Effects of Blocking $\alpha_v\beta_3$ integrin by a recombinant RGD disintegrin on remodeling of wound healing after induction of incisional hernia in rats[1]

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ABSTRACT

PURPOSE: Incisional hernia (IH) is characterized by defective wound healing process. Disba-01, a $\alpha_v b_3$ integrin blocker has shown to control the rate of wound repair and therefore it could be a target for new wound healing therapies.The objective of the study was to determine the changes induced by Disba-01 on repair of wound healing after induced IH in rats.

METHODS: Thirty two male albino rats were submitted to IH and divided into 4 experimental groups: G1, placebo control; G2, DisBa-01-treated; G3, anti-$\alpha_v\beta_3$ antibodies-treated and G4, anti-α_2 antibodies-treated. Histological. biochemical and extracellular matrix remodeling analysis of abdominal wall were evaluated.

RESULTS: After 14 days, 100% of the G2 did not present hernia, and the hernia ring was closed by a thin membrane. In contrast, all groups maintained incisional hernia. DisBa-01 also increased the number macrophages and fibroblasts and induced the formation of new vessels. Additionally, MMP-2 was strongly activated only in G2 ($P<0.05$). Anti- $\alpha_v\beta_3$-integrin antibodies produced similar results than Disba-01 but not anti-α_2 integrin blocking antibodies.

CONCLUSION: These results strongly indicate that Disba-01 has an important role in the control of wound healing and the blocking of this integrin may be an interesting therapeutical strategy in IH.

Key words: incisional hernia, disintegrin, wound healing, MMP-2, $\alpha_v\beta_3$ integrin

Introduction

Incisional hernias (IH) are usually found as a complication of about 11% of abdominal wall closures. Approximately 200.000 IH are repaired in the USA each year. Mechanical advances in mesh, suture material and closure techniques did not reduce the high rate of this complication.[1,2] Therefore, IH is considered a connective tissue disease characterized by defective wound healing process.[3]

The distinct phases of wound healing process are well described and include local hemorrhage with extravasation of platelets and platelet-derived growth factors such as PDGF (platelet derived growth factor) and EGF (epidermal growth factor). These mitogens stimulate FGF-7 (fibroblast growth factor-7) expression by fibroblasts. In addition, invading neutrophils and macrophages will secrete several proinflammatory cytokines and growth factors resulting in angiogenesis and fibroplasia. These events will culminate with the synthesis of a provisional matrix that must be able to support the biomechanical forces of the abdominal wall.[2]

IH is believed to be due to a combination of biomechanical and biochemical failures of the fascial wound after chirurgical procedures. Biomechanical failure may happen early during the post-operative period when the wound depends on the suture quality and integrity to support the increasing demands during the recovery period. On the other hand, abnormal collagen metabolism has also been correlated to the incidence of IH. A significant decrease in the ratio of collagen I to collagen III (coll I/III) is implicated in modifications of structural integrity and mechanical stability of the connective tissue in experimental hernias.[4]

Matrix metalopeptidases (MMPs) were also suggested to have an important role in the pathogenesis of IH. MMPs comprise a family of enzymes that play a central role in extracellular matrix (ECM) turnover and remodeling.[5-7]

MMPs are zinc and calcium dependent enzymes, synthesized as zymogens in connective tissue. Under normal conditions, MMPs are present at low levels, usually in the latent form, and are responsible for normal physiological tissue turnover.[8-10]

In pathological conditions, there is an imbalance between the synthesis and degradation of matrix leading to net tissue degradation. After injury, proteolysis is required to remove the damaged matrix and to help the synthesis of the new healing tissue. An increase in net MMP activity indicates matrix degradation as well as tissue repair, and so it is needed for the remodeling process in wound healing.[11,12] Despite the importance of MMPs in the wound healing process, the role of these enzymes in IH repair is not well understood. MMP-1 and MMP-13 were not different in patients with inguinal hernia and the controls[4] and the expression of MMP-2 was demonstrated to be dependent on the mesh material. However, a correlation between the ratios of coll I/III and levels of MMP-2 activity remains to be determined yet.

The role of adhesion receptors such as the integrins in the progression of IH has not been deeply addressed yet. Integrins are heterodimeric transmembrane proteins, which connect the ECM components and the cell cytoskeleton.[13] Cell adhesion to the ECM may be mediated by binding of integrin to an integrin-recognition RGD motif found in some ECM components such as fibronectin, vitronectin and fibrinogen.[14] The α$_2$β$_1$ is required for keratinocyte adhesion to collagen I for proper healing of epidermis.[15] The α$_v$β$_3$ integrin is over expressed in several cell types involved in wound healing such as platelets, endothelial cells, macrophages and fibroblasts. Antibody inhibition of α$_v$β$_3$ integrin decreased the migration of these cells and angiogenesis as well at the wound site.[16,17] However, b$_3$-integrin deficient mice showed accelerated re-epithelialization associated with enhanced TGF-b$_1$ signaling. These results suggest that the α$_v$β$_3$ integrin controls the rate of wound repair, and, therefore, it could be a target for new wound healing therapies.

Exogenous proteins having the RGD motif such as the disintegrins, may also bind to integrins and block their functions. Disintegrins are small proteins isolated from snake venom, usually derived by proteolysis of precursors having metalloprotease activity. Disintegrins having the RGD motif are very potent inhibitors of platelet aggregation by acting as antagonists of the fibrinogen binding to its platelet receptor, the α$_2$β$_3$ integrin.[19-21] Some RGD-disintegrins also bind to a$_5$b$_1$ integrin and inhibit cell adhesion to fibronectin as well as the downstream intracellular signaling events such as a phosphorylation cascade.[22,23] Therefore, disintegrins have been used as prototypes for drug design of new therapies targeting the integrins.

We have recently reported the production and isolation of a novel recombinant RGD disintegrin, DisBa-01 (Genbank accession no. AY259516) from the Brazilian snake Bothrops alternates.[24] DisBa-01 is a 78-residue protein with an RGD adhesive motif. The recombinant protein is fused with a His Tag and has a molecular weight of 11,637 Da deduced by mass spectrometry. In silico studies predicted the preferential interaction of the toxin with b$_3$ integrins and biospecific interaction analysis confirmed the specific binding of DisBa-01 to immobilized purified α$_v$β$_3$ integrin in a dose dependent manner.[25] DisBa-01 inhibited α$_v$β$_3$-dependent cell adhesion in vitro and potently inhibited angiogenesis and metastasis in vivo.

The present study was designed to determine the histological, biochemical and extracellular matrix remodeling analysis of abdominal wall induced by Disba-01 on wound healing after incisional hernia in rats. The hypothesis is that the Disba-01 would be helpful in the tissue repair by the blockage of the $\alpha_v\beta_3$ integrin and increasing MMP-2 activity and tissue remodeling.

Methods

DisBa-01 expression, purification and characterization. Recombinant DisBa-01 was produced from the mRNA fraction purified from the venom gland of a Bothrops alternatus specimen as recently described.[24] The coding region corresponds to a medium disintegrin (78 amino acid residues) with an RGD adhesive motif. The His-Tag fusion protein produced in E. coli is a 12 kDa protein as estimated by mass spectrometry and SDS-PAGE.

Thirty two male rats (Wistar, Rattus novergicus albinus, ± 250g) were grouped in plastic cages at room temperature and allowed to food and water ad libitum. All animal procedures were performed in accordance with the U.S.A. National Research Council's guide for care and use of laboratory animals (National Research Council, 1996). The experimental procedures were also approved by the Ethics Committee in Animal Research of Federal University of São Carlos (protocol number 001/2008). Rats were randomly distributed into 4 experimental groups: G1, placebo control; G2, DisBa-01-treated; G3, animals treated with anti- $\alpha_v\beta_3$ antibodies (MAB1976, CHEMICON, USA); and G4, animals treated with anti-a_2 antibodies (MAB1233, B&D, USA).

Incisional hernia model:

The experimental IH model was made as previously described[1] with minor modifications as follows. Rats were anaesthetized and the abdomen was trichotomized and cleaned with alcohol. A ventral midline incision muscle and peritoneum was made from just below the level of the rib cage, extending approximately 15 mm distally. Skin was elevated and retracted to allow access to a site at the mid-lateral aspect of the caudal peritoneal wall. Using a template, a 3 X 0.5 cm piece of peritoneal wall was excised to leave the peritoneum intact.

Experimental procedures

Subsequently, animals of G1 group received placebo (PBS); G2 group were treated with topical application of 1.0 ml DisBa-01 PBS sterile solution (0.5mg/Kg), G3 treated with anti-

$\alpha_v\beta_3$ antibodies (7µg/ml) and G4 treated with anti-a_2 antibodies (10µg/ml). The cutaneus incision was sutured across the wound with catgut 3-0 placed about 1 cm apart. The animals were replaced to the cages and observed until completely recovered.

Tissue preparation and histology

After 14 days of postoperative animals were killed and fragments of tissues were removed from the abdominal muscle and lesion areas for histological analysis. Fragments were divided in two parts. The upper part of the samples were frozen in liquid nitrogen and stored at -80ºC for later activity assays. The remaining of tissue samples were fixed in buffered paraformaldehyde (4%), embedded in paraffin, sectioned (5 μm thick) and stained with hematoxilin-eosin (H&E), reticulin and Masson´s trichome. Three non-consecutive digital images per animal were acquired with a SV Micro Sound Vision camera and an Olympus BX51 epifluorescence microscope and used for cell counting in a test frame area of $0.0625 mm^2$ equipped with a SPOT RT slider chilled charge-coupled device digital camera (Diagnostic Instruments, Sterling Heights, MI). Semi-quantitative analysis included quantification of inflammatory cells, neo-vascularisation and cellular density (non-inflammatory host cells, mainly fibroblasts). A minimum of seven fields per slide were counted at an objective magnification of 40x. Fields were randomly selected within the surrounding host tissue. For descriptive purposes, a semi-quantitative histological scoring criterion was generated.[25]

Collagen determination: Tissue samples were fixed in buffered paraformaldehyde (4%), embedded in paraffin, sectioned (5 μm thick) and stained with 0.025 toluidine-blue aqueous solution at pH 4.0. Three non-consecutive digital images (40x magnification) per animal were photographed with a Nikon Eclipse E-400 photomicroscope and used for collagen counting in a test frame area of $0.0625 mm^2$.

Protein extraction and analysis: Tissue samples were prepared for protein analysis by SDSPAGE and N-terminal sequencing. Samples were dissolved in extraction buffer (4M guanidine chloride, 50mM sodium acetate, 50mM EDTA and 1mM PMSF) for 24h at 4ºC. After, protein content was precipitated with 1M acetate-ethanol buffer, pH7.4, centrifuged at 8,000 x g and the pellet was dried before being dissolved in sample buffer. Samples were resolved in a 10% polyacrylamide gel and stained with Coomassie Blue. Alternatively, after running, protein bands were transferred to a PVDF membrane, stained and cut for N-terminal sequencing in a PPSQ 23A Protein Sequencer (Shimadzu, Japan).

Gelatin zymography: The tissue samples were treated

as previously described for muscle extracts (27). Tissues were homogenized and incubated in 0.5 ml of extraction buffer (10Mm cacodylic acid pH 5.0; 0.15M NaCl; 1μM $ZnCl_2$, 20mM $CaCl_2$, 1.5mM NaN_3; 0.01% Triton X-100 [v/v]), at 4°C for 24 hours. After this period the solution was centrifuged (10 min., 13.000x g at 4°C), and the pH of the supernatant was adjusted to 7.4 with 0.5 M NaOH. Control and DisBa01 group's samples were concentrated in order to load 10 μg of protein, whereas the samples of anti- $α_vβ_3$ and anti-a_2 groups were concentrated in 2 ug of total protein. The samples were applied on a SDS-10% polyacrylamide gels prepared with 1mg/mL gelatin in the presence of sodium dodecyl sulfate under nonreducing conditions. After electrophoresis, the gels were washed twice for 20 min in a 2.5% of Triton X-100 solution to remove SDS and incubated in substrate buffer (50mM Tris-HCl pH 8.0; 5mM of $CaCl_2$ and 0.02% NaN_3) at 37ºC for 20 h. Gels were stained with Coomassie Brilliant Blue for 1.5h and destained with acetic acid:metanol:water (1:4:5; v:v:v). All samples were evaluated in triplicate, to guarantee the precision and linearity of the analysis and each sample was normalized for the total amount of protein included. Gelatinase activity was visualized as clear bands in the stained gel. The gels were photographed with a Canon G6 Power Shot 7.1 mega pixels camera (Virginia, USA). The averages of band intensity were measured using Gene Tools software (Syngene, Cambridge, UK). Data are expressed as concentration of MMP-2 (i.e. the totality of arbitrary unit for the MMP-2 intermediate and active forms) and MMP-2 active form.

Statistical analysis

Results were presented as mean ± 1SE. Kolmogorov – Smirnov and Levene's tests were used to analyze the normality and homogeneity of variance. Student's t test was performed to analyze differences between control and DisBa-01 groups. For all comparisons, statistical significance was considered at a 5% level ($p < 0.05$).

Results

After the experimental procedure, the progression of IH was followed by daily animal observation up to day 14, as shown in Fig. 1A. After this time period all control animals developed IH, which was also demonstrated by the persistency of the herniation ring (Fig. 1B). In contrast, in all DisBa-01-treated animals there was no evidence of IH. Instead, a thin membrane completely closed the hernia ring, which avoided the IH (Fig. 1C). The presence of adherences was observed in this group but not in the controls. Interestingly, animals that were treated with anti-$α_vβ_3$ antibodies presented similar results (Fig.1D-E). The hernia ring was almost closed by a fibrotic tissue and muscle retraction (Fig. 1F).
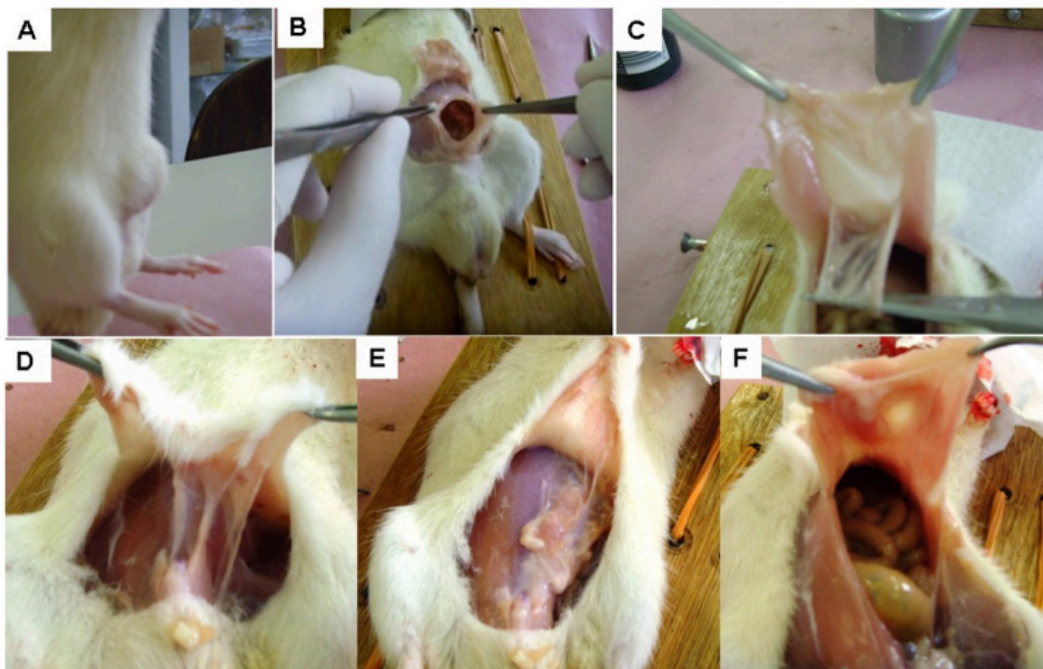

Figure 1 - Prevention of incisional hernia by DisBa-01 in the post-operative day 14. (A) Incisional hernia developed (100% control animals) after 14 days. (B) Presence of opened hernia ring in control group. (C) Presence of membrane occluding the hernia ring in DisBa-01 group. (D-F) It is observed in the anti-avb3 group: Adhesions between the abdominal subcutaneous tissue and muscle (D); Retraction and fibrosis in abdominal muscle (E); Partial occlusion of hernia ring.

Adherences were also observed. In contrast, in the animals treated with anti-α$_2$ antibodies the progression of IH was observed, as well as the presences of adherences (not shown).

Histological analysis showed that DisBa-01 at day 14 significanty increased the number of mononuclear cells (Fig. 2A), as well as the fibroblasts density (Fig. 2B), increased the number of new vessels in the injured area (Fig. 2D) but not the number of polimorfonuclear cells (Fig. 2C).

Morphometrical Masson´s trichome analysis of stained sections demonstrated that this disintegrin increased the content of collagen (Fig. 3A-C).

In parallel, DisBa-01 strongly activated MMP-2 activity as demonstrated by gelatin zymography gels (Fig. 4A-B). Activity bands corresponding to the pro-enzyme, intermediate and active enzyme were observed. Bands corresponding to the active form were quantified by densitometry (Fig. 4B). In contrast, only traces of activity, corresponding to the intermediate band, were found for the controls (Fig. 4A). Interestingly, animal treatment with anti-α$_v$β$_3$ and anti-α$_2$ antibodies also strongly activated MMP-2 (Fig. 4C-D).
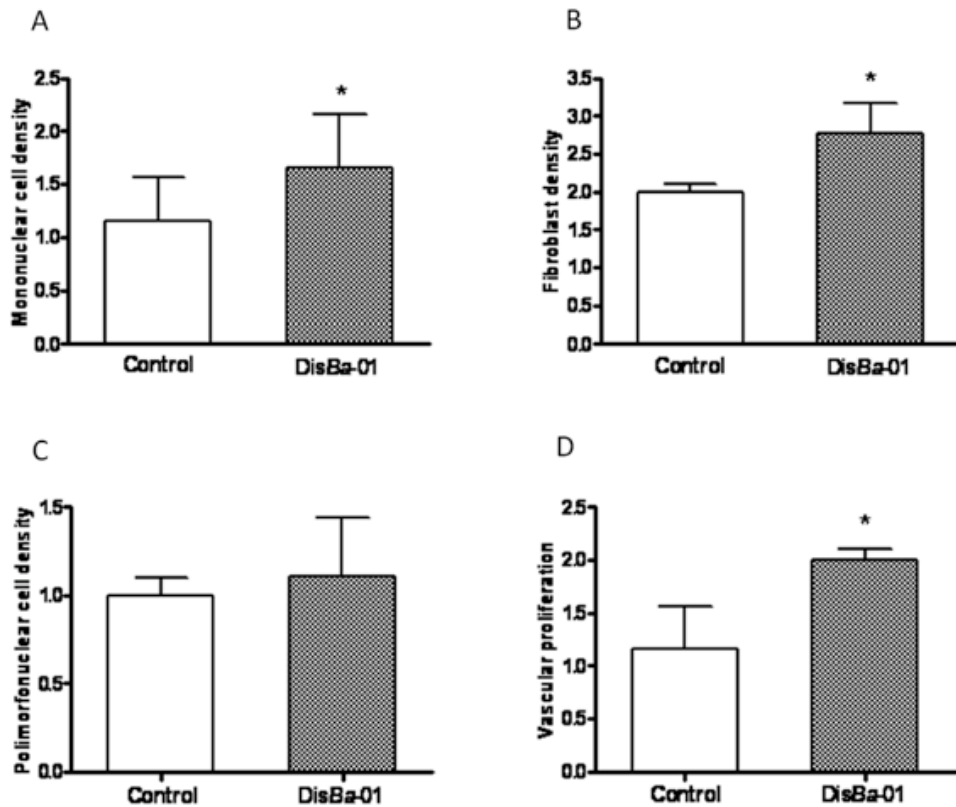

Figure 2 - Significant increase of mononuclear cells (A), fibroblasts (B), and vascular proliferation (D) induced by DisBa-01 after the postoperative day 14. (*p <0.05). Cells were counted in HE-stained slides of lesion tissue.
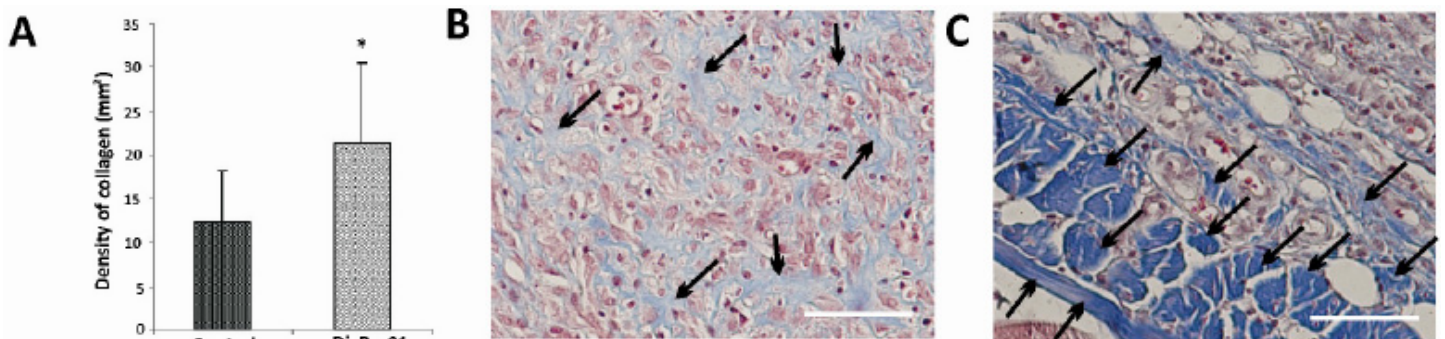

Figure 3 - The collagen density increased in G2 group treated with DisBa-01 (p< 0.05) compared with G1 group (control) (A); Photomicrographs of the tissue on the 14th day after induction of incisional hernia at G1 group (B) and G2 group treated with DisBa-01 (C) (Toluidine Blue, x 40 magnification). Arrows indicate collagen fibers formed in the regenerating 14 days after injury. UA: Arbitrary Unit.
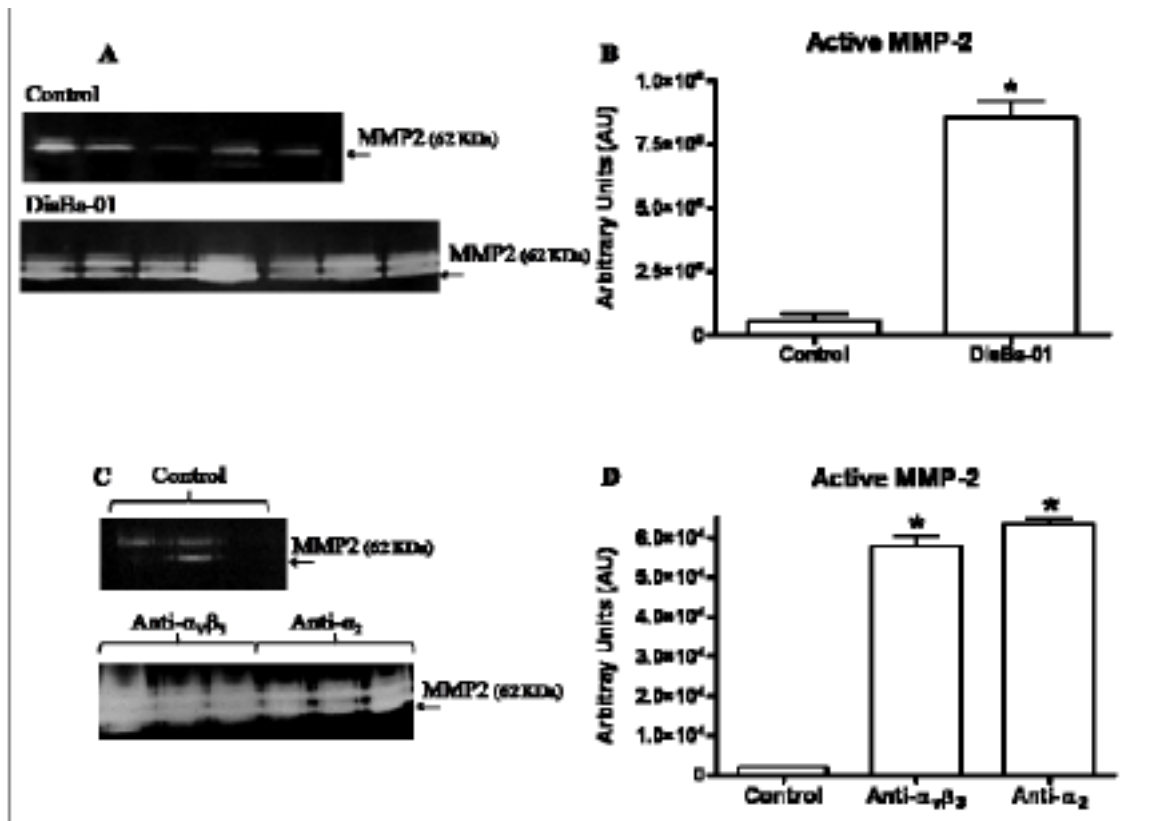Scale bar = 100x.

Figure 4 -

Discussion

The knowledge of the molecular mechanisms of herniation is important for diagnostic improvement, prognostic definition and hernia prevention. Connective tissue disorders involved in herniation are probably responsible for failed wound healing[2] The development of new techniques for IH prevention included the direct application of a growth factor. TGF-β_2 was either directly applied on the incision or immobilized in a mesh for delayed delivery[28] both with good results. TGF-β_2 stimulated macrophage and fibroblast chemotaxis as well as an increase in collagen production. bFGF immobilized in a polygalactone rod polymer was very effective in prevention of IH development.[1] On the other hand, TGF-b$_1$ did not increase the tensile strength. Searches for new strategies for IH prevention would help to avoid this common surgical complication.

The experimental model of IH used here was 100% efficient since all control animals developed herniation after the experimental period. Dubay et al.[1] reported an incidence of 80% of IH in a very similar model but the observation was made at day 28 which could explain the difference between our results. In addition to hernia ring closure, DisBa-01 stimulated macrophage and fibroblast migration, which probably secrete a set of growth factors that would improve the wound healing process. The latter cells are probably responsible for the synthesis of the membrane that closed the hernia. Despite the presence of collagen I in this membrane, as confirmed by SDS-PAGE and N-terminal sequencing, other components of lower molecular mass could be found suggesting that collagen was not the predominant component. These results are in agreement with the observation that tissue from DisBa-01-treated animals had lower levels of collagen on the trichrome-stained sections. However, the identity of the other membrane components remains to be determined in the future.

Apparently, DisBa-01 stimulated angiogenesis at the wound site, probably by the higher number of fibroblasts which would secrete angiogenic growth factors such as VEGF and FGF (29). Interestingly, we previously shown that DisBa-01 was shown to inhibit the angiogenic effect of βFGF in the matrigel model in nude mice.[24] This apparent controversy could be explained by the fact that the matrigel model is made in athymic mice which have impaired immunological response. In normal rats, DisBa-01 could induce migration of macrophages and fibroblasts that in turn would secrete the growth factors responsible for the angiogenic effect. Since fibroblasts are key cells in the wound healing process, the

chemotatic effect of DisBa-01 on these cells would significantly increase tissue repair.

Interestingly, it has been demonstrated that low concentrations of ADAMTS1, a metalloprotease with disintegrin and thrombospondin motifs, stimulate fibroblast and endothelial cell migration in healing skin wounds.[30] However, in higher concentrations this effect is inhibited due to FGF-2 binding and excessive proteolysis. It is suggested that this protein would have a role in the regulation of bioavailability of growth factors and their diffusion into the granulation tissue. However, the role of the adhesive domains in these activities has not been addressed yet.

One of the most striking observations in the present study was the strong activation of MMP-2 in DisBa-01 treated animals thus suggesting accelerated tissue remodeling. Under normal physiological conditions, MMP activity is precisely controlled at the levels of transcription, activation of precursor zymogens, binding to ECM components and inhibition by endogenous inhibitors such as the TIMPs and RECKS.[28] When activated, MMPs can degrade ECM components with the subsequent release of growth factors, including those involved in angiogenesis.[28]

These enzymes may also participate in the shedding of cell receptors therefore regulating cell activity. MMP-2 may be also activated by collagen I and fibronectin. Interestingly, fibroblasts from patients with recurrent IH presented lower levels of MMP-2 activity in the presence of mesh biomaterials.[31]

In addition, a close association between MMP-2 and $\alpha_v\beta_3$ integrin has been suggested. Both MMP-2 and MMP-9 localize to the membrane by binding to avb3 integrin and CD44, respectively.[32] Also, the $\alpha_v\beta_3$ integrin selectively suppressed the collagen I-induced MMP-2 activation in vitro and in vivo[33] MMP-2 activation by fibrillar collagen was reduced in b_3- overexpressing cell lines and in human breast cancer cells.[33] However, the molecular mechanism of this inhibition is not completely understood yet.

The current study provides additional evidence for the inhibitory effect of $\alpha_v\beta_3$ integrin on MMP-2 activation by collagen I. DisBa-01 antagonizes $\alpha_v\beta_3$ integrin, which could release pro-MMP-2 to be activated by collagen I. This observation may explain the strong MMP-2 activity seen in the zymography gels. This high level of MMP-2 activity could in turn explain the higher collagen content in the tissue from DisBa-01-treated animals, which indicates an accelerated tissue remodeling.

This hypothesis was also confirmed by experiments with anti- $\alpha_v\beta_3$ antibodies, which produced similar results, although significant differences were also observed. The hernia ring was almost closed but there was no formation of membrane; instead, muscle retraction occluded the area. Conversely, antibodies to the α_2 integrin produced results that were similar to the controls, with evident progression of IH. These results strengthen our data on the DisBa-01 specific effect on $\alpha_v\beta_3$ integrin. In addition, MMP-2 was also strongly activated in the animals from both antibodies-treated animals. These results are in agreement with previous demonstration that $\alpha_v\beta_3$-integrin binding by blocking antibodies increased secretion of MMP-2.[34]

Collectively, our results demonstrate, for the first time, that $\alpha_v\beta_3$ integrin blocking by an RGD disintegrin may be very helpful in prevention of IH, by attracting macrophages and fibroblasts, increasing MMP-2 activity and tissue remodeling.

The data presented in this manuscript expand the current understanding of the Disba-01 effects on surgical wound repair following IH. However, future studies addressing the contribution of this blocking $\alpha_v\beta_3$ integrin in different phases of wound healing deserves to be analyzed. For this reason, this investigation is also limited by nature of the 14-day survival time course studied. In addition, dose-response deserves to be investigated in future trials. Finally, DisBa-01 application may be extended in the future for other clinical situations in tissues with relatively poor blood supply such as fractured bone healing and tendon repair.

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