

Research article

A role for the collagen I/III and MMP-I/-13 genes in primary inguinal hernia?

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Abstract

Background: Abnormal collagen metabolism is thought to play an important role in the development of primary inguinal hernia. This is underlined by detection of altered collagen metabolism and structural changes of the tissue in patients with primary inguinal hernia. However, it is still unknown whether these alterations reflect a basic dysfunction of the collagen synthesis, or of collagen degradation.

Methods: In the present study, we analysed type I and type III procollagen messenger ribonucleic acid (mRNA) and MMP-I and MMP-13 mRNA in cultured fibroblasts from the skin of patients with primary inguinal hernia, and from patients without hernia (controls) by reverse transcription polymerase chain reaction (RT-PCR) and Northern Blot.

Results: The results indicated that the ratio of type I to type III procollagen mRNA was decreased in patients with primary hernia, showing significant differences as compared to controls ($p = 0.01$). This decrease was mainly due to the increase of type III procollagen mRNA. Furthermore, RT-PCR analysis revealed that the expression of MMP-I mRNA in patients with primary hernia is equivalent to that of controls ($p > 0.05$). In addition, MMP-13 mRNA is expressed neither in patients with primary hernia nor in controls.

Conclusion: We concluded that abnormal change of type I and type III collagen mRNAs contribute to the development of primary inguinal hernia, whereas the expressions of MMP-I and MMP-13 mRNA appears not to be involved in the development of primary inguinal hernia. Thus, the knowledge on the transcriptional regulation of collagen in patients with primary inguinal hernia may help to understand the pathogenesis of primary inguinal hernia, and implies new therapeutic strategies for this disease.

Background

Several connective tissue diseases have been related to an abnormal collagen metabolism. Although for the development of an abdominal wall hernia evident molecular-biological links are missing. A disease of the soft tissues

has been accused to be a decisive factor. Correspondingly, the patients with an aortic abdominal aneurysm [1,2], with an Ehlers-Danlos syndrome [3] or with a poly cystic kidney disease [4] show an increased risk for inguinal herniation. Furthermore, previous studies on protein level in-

dicating that patients with an inguinal hernia present a disturbed collagen proportion with a reduced ratio of type I and type III collagen as well as abnormal ultra-structural changes of the deposited collagen [5–7]. Friedman et al. showed increases in type III collagen gene expression and protein synthesis in patients with inguinal hernias [6]. A defective collagen metabolism contributes to a decreased tensile strength and mechanical stability of both the connective tissues and the induced scar tissue [8–11]. Therefore these alterations in collagen formation should be of central relevance in the pathophysiology of hernias.

The altered ratio of the collagen subtypes can result either by a modified synthesis or by an imbalanced breakdown. The cleavage is regulated by the activity of the matrix metallo-proteinases (MMPs), proteins of a family of zinc-dependent endopeptidases. Among them MMP-1 and MMP-13 are the principal matrix enzymes cleaving fibrillar type I, II and III collagen [12–14]. It has been shown that these MMPs can play a pathologic role in excessive breakdown of some connective tissue components, e.g. in rheumatoid arthritis [15], osteoarthritis [16], in atherosclerosis, in tumor cell invasion and metastasis [12,17]. In particular, the alterations in MMP-1 and MMP-13 protein expressions could have been responsible for the changed ratio of type I to type III collagen on the protein level. Nevertheless, as firstly shown in investigations by Bellon et al. in 1997, cultured fibroblasts in fascia transversalis from patients with inguinal hernia showed no differences in the expression of matrix metallo-proteinase-1, whereas the same author later detected a MMP-2 overexpression in these patients [18,19].

In our own previous studies, we determined collagen type I and type III in the skin of patients with indirect and direct inguinal hernia both by immunohistochemistry and Western blot analysis and quantified the immunohistochemical expression of MMP-1 and MMP-13. The results indicated that the ratio of collagen type I/III was significantly decreased in the skin of patients with either indirect or direct inguinal hernia, with a concomitant increase in collagen type III ($p < 0,001$ vs. control). Likewise the results of Bellon et al., we found no significant differences in the expression of MMP-1. No MMP-13 was detected in any of the skin samples investigated [20].

In the present study we examined cultured fibroblasts from the skin of patients with primary inguinal hernia and analysed the mRNA expressions of types I and III collagen to verify the up- or downregulation of the collagen synthesis. In addition to that, MMP-1- and MMP-13-mRNA were analysed in order to confirm the previous results on protein level that these components of the degrading system are not involved in the pathogenesis of herniation.

The confirmation of a fibroblast malfunction should give strong evidence of a systemic or genetic connective tissue disease of patients with an inguinal hernia.

Materials and methods

Patients, tissue samples and fibroblast cultures

A total of 10 patients participated in this study of the Department of Surgery. Informed consent was obtained from all patients. Skin tissue samples were taken from patients with primary inguinal hernia during the operation ($n = 5$, mean age 58 years, range 50–72 years) and from a group of patients undergoing interventions for intra-abdominal inflammatory diseases without any pre-existing scar and clinical signs of herniation (control, $n = 5$, mean age 61 years, range 52–70 years). Patients with steroid therapy, previous history of any abdominal surgery, diabetes, abdominal aortic aneurysm and connective tissue diseases were excluded from the study.

All skin specimens were obtained under strictly sterile conditions and transferred to a fibroblast culture system as previously described [21].

Extraction of total RNA

The chemicals and reagents used for extraction of total RNA and RT-PCR were purchased from Promega (Promega, USA) unless otherwise stated. Total RNA was extracted from confluent fibroblast cultures. About 5×10^6 cells were homogenised in 600 μ l ice cold denaturing solution, 60 μ l 2 M sodium acetate, pH 7.4 and 600 μ l phenol: chloroform :isoamyl alcohol (125:24:1), pH 4.7. The supernatant then was collected at 12000 revolutions per minute and the remaining phenol was removed after adding chloroform to the aqueous phase. Afterwards, the total RNA was precipitated with an equal volume of ice cold isopropanol at -20°C overnight. The pellet was washed in 70% ethanol (-20°C) and resuspended in RNase-free water. The concentration, purity and integrity of total RNA were determined by GeneQuant II (Pharmacia Biotech, England) and visualised electrophoretically by separation in a denaturing agarose gel (1%) containing ethidium bromide. Total RNA used for RT-PCR was treated with RQ1 RNase-free DNase (1 μ / μ g total RNA) at 37°C for 30 minutes prior to RT-PCR. Then phenol: chloroform :isoamyl alcohol was added and the total RNA was participated with iso-propanol to remove DNase as described above.

RT-PCR

Oligonucleotide primers were designed using the published sequences of type collagen I and III genes [22], MMP-1 and MMP-13 genes [13], and β -actin gene. They were synthesised by Genset (Genset, France). Table 1 lists the primers employed in the present experiment.

Table 1: Primer sets used for RT-PCR

mRNA template	Primer sequence (sense/anti-sense)	PCR product size (bp)	Annealing temperature
type I collagen	5'CCCCCTCCCCAGCCACAAAG-3' 5'- TCTTGGTCGGTGGGTGACTCT-3'	360	44°C
type III collagen	5'-CCAAACTCTATCTGAA-3' 5'-GGACT- CATAGAATACA-3'	449	44°C
MMP-1	5'-AGGTTATCCCAAATGATAG-3' 5'- TGCAGTTGAACCAGCTATTA-3'	173	55°C
MMP-13	5'-TTCTGGCACATGCTTTTCCTC-3' 5'- GGTTGGGTCTTCATCTCCTG-3'	273	55°C
β -actin	5'-ATCTGGCACCACACTTCTACA-3' 5'- GTTTCGTGGATGCCACAGGACT-3'	577	55°C

Synthesis of cDNA was carried out at 42°C for 55 minutes in 20 μ l final volume containing 1 μ g total RNA, 5 mM MgCl, 1 \times reverse transcription buffer, 1 mM each dNTP, 1 U/ μ l recombinant RNasin[®] ribonuclease inhibitor, 15 U/ μ g (total RNA) AMV reverse transcriptase and 0.5 μ g oligo(dT)15 primer. Afterwards, 2 μ l of cDNA was added to the PCR reaction. The PCR reaction volume of 50 μ l consisted of 1.5 mM MgCl₂, 1 \times thermophilic DNA polymerase reaction buffer (MgCl₂-free), 200 μ M each dNTP, 1.25 U taq DNA polymerase. The optimal concentration of primers used was 5 pmol for β -actin, 50 pmol for type I and III collagen, MMP-1 and MMP-13. PCR amplification was performed for 30–40 cycles with denaturation at 95°C for 60 seconds, annealing at the indicated temperature (Table 1), each for 60 seconds, and extension at 72°C for 90 seconds. PCR was repeated thrice with each sample. PCR products were electrophoretically fractionated on a 1.2–1.8% agarose gel containing ethidium bromide.

Semiquantitative determination was performed densitometrically using the Multi-Analyst from Biorad (Biorad, USA). The size and intensity of each band was assessed and the results were integrated as follows: relative amount of PCR products = band area \times band specific intensity. All values were corrected by β -actin mRNA levels as an internal standard.

Northern Blot

The chemicals and reagents used for Northern Blot were purchased from Boehringer Mannheim (Germany) unless otherwise stated. Total RNA, 18 μ g/lane, was fractionated on 1% agarose gel containing 2.2 M formaldehyde. The RNA was transferred to a charged nylon membrane by capillary method overnight in 20 \times SSC transfer buffer at room temperature and fixed by baking at 120°C for 30 minutes. Prehybridisation and hybridisation were performed at 10°C below the evaluated melting temperature of oligonucleotides in Dig Easy Hyb. The specific internal

oligonucleotides used in the present study can be hybridised to unique sequences within type I collagen (5'-AACCTCAAGAAGGCCCTGCT-3', melting temperature 62°C) [23], type III collagen (5'-AGAAACTGCAGACCTGAA-3', melting temperature 58°C) [24] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 5'-ATGACATCAAGAA-GGTGGTGAA-3', melting temperature 51°C) [13] respectively. These three oligonucleotide probes, and their respective anti-sense probes were synthesised (Genset, France) and, afterwards, were 3' end-labelled with digoxigenin-11-dUTP using terminal transferase at 37°C for 15 minutes (10 pmol/ml). The sense oligonucleotide probes served as negative controls, whereas the anti-sense oligonucleotide probe were used for the investigation of type I collagen mRNA, type III collagen mRNA and GAPDH mRNA, respectively.

After stringent washes, the membrane was blocked by incubation with 1% blocking reagent for 1 hour at room temperature. A 1:20000 dilution of anti-Dig-alkaline phosphatase was incubated with the membrane for 30 minutes at room temperature. Finally, hybridization products were exposed to chemoluminescent detection film for up to 10 minutes.

The relative amount of type I and type III collagen mRNA were determined densitometrically using the Multi-Analyst from Biorad (Biorad, USA) after standardisation of both type I and type III collagen mRNA levels with their respective GAPDH mRNA levels.

Statistical Methods

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) – software. Student's two-tail t-test was used to determine statistically the differences between controls and primary hernias. All data were presented as mean \pm SD (standard deviation). Values of $p < 0.05$ were considered to be statistically significant.

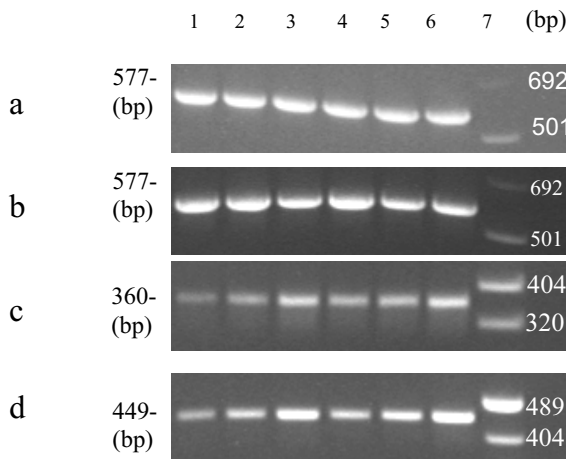


Figure 1
PCR products analysis of type I and type III collagen samples and β -actin optimisation. 1, 2 and 3) controls; 4, 5 and 6) inguinal hernias; 7) standard marker, a) β -actin amplification with annealing temperature at 60°C of type I collagen; b) β -actin amplification with annealing temperature at 44°C of type III collagen; c) type I collagen; d) type III collagen

Results

Expression of collagen type I and type III

The product size and intensities for each of the amplified products by using RT-PCR are shown in figure 1. The amplified product size for each are 577 bp for β -actin, 360 bp for type I collagen, and 449 bp for type III collagen. The respective intensities of β -actin are almost equivalent in the same PCR reaction. The densitometric analysis showed the variation between lanes to be less than 10%, both for each individual experiment and between experiments. Semiquantitative analysis of PCR products showed that the densitometry units of type I and type III collagen were 0.46 ± 0.06 and 0.51 ± 0.07 in controls, respectively; 0.51 ± 0.06 and 0.87 ± 0.10 in patients with primary hernia, respectively (all values were derived after standardization of their mRNA levels with the respective β -actin mRNA levels). Due to the apparent increase in type III collagen mRNA levels in patients with primary inguinal hernia the ratio of type I to type III collagen mRNA levels in these patients were markedly reduced and showed highly significant differences compared to the controls (0.59 ± 0.06 versus 0.90 ± 0.08 ; $p < 0.01$).

In the Northern Blot analysis the corresponding expressions of type I and type III collagen mRNA in total RNA are shown in figure 2. Semi-quantitative analysis revealed that the relative amount of type I collagen mRNA and type III collagen mRNA was 1.9 ± 0.2 and 1.7 ± 0.2 in controls, respectively; 1.8 ± 0.2 and 2.2 ± 0.2 in patients with primary hernia, respectively (all values were derived after

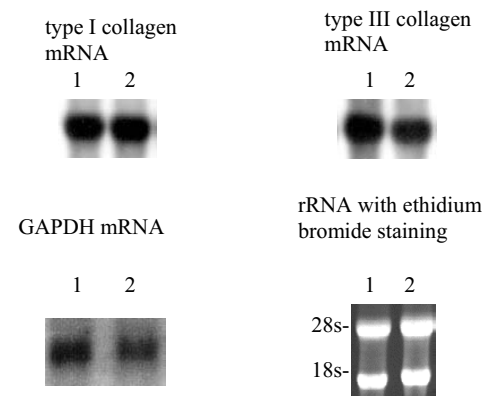


Figure 2
Northern Blot analysis of type I and type III collagen mRNA from cultured fibroblasts. The ratio of 28S to 18S rRNA (ribosomal RNA) was approximately 2 : 1 by ethidium bromide staining of denaturing agarose gel electrophoresis, indicating that no gross degradation of extracted RNA occurred. 1. primary inguinal hernia; 2 control

standardisation of their mRNA levels with the respective GAPDH mRNA levels). As a consequence of the significant increase of type III collagen mRNA in patients with primary hernia the ratio of type I to type III collagen mRNA levels (0.83 ± 0.09) was apparently decreased and showed a highly significant difference in comparison with the ratio of controls (1.12 ± 0.13 ; $p < 0.01$).

Expression of MMP-1 and MMP-13

The amplified product size is 173 bp for MMP-1 by using RT-PCR (table 1; Fig. 3). Although the expression of MMP-1 PCR products were observed in all samples, their intensities in patients with primary hernia were almost equivalent to that of controls. The semiquantitative analysis confirmed this finding, i. e. the expression of MMP-1 mRNA in patients with primary inguinal hernia did not show any significant difference as compared to that of controls (0.40 ± 0.04 versus 0.41 ± 0.04 ; $p > 0.05$) (all values were derived after standardisation of their mRNA levels with the respective β -actin mRNA levels).

However, in contrast to the expression of MMP-1, an expression of MMP-13 PCR products was detected neither in the patients with primary hernia nor in the controls (data not shown), even using amplified reactions of 40 cycles.

Discussion

Abdominal wall stability depends on the integrity of the muscular and fascial framework. The mechanical properties of each fascia relies on the connective tissue and its

collagens. The predominant collagen type in skin is the mature and mechanically stable type I collagen, whereas the immature and instable type III collagen is typically seen during the early phases of wound healing [25]. Thus, in normal skin type I and III collagen are known to exist in a ratio of up to 4:1 [26]. Earlier observations confirmed a correlation between collagen production and their cellular mRNA levels in cells and tissues [10,11,27].

In a previous study, immunohistochemical and Western blot analysis indicated that the ratio of collagen type I/III was significantly decreased in the skin of patients with incisional and with either indirect or direct inguinal hernia, with a concomitant increase in collagen type III [20,21]. These findings correspond to the results of the present study. Both RT-PCR and Northern Blot analysis revealed that the ratio of type I to type III collagen mRNA in fibroblast cultures from skin of patients with primary inguinal hernia was markedly reduced compared to normal controls. The decrease is caused by a significantly increased expression of type III collagen mRNA, whereas type I collagen mRNA level was unchanged or only slightly elevated. Furthermore, these data confirmed the results of Friedman et al. in 1993 describing a rise in the levels of type III collagen mRNA and in type III collagen protein in patients with primary inguinal hernia [6]. Accordingly, in 1992 Ajabnoor et al. detected in-vitro a reduced ¹⁴C-proline uptake in muscle cells of patients with an inguinal hernia [5]. The detection of a lowered ratio of type I to type III collagen in cultured fibroblasts disproves the supposition that this collagen disproportion is only a secondary consequence to the mechanical strain or other local influences. The detection of an impaired collagen balance both in the tissue as well as in cultured fibroblasts underlines the suspicion that the development of an inguinal hernia is likely to be implemented by a primarily disturbance of the fibroblast function and their collagen genes. Based on these results we assume that the altered collagen synthesis in hernia patients can be regarded as basic genetically caused dysregulation serving as an initiating or promoting factor for the development of primary inguinal hernias.

Furthermore, MMP-1 and MMP-13 were assessed as the principal enzymes cleaving fibrillar type I, II and III collagen [12–14]. Their activities are predominantly responsible for the content and proportion of type I and III collagens. In this study, our results revealed that the expression of MMP-1 mRNA in fibroblasts from the skin did not show any significant differences between the patients with primary hernias and the controls. In contrast, MMP-13 gene expression was not detected at all, neither in the fibroblasts from the skin of patients with primary inguinal hernias nor in the controls. This is in accordance with previous observation at the protein level [21], in which we

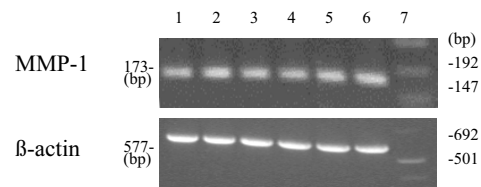


Figure 3
PCR products analysis of MMP-1 samples. 1, 2 and 3) controls; 4, 5 and 6) primary hernias; 7) standard marker.

found a similar immuno staining reaction and intensity against MMP-1 in the skin of primary hernia patients as compared to controls. Furthermore, no MMP-13 protein expression was observed by immunohistochemistry, neither in the samples of primary hernia patients nor in the controls.

Ajabnoor et al. [5] investigated the susceptibility of the fibroblast extracellular matrix to degradation by collagenase and found no difference in collagenase activity between inguinal hernia patients and controls. Interestingly, recent investigations by Bellon et al. showed significant active MMP-2 expression by cultured fibroblasts from the fascia transversalis of patients with inguinal hernias, hereby confirming previous in vivo results with immunosorbent assay, immunoblotting and zymography [18,19]. These results on protein level appear to suggest that in comparison to MMP-1 and MMP-13, MMP-2 is an active part of degradation system of the extracellular matrix in hernia patients. If this enhanced MMP-2 activity is regulated through a direct increase of MMP-2 mRNA or the answer of mediating factors of the extracellular matrix must be investigated in further studies.

Our data confirm that patients with primary inguinal hernia display an increase of type III collagen mRNA in fibroblasts, which leads to a decreased ratio of type I to type III collagen mRNA. The amount of MMP-1 and MMP-13 mRNA in patients with primary inguinal hernias is not significantly different from that of the controls. The data give strong evidence of a disorder of the collagen tissues in patients with a primary inguinal hernia. Furthermore, the presence of a basic impairment of the soft tissue explains the frequently seen appearance of multiple hernias as well as the high rate of recurrences after suture repair of about 10 % to 15%, being almost constant in many countries [28]. As a consequence, a reinforcement of the surgical repair, e.g. with alloplastic material, seems to be imperative, at least for some patients. Larger studies are necessary to define the proportion of patients who suffer from this col-

lagen imbalance. A better determination of the transcriptional control of collagens in various populations with and without hernia should help to understand the pathophysiology and the genetic background of the development of primary inguinal hernia and to select the patients at risk, who probably profit from surgical repair with prosthetic material.

Competing interests

None declared.

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