extraction technique.[16] Northern and slot–blot hybridization analyses of RNA preparations were then performed using a published procedure.[17] We performed phosphate-32 labeling of DNA fragments by nick translation of complementary DNA sequences coding for γ-actin and the carboxyl propeptide domains of α1(I) and α1(III) procollagen using a commercially available kit (Boehringer-Mannheim, Mannheim, Germany) and ^{32}P-deoxycytidine triphosphate at a specific activity of 3000 Ci/mmol (ICN, Irvine, CA). A 700-base pair Eco RI/Ava I fragment coding for human α1(I) procollagen,[18] a 700-base pair Eco RI/Xho I fragment coding for human α1(III) procollagen (Deak SB and Boyd CD, unpublished data), and a 1000-base pair Bam HI/Hind III fragment coding for human γ-actin[17] were used for radiolabeling.

Joint Flexibility

Joint mobility was determined by using modified Carter-Wilkinson criteria as demonstrated by the ability to oppose passively both thumbs to the volar aspect of the forearms, the ability to hyperextend passively the fifth digits to $\geq 55°$, and the ability to hyperextend actively the elbows to $> 190°$. Those patients exhibiting any two of these three criteria were defined as hypermobile.[19]

Statistical Analysis

Student's two-tailed t test was used to determine statistically significant differences between groups. All data are presented as the mean ± the standard error of the mean.

RESULTS

Ratios of Type I/III Collagen

Figure 1 is a representative fluorogram of secreted procollagens isolated from control and hernia fibroblast cultures. The ratio of α1(I)/α1(III) procollagen recovered from each fibroblast preparation was quantified by laser densitometry. A typical densitometric tracing obtained from both control and hernia fibroblast cultures is presented in Figure 2.

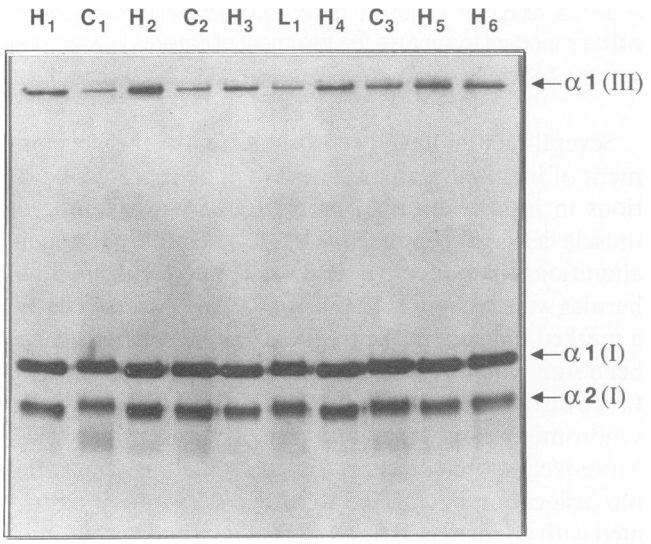

Figure 1. Fluorogram of radiolabeled fibroblast collagens digested with trypsin and chymotrypsin. Note the approximately equivalent amounts of α1(I) and α2(I) procollagens in all specimens and the increased amounts of radiolabeled α1(III)3 procollagen from the patients with hernias. C = control, H = hernia, L = lipoma of the cord.

To ensure that α1(I)/α1(III) collagen ratios were determined within the linear response range of x-ray film, fluorograms were selected in which the α1(I)/α2(I) ratio was 2:1. This internal control α1(I)/α2(I) collagen ratio was established from a least-squares regression analysis of fluorograms exposed for varying amounts of time (Fig. 3). A 2:1 ratio was selected because previous studies have

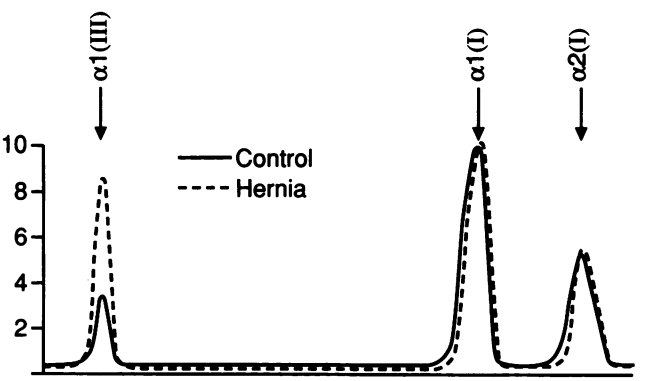

Figure 2. Laser densitometric tracing of fluorograms of procollagens secreted by cultured skin fibroblasts. Note the approximately equivalent amounts of α1(I) and α2(I) procollagens from both the controls and patients with hernias and the increased amount of α1(III) procollagen from the latter (comparison of peak areas).

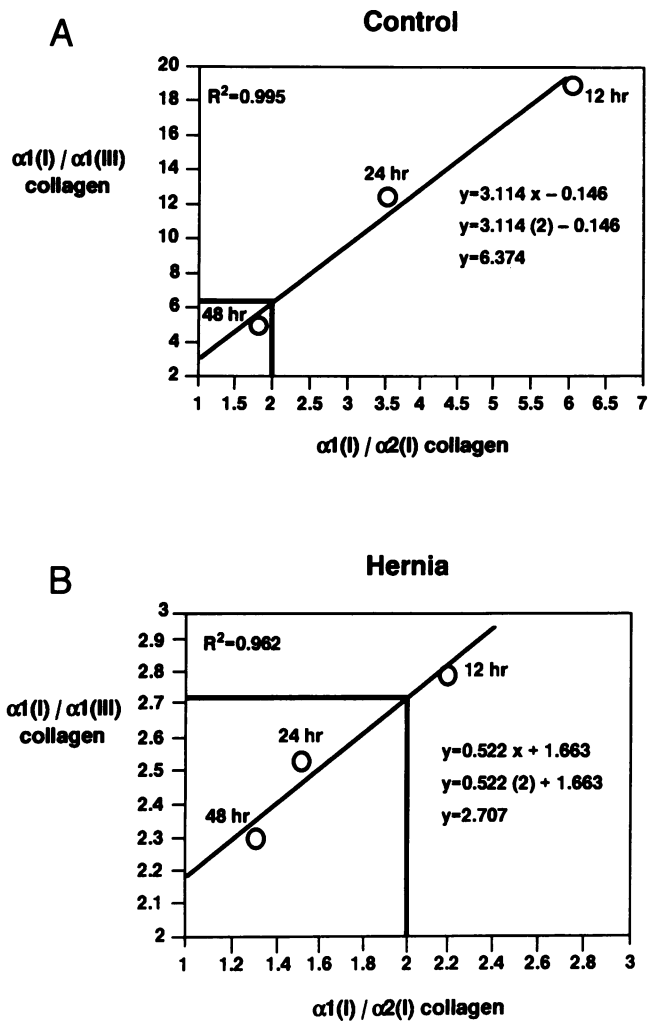

Figure 3. Typical plots, obtained after multiple exposures of fluorograms to x-ray film. Varying exposure times were used to determine α1(I)/α1(III) collagen ratios by least-squares regression analysis.

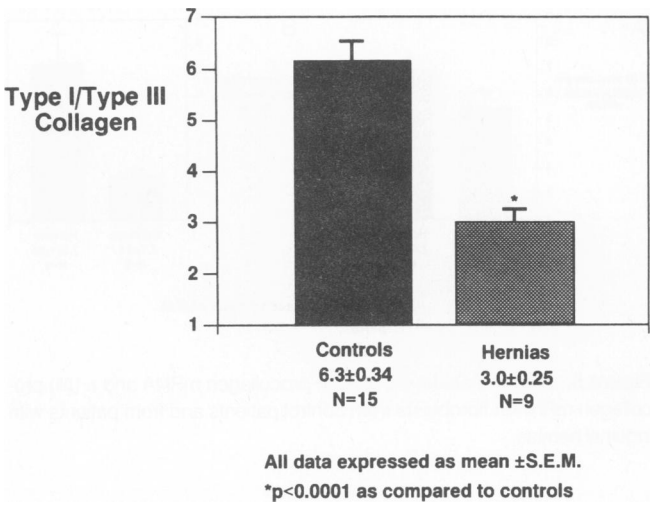

Figure 4. Type I/type III collagen ratios in fibroblast cultures from control patients and from patients with inguinal hernias.

demonstrated such a ratio in secreted type I heterotrimers from skin fibroblast cultures.[15]

Under these conditions, the α1(I)/α1(III) collagen ratio in control fibroblasts (n = 15) was 6.3 ± 0.34; the α1(I)/α1(III) collagen ratio in fibroblast cultures obtained from patients with hernias was 3.0 ± 0.25 (n = 9). There was a statistically significant difference in this ratio between fibroblast cultures obtained from patients and those obtained from control individuals ($p < 0.0001$, Fig. 4). No differences were observed in the α1(I)/α1(III) collagen ratios from fibroblast cultures obtained from either patients exhibiting direct (n = 4, 3.0 ± 0.29) or indirect (n = 5, 2.9 ± 0.41) hernias. This reduced α1(I)/α1(III) collagen ratio observed in fibroblast cultures obtained from either direct or indirect hernia patients was the result of the increased recovery of α1(III) collagen in the media from these fibroblast preparations.

mRNA Coding for α1(I) and α1(III) Procollagen

Northern blot analysis with both α1(I) procollagen and γ-actin complementary DNA probes revealed the expected 4.8- and 5.8-kilobase pro-α1(I) collagen mRNA transcripts and a 1.9-kilobase γ-actin mRNA transcript in all fibroblast mRNA samples.[17,20] Northern blot hybridization analysis of total fibroblast RNA using a radiolabeled α1(III) procollagen complementary DNA revealed the presence of 4.8- and 5.4-kilobase mRNA species; this finding was in agreement with previous estimations of the size of human α1(III) procollagen mRNA.[14,20]

Slot–blot analysis demonstrated that steady-state levels of mRNA coding for γ-actin were similar in controls (n = 8, 0.008 ± 0.001) and patients with hernias (n = 4, 0.010 ± 0.002). An α1(I) procollagen/γ-actin mRNA ratio of 5.5 ± 0.65 was found in controls, and a similar ratio of 7.0 ± 0.52, in patients with hernias (no significant difference, Fig. 5A). When α1(III) procollagen mRNA levels were compared with levels of γ-actin mRNA, a ratio of 2.7 ± 0.27 was found in controls compared with a ratio of 7.3 ± 1.20 in patients with hernias ($p < 0.0004$, Fig. 5B). The increased α1(III) procollagen/γ-actin mRNA ratio was the result of an increased recovery of α1(III) procollagen mRNA (Fig. 6).

Joint Mobility

Three of the nine patients with inguinal herniation were found to be hypermobile, an incidence of 33%, *versus* 5% for the general population.[19]. All these patients had indirect inguinal hernias. The hypermobile patients

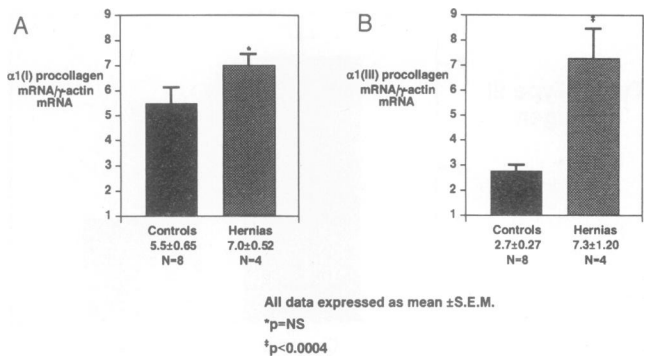

Figure 5. Steady-state levels of α1(I) procollagen mRNA and α1(III) procollagen mRNA in fibroblasts from control patients and from patients with inguinal hernias.

had a type I/type III collagen ratio of 2.3 ± 0.07, which was somewhat lower than the ratio of 3.3 ± 0.29 found in those with normal mobility ($p < 0.06$).

DISCUSSION

We found an approximately twofold increase in the amount of α1(III) procollagen mRNA in fibroblasts isolated from the skin of patients with inguinal hernias compared with that in normal controls. This increase was paralleled by a concomitant increase in type III collagen as evidenced by a decrease in the type I/type III collagen ratio in skin fibroblast cultures obtained from these patients. Prior studies, however, detected decreased amounts of insoluble (polymeric) collagen in patients with inguinal herniation.[5,7,10] We, on the other hand, found an increase in the amount of type III collagen relative to type I. An overabundance of type III collagen fibers relative to type I fibers may lead to an increase in nonpolymeric collagen levels by inhibiting the associative properties of type I collagen. This can lead to a diminished amount of insoluble (polymeric) collagen. The overall collagen content, however, may remain unchanged (or may even be elevated) because of the increased amount of isolated collagen not in the form of polymerized collagen fibrils. The increase in type III collagen may result, as described previously, in collagen fibrils that do not meet the mechanical requirements of the tissue and may predispose certain individuals to the development of inguinal herniation and to recurrence after corrective surgery.

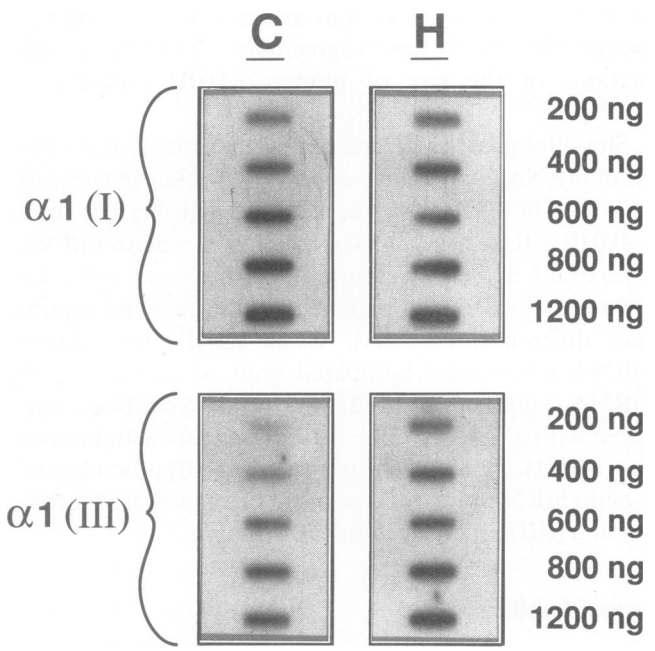

Figure 6. Slot blots obtained from total RNA from fibroblasts of a control patient (C) and a patient with inguinal herniation (H). Filters were probed with α1(I) and α1(III) procollagen complementary DNA, then dried, and exposed to x-ray film.

Type I and type III collagen fibers coexist within individual fibrils, and their relative ratios play an important role in the regulation of fibrillogenesis and the determination of the final fibril diameter and bundle architecture.[21-23] Type I collagen forms a network of thick fiber bundles, while type III forms thin isolated fibers, and mixtures of the two form bundles of intermediate sizes.[22-24] An increase in the amount of type I collagen is found in healing wounds,[25] hypertensive arteries,[25] and fibrotic lungs.[26] A higher proportion of type III collagen can be found in the walls of blood vessels and in the papillary dermis, accounting for their laxity and distensibility.[23] In patients with inguinal hernia, we demonstrated a decrease in the type I/type III collagen ratio. This altered ratio was the result of an increase in the levels of type III procollagen mRNA and a concomitant increase in the synthesis of type III collagen. This increase in type III collagen synthesis may alter the physical properties of the collagen matrix in the abdominal wall and predispose individuals to the development of inguinal hernias.

Inguinal hernias can recur as late as 15 years after initial surgery. Those that recur within the first 9 months are most likely to be technically related and should be dealt with by performing an appropriate operation. Those recurring after 9 months may be the result of ongoing metabolic abnormalities in collagen production.[27] This may explain the low (70%) success rate associated with the classic repair of recurrent inguinal hernias.[12] If metabolic defects are present, indirect inguinal hernias can recur despite adequate ligation and removal of the peritoneal sac.[12,25,28] Furthermore, it seems likely that individuals with altered collagen metabolism may also be predisposed to the development of incisional and other

types of hernia defects. Further studies are needed in this area.

The use of autogenous tissue, including anterior rectus sheath,[29,30] to induce normal collagen production has been proposed. The inductive influence of autogenous fascial transplants, namely fascia lata, results in a tenfold increase in net collagen synthesis and deposition for at least 2 years.[2,8,31] In addition, Marlex (Bard Vascular Systems, Billerica, MA), by inducing an intense inflammatory response, sets up scaffolding that, in turn, induces collagen synthesis.[2,8]

The cause of inguinal hernia is most likely multifactorial. Altered collagen metabolism may play an important role in predisposing an individual to hernia formation. In addition to the overproduction of type III collagen, we found a tendency for patients with indirect inguinal herniation to be hypermobile. Furthermore, those that had increased joint mobility tended to have an even lower type I/type III collagen ratio, suggesting that these patients produce a further increased amount of type III collagen compared with type I. This leads us to believe that the clinically evident inguinal hernia may simply be a locus resistentiae minoris of a more generalized defect in collagen metabolism.

To treat patients with inguinal herniation effectively, we must first understand the cause and pathophysiology underlying this problem. A generalized defect in collagen metabolism may cause an alteration in fibrillogenesis, resulting in tissues that are unable to withstand the stresses placed on them. Therefore, the use of autogenous tissues may not be the ideal method to repair this defect because these tissues would express similar abnormalities in collagen production. Perhaps the use of synthetic materials, or even allografts, that are able to stimulate normal collagen production are more suitable. Furthermore, we may soon be able to screen asymptomatic patients for various defects in collagen metabolism. This would allow us not only to become more aware of possible problems arising in those patients, but it would also allow us to treat them more effectively after a problem did arise. Further studies are needed to understand this complex metabolic problem. We eventually hope to be able to restore a normal balance of collagen in those individuals expressing altered collagen metabolism and, thereby, allow for the production of an extracellular matrix suitable to withstand the forces exerted on it.

Acknowledgments

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References

1. Nyhus LM. Historical survey of the treatment of hernia. *In* Nyhus LM, Condon RE, eds. Hernia, 2nd ed. Philadelphia: JB Lippincott, 1964, pp 10–14.
2. Keith A. On the origin and nature of hernia. Br J Surg 1924; 11: 455–475.
3. Uitto J, Perejda A, eds. Connective Tissue Disease: Molecular Pathology of the Extracellular Matrix. New York: Marcel Dekker, 1986.
4. Uden A, Lindhagen T. Inguinal hernia in patients with congenital dislocation of the hip: a sign of general connective tissue disorder. Acta Orthop Scand 1988; 59:667–668.
5. Wagh PV, Leverich AP, Sun CN, et al. Direct inguinal herniation in men: a disease of collagen. J Surg Res 1974; 17:425–433.
6. Read RC. Pre-extraperitoneal approach to inguinofemoral herniorrhaphy. Am J Surg 1967; 113:672–678.
7. Wagh PV, Read RC. Collagen deficiency in the rectus sheath of patients with inguinal herniation. Proc Soc Exp Biol Med 1971; 137:382–384.
8. Read RC. Attenuation of the rectus sheath in inguinal herniation. Am J Surg 1970; 120:610–614.
9. Read RC. Preperitoneal exposure. Curr Probl Surg 1967; 4:17–39.
10. Wagh PV, Read RC. Defective collagen synthesis in inguinal herniation. J Surg 1972; 124:819–822.
11. White HJ, Sun CN, Read RC. Inguinal hernia: a true collagen disease. Lab Invest 1977; 36:359.
12. Peacock EE Jr, Madden JW. Studies on the biology and treatment of recurrent inguinal hernia: II. Morphological changes. Ann Surg 1974; 179:567–571.
13. Conner WT, Peacock, EE Jr. The etiology of inguinal hernia. Surg Forum 1971; 22:69–71.
14. Deak SB, Ricotta JJ, Mariani, TJ, et al. Abnormalities in the biosynthesis of type III procollagen in cultured skin fibroblasts from two patients with multiple aneurysms. Matrix 1992; 12:92–100.
15. Deak SB, Nicholls AC, Pope FM, Prockop DJ. The molecular defect in a nonlethal variant of osteogenesis imperfecta: synthesis of pro α2(I) chains which are not incorporated into trimers of type I procollagen. J Biol Chem 1983; 258:15192–15197.
16. Glisin VR, Crkvenjakov R, Byus C. Ribonucleic acid isolation by cesium chloride centrifugation. Biochemistry 1974; 13:2633–2637.
17. Pierce RA, Glaug MR, Greco RS, et al. Increased procollagen mRNA levels in carbon tetrachloride-induced liver fibrosis in rats. J Biol Chem 1987; 262:1652–1658.
18. Deak SB, Scholz PM, Amenta PS, et al. The substitution of arginine for glycine of the α1(I) procollagen chain results in mild osteogenesis imperfecta. J Biol Chem 1991; 266:21827–21832.
19. Jessee EF, Owen DS, Sagar KD. The benign hypermobile joint syndrome. Arthritis Rheum 1980; 23:1053–1056.
20. Uitto J, Perejda AJ, Abergel RP, et al. Altered steady-state ratio of type I/III procollagen mRNA's correlates with selectively increased type I procollagen biosynthesis in cultured keloid fibroblasts. Proc Natl Acad Sci U S A 1985; 82:5935–5939.
21. Fleischmajer R, Perlish JS, Burgeson RE, et al. Type I and type III collagen interactions during fibrillogenesis. Ann N Y Acad Sci 1990; 580:161–175.
22. Henkel W, Glanville RW. Covalent crosslinking between molecules of type I and type III collagen. Eur J Biochem 1982; 122:205–213.
23. Lapiere CM, Nusgens B, Pierard GE. Interaction between collagen type I and type III in conditioning bundles organization. Connect Tissue Res 1977; 5:21–29.

24. Shuttleworth CA, Forrest L, Jackson DS. Comparison of the cyanogen bromide peptides of insoluble guinea-pig skin and scar collagen. Biochim Biophys Acta 1975; 379:207–216.
25. McCullagh KA, Balian G. Collagen characterisation and cell transformation in human atherosclerosis. Nature 1975; 258:73–75.
26. Seyer JM, Hutcheson ET, Kang AH. Collagen polymorphism in idiopathic chronic pulmonary fibrosis. J Clin Invest 1976; 57: 1498–1507.
27. Postlethwait RW. Causes of recurrence after inguinal herniorrhaphy. Surgery 1971; 69:772–775.
28. Thieme ET. Recurrent inguinal hernia. Arch Surg 1971; 103:238–241.
29. Oughterson AW. The hypertrophy of fascia and its uses in the repair of large scrotal herniae. Surg Gynecol Obstet 1929; 49:387–391.
30. Poth EJ. Inguinal hernia repair using the rectus-pyramidalis sheath. Am J Surg 1971; 122:699–702.
31. Peacock EE Jr. Subcutaneous extraperitoneal repair of ventral hernias: a biological basis for fascial transplantation. Ann Surg 1975; 181:722–727.