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Fibroblasts From the Transversalis Fascia of Young Patients With Direct Inguinal Hernias Show Constitutive MMP-2 Overexpression

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Objective
To determine the expression pattern of certain metalloproteinases (MMPs) known to be involved in the degradation of the extracellular matrix in cultured fibroblasts from the transversalis fascia (TF) of patients with inguinal hernia.

Summary Background Data
Inguinal hernia is a common pathology, the cause of which remains unknown. It is, however, clear that the TF is one of the anatomical structures that may impede the formation of hernias, and particularly the direct type of hernia. In previous studies the authors found enhanced MMP-2 expression in TF specimens in vivo. The persistence of increased expression in cultured fibroblasts might support the idea of a genetic defect as the cause for this pathology.

Methods
Fibroblasts from the TF of patients with direct and indirect inguinal hernia were cultured and compared with those obtained from control TF in terms of MMP (MMP-2 and MMP-9) expression.

Results
Significant active MMP-2 expression was shown by TF fibroblasts from young patients with direct hernias. These findings were confirmed by immunosorbent assay, immunoblotting, and zymography of the fibroblast culture media. No MMP-9 expression was detected.

Conclusion
These results indicate that MMP-2 may be involved in the TF matrix degradative process in patients with direct hernia. The persistence of changes in MMP-2 levels in the cell cultures appears to suggest a genetic defect or irreversible change as the origin of this pathology rather than environmental factors, which may later participate in the development of the hernial process.

Inguinal hernias are among the disorders that most frequently require surgery: their repair accounts for 10% to 15% of all general surgical procedures.[1] Although the cause remains unknown, it has been established that the integrity of the abdominal wall in the groin area is dependent on the transversalis fascia (TF), the oblique orientation of the inguinal canal, and a sphincterlike structure of the internal ring.[2]

Despite numerous predisposing factors, including anatomical features (persistence of the peritoneal-vaginal conduit, high insertion point of the transverse arch) and those associated with other diseases (obesity, chronic obstructive pulmonary disease, constipation), the underlying cause of the development of the different types of hernias is of a biologic nature. Research aimed at evaluating the role played by biologic factors has centered on possible alterations in connective tissue metabolism. This idea is also supported by the fact that diseases such as Marfan and Ehlers-Danlos syndromes, cutis laxa, osteogenesis imperfecta,[3] and congenital hip dislocation[4] have been associated with hernial processes.

Tissue specimens from patients with hernias for this type of experimental study include the abdominal anterior rectus muscle sheath,[5] cremaster, hernial sac,[6] and even skin tissue.[7] The expression pattern of certain types of collagen[8] and certain enzyme dysfunctions[9] have also been the subject of several of these studies.

Concerned about the cause of hernias, we have conducted several investigations on TF biopsy specimens from patients with hernias. We correlated the ultrastructural features of the TF with collagen activity and degradation through the study of certain metalloproteinases (MMPs) and observed an increase in MMP-2 expression in TF specimens from patients with direct hernias.[10] This enzyme degrades and participates in the turnover of the extracellular matrix, acting on certain types of collagen and elastin. Its expression is enhanced in

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processes such as genitourinary prolapse[11] and aortic aneurysm.[12,13] Further, it has been reported that patients with this last pathology have an increased incidence of hernias.[14,15] These findings prompted us to propose a hypothesis for the behavior of MMP-2 in TF specimens of patients with hernias, based on the possibility of a similar mechanism that might explain why in some patients the posterior wall of the inguinal canal of the TF remains unaltered, and in others it presents defects that lead to hernial processes.

To this end, we designed an in vitro model using cultured fibroblasts from the TF to determine whether the modifications in MMP expression found in tissue are maintained in culture. If this were the case, it could point to a genetic predisposing factor for this pathology, and thus hernia types showing some alteration at the level of the TF could be candidates for primary repair with a prosthetic material in a effort to prevent future relapses.

PATIENTS AND METHODS Patients

Control TF specimens were obtained from donor patients at the time of multiorgan extraction for transplant. Pathologic specimens were obtained from patients undergoing primary inguinal hernia repair with no other disease. In each case, biopsy specimens were taken from the same central area of the posterior wall of the inguinal canal.

All patients were men age 20 and 60 years. The TF specimens were used to establish the study groups: control (n 5 10), indirect hernia (n 5 36), and direct hernia (n 5 32). These were further divided according to patient age: 20 to 40 years (young; mean 30.6 years) and 41 to 60 years (aged; mean 51.6 years).

Methods

Immediately after surgery, the biopsy specimens were introduced into sterile minimum essential medium (Gibco BRL, Life Technologie, Barcelona, Spain) supplemented with 100 IU penicillin/100 mg streptomycin (Gibco BRL) for transport to the laboratory. Specimens were then frozen (-20°C) for processing and later biochemical analysis.

The study was approved by the local ethics committee. Informed consent was obtained from each patient.

Fibroblast Cultures

The TF specimens were processed under sterile conditions in a Clase II laminar flow cabinet (Telstar AV 30/70, Telstar SA, Madrid, Spain). The TF was separated from associated fatty tissue by dissection using a scalpel blade and cut into 1-mm^2 pieces. These explants were placed on the culture surface of 25-cm^2 Roux flasks (Nuclon-Intermed, Nunc A/S, Roskildo, Denmark), to which 0.5 mL BIO-AMF 2 medium (Biologic Industries, Kibbutz Beit Haemek, Israel) had been added to maintain the humidity of the culture surface and to improve the adherence of explants.

The culture flasks were then incubated in a vertical position at 37°C in the presence of 5% CO_2 in a culture oven (Forma Scientific Inc., Marietta, OH) for 2 hours. Next, 2.5 mL BIO-AMF 2 medium was added per flask, and the flasks were incubated horizontally under the previous conditions. Care was taken to avoid movements that might cause the explants to become unstuck. The culture medium was carefully replaced twice a week.

Once the cells had grown to confluence, fibroblasts were subcultured by enzyme treatment. This involved withdrawing the medium and rinsing three times in 2 mL 13 Hank's balanced salt solution (Gibco BRL), followed by the addition 2 mL 13 trypsin-ethylenediaminetatraacetic acid solution at 1:250 (Gibco BRL) and incubation at 37°C for 5 minutes. The enzyme reaction was stopped by the addition of 4 mL culture medium. The resultant cell suspension was centrifuged at 200*g* for 7 minutes and the cell pellet was resuspended in 9 mL BIO-AMF 2 medium. These cells in suspension were once again placed in culture at a density of 3 mL per 25-cm^2 Roux flask until a confluent monolayer was obtained.

Fibroblasts from the first or second passage were used in all the subsequent experiments.

Immunosorbent Assay

Once the fibroblasts had grown to confluence, the culture medium was withdrawn and the cells were centrifuged at 200*g* for 7 minutes. The supernatant was kept at 270°C until further processing. Next, the cells were lifted from the culture surface by trypsin treatment and enumerated in a Neubauer chamber (Weber Scientific International Ltd., Marienfeld, Germany). Levels of the metalloproteinases MMP-2 and MMP-9 (The Binding Site, Birmingham, UK) were determined in the culture media by enzyme-linked immunosorbent assay.

The wells of the plate supplied with the kit were filled with 100 mL of each sample and incubated at room temperature for 1 hour. Once each well had been washed three times with 250 to 300 mL rinsing solution, 100 mL biotinated antibody was added to each well. After incubation at room temperature for 1 hour, excess antibody was withdrawn and the wells were washed again. Next, 100 mL streptavidin peroxidase was added to each well, followed by incubation at room temperature for 30 minutes. Washing was conducted as in the previous steps, and 100 mL chromogenic substrate 3,39,5,59-tetramethylbenzidine was added to each well, followed by incubation at room temperature for 10 minutes. The enzyme reaction was stopped with 100 mL stopping solution. The plate was read in an ELX 800 microplate counter (Bio-Tek Instruments Inc., Izasa, Barcelona, Spain) at an optical density of 450 nm. Results were expressed as ng/10^6 cells.

Immunocytochemical Analysis

Fibroblasts were fixed in 10% formalin, washed twice in phosphate-buffered saline (pH 7.4), and processed for the detection of MMP-2 and MMP-9 using the monoclonal antibodies antihuman matrix MMP-2 and antihuman MMP-9 (The Binding Site), respectively. The antigen–antibody reaction was detected by the peroxidase–antiperoxidase technique. The chromogenic substrate used was 3-amino9ethyl carbazole in N,N-dimethylformamide. Fibroblasts were examined under a light microscope (Zeiss, Jena, Germany).

Immunoblotting

Each culture medium was mixed with an equal volume of sample buffer (0.12 Tris-base, pH 6.8, containing 20% glycerol, 0.04% bromophenol blue, 6% sodium dodecyl sulfate in the absence or presence of 10% 2-mercaptoethanol) and subjected to 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the modified method of Laemmli.[16]

After SDS-PAGE, the gels were blotted onto a nitrocellulose membrane (BioRad, Hercules, CA) as described by Towbin et al.[17] Excess protein-binding sites were saturated with Tris-buffered saline (20 mmol/L Tris-base and 137 mmol/L NaCl, pH 7.6) containing 5% nonfat dried milk and 0.1% Tween 20. The blotted membranes were incubated with sheep polyclonal antihuman MMP-2 antibodies (1: 1,000 dilution in Tris-buffered saline) for 1 hour at room temperature. The immunoreactive protein was revealed with peroxidase-conjugated rabbit antigoat IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) (1:1,000 dilution in Tris-buffered saline) and subjected to luminescence analysis according to a standard protocol.[18]

Gelatinolytic Activity

Conditioned media were diluted 1:1 in sample buffer (0.12 mol/L Tris-base, pH 6.8, containing 20% glycerol, 0.04% bromophenol blue, and 6% SDS). The samples were analyzed by a zymographic technique using 10% SDSPAGE containing 0.1% (w/v) gelatin (Sigma Chemical, Madrid, Spain) as the substrate. Each lane was loaded with a total protein concentration of 3 mg and subjected to electrophoresis using a 25-mA/gel constant current at 4°C. Gels were washed twice in 50 mmol/L Tris (pH 7.4) containing 2.5% (v/v) Triton X-100 for 1 hour, followed by two 10-minute rinses in 50 mmol/L Tris (pH 7.4). After removal of the SDS, the gels were incubated overnight in 50 mmol/L Tris (pH 7.5) containing 10 mmol/L $CaCl_2$, 0.15 mol/L NaCl, 0.1% (v/v) Triton X-100, and 0.02% sodium azide at 37°C under constant gentle shaking. After incubation, the gels were stained with 0.25% coomasie brilliant blue R-250 (Sigma Chemical) and destained in 7.5% acetic acid with 20% methanol. The gelatinase bands appear white on a blue background. The degradative activity of MMP-2 and MMP-9 was semiquantitatively determined by densiometry.

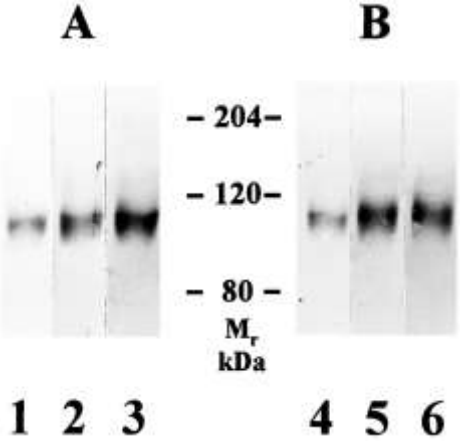

Figure 1. MMP-2 expression by immunoblot analysis. (A) 20- to 40year-old group. (B) 41- to 60-year-old group. Lanes 1 and 4: control; lanes 2 and 5: indirect hernia; lanes 3 and 6: direct hernia. An MMP-2 immunoreactive band was observed at approximately 110 kd. Molecular mass markers are indicated (myosin, 204 kd; b-galactosidase, 120 kd; bovine serum albumin, 80 kd).

Statistical Analysis

Data corresponding to the different groups were compared using the nonparametric Mann-Whitney test for independent samples.

RESULTS

MMP-2 expression could be detected in fibroblasts from each of the study groups. The greatest MMP-2 levels were found in the young patients with direct hernia. Fibroblasts from the older patients showed less intense MMP-2 labeling than those from the younger group. No MMP-9 expression was detected in any of the study groups.

Both control and pathologic samples subjected to immunoblotting showed MMP-2 (Fig. 1) but not MMP-9 in the culture media, irrespective of age. In the presence of reducing agents (2-mercaptoetanol), the band characteristic of

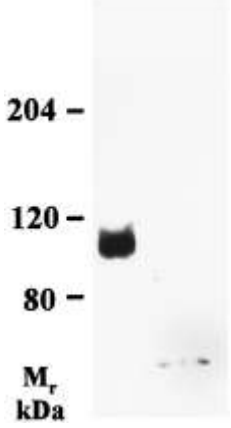

Figure 2. MMP-2 expression by immunoblot analysis in the presence (right panel) and absence (left panel) of 2-mercaptoethanol. Molecular mass markers are indicated (myosin, 204 kd; b-galactosidase, 120 kd; bovine serum albumin, 80 kd).

MMP-2 (approximately 110 kd) was replaced by a band corresponding to a molecular weight of 67 kd (Fig. 2).

The use of enzyme-linked immunosorbent assay to quantify MMP-2 (Fig. 3) in the culture media showed a significant increase in the levels of this enzyme in the young patients with direct hernia compared with the samples from the control subjects of similar age and patients with indirect hernia (P , .05). However, in the older group, the amount of MMP-2 secreted into the medium in patients with direct hernia was significantly lower (P , .05) compared with the samples from control subjects and patients with indirect hernia. Comparison by age yielded significant differences only between the direct hernia subgroups (P , .05).

The gelatinolytic activity of active MMP-2 is shown in Figure 4. Quantification of this activity (Fig. 5) showed a significant increase in the younger group compared with

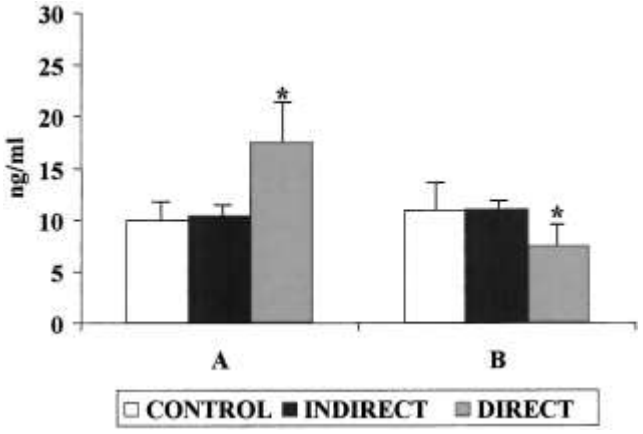

Figure 3. Quantification of MMP-2 by enzyme-linked immunosorbent assay. Results are expressed in ng/mL. In both age groups, significant differences were found in the direct hernia patients compared with the other groups (*P, .05). (A) 20- to 40-year-old group. (B) 41- to 60year-old group.

Figure 4. Gelatinolytic activity determined by gelatin zymography. (A) 20- to 40-year-old group. (B) 41- to 60-year-old group. Lanes 1 and 4: control; lanes 2 and 5: indirect hernia; lanes 3 and 6: direct hernia. A white band corresponding pro-MMP-2 was observed at approximately 68 kd.

control subjects of similar age, irrespective of hernia type. In the older group, the quantity of active enzyme showed a similar pattern to that observed by immunosorbent assay, with a significant reduction shown for patients with direct hernia (P , .05) compared with samples from control subjects and patients with indirect hernia.

DISCUSSION

A balance between extracellular matrix synthesis and degradation is important for tissue integrity because remodeling occurs continuously. Changes or defects in matrix molecules may also alter tissue architecture, resulting in the impairment of the proper assembly of the components and modifying the mechanical properties of the tissue. To extend this idea, we had previously investigated the role of MMP-2 in tissue from the TF and found increased expression of this degradative enzyme in patients with direct hernias.[10]

In this study, we attempted to find out whether the mod-

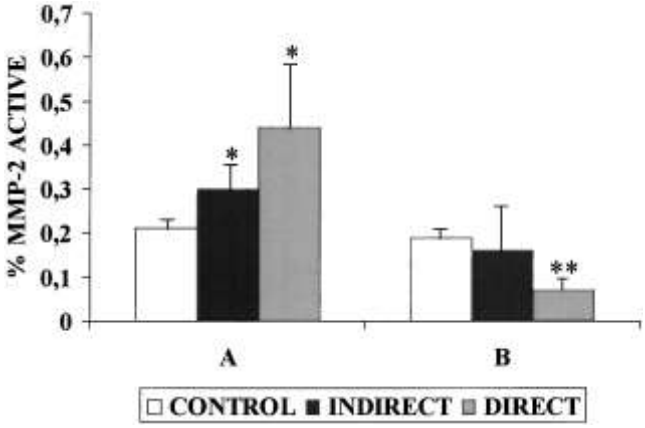

Figure 5. Percentage of active MMP-2 levels based on gelatin zymography optical density measurements. (A) 20- to 40-year-old group: control/indirect, *P, .05; control/direct, *P, .05; indirect/direct, not significant. (B) 41- to 60-year-old group: control/indirect, not significant; control/direct, **P, .05; indirect/direct, not significant.

ification in MMP-2 levels could be related to environmental factors or whether there was a more stable component inducing permanent changes in the fibroblasts of the TF affected by this pathology. The use of fibroblast cultures ensured that all the cells were under the same environmental conditions, permitting us to determine whether the changes shown in vivo persisted under such controlled conditions.

Comparison of MMP-2 levels in the culture media of fibroblasts from control subjects with those from patients with hernias demonstrated significant changes in MMP-2 expression. In each case, immunoblotting yielded a band of molecular weight close to 110 kd. However, in the presence of the reducing agent mercaptoethanol, this band was replaced by one corresponding to a weight of approximately 67 kd. These findings suggest the presence of MMP-2 in the form of dimers.

Immunosorbent assay revealed that the amount of MMP-2 secreted by the fibroblasts from patients with direct hernias was significantly greater in the patients age 20 to 40 years. When we evaluated the amount of MMP-2 in the older group (41–60 years), significant differences were observed only in patients with direct hernias. If we consider the age factor, a large reduction in MMP-2 secretion was shown in patients with direct hernias in the older group. We thus propose that the hernia pathologic process is related to MMP-2 secretion. Further, the significant increase in the level of this enzyme shown by fibroblasts from young patients with direct hernia could be in line with the macroscopic weakness characteristics shown by the TF in these patients.

These findings would not be conclusive without an estimation the amount of active MMP-2, the form of the enzyme capable of degrading the extracellular matrix. This was the basis for the semiquantitative analysis of gelatinolytic activity, which yielded statistically significant changes for young patients with direct hernia. This is partly consistent with the former data because it confirms that in the population of young patients with direct hernia, there is a greater expression of not only MMP-2 but also its active form. This finding is of great interest, given the infrequent appearance of this type of hernia in young patients.

A further finding of note is the overexpression of this enzyme in other pathologies such as aneurysms[12,13] and genitourinary prolapses,[11] which could indicate an imbalance between matrix synthesis and degradation in all these pathologies.

As expected, MMP-9 and other enzymes mainly secreted by neutrophils, macrophages, and other inflammatory response cells was not detected in the fibroblast culture media. Thus, that activity reported in other tissues[9,19] is probably attributable to cell types other than fibroblasts. We believe that these secondary factors may accelerate the hernia process, especially in elderly patients.

In conclusion, fibroblasts from the TF of young patients with direct hernia show increased active MMP-2 expression. This enzyme may be intimately involved in the extracellular matrix degradative process. The persistence of this alteration in the fibroblast cultures appears to indicate a genetic defect or perhaps later transformation as the underlying cause of this pathology, ruling out environmental factors as the main cause.

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