Effect of topical application of fibronectin in duodenal wound healing in rats[1]

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ABSTRACT - Fibronectin (FN), a large family of plasma and extracellular matrix glycoproteins, plays an important role in wound healing. **Purpose:** To evaluate the effect of fibronectin on the healing of sutured duodenal wounds, correlating with the serum and tissue level of the substance. **Methods:** An experimental study was done in 30 adult Wistar rats divided into two group. In the control group (n=15) a duodenal suture was treated with saline solution 0,9% and in the test group the duodenal wounds were treated with 1% FN. The duodenal wound healing process was studied in the 5th, 7tn and 10th postoperative days, by histological sections stained by hematoxylin-eosin, Masson trichromic and immunohistochemical reaction for FN. A digital histological grading system was used to obtain a score for each group and to observe the healing process. **Results:** the FN was present in the several layers of the duodenum and the cellular and plasmatic FN increased with the evolution of healing. In the test group the FN enhanced the wound healing within 5, 7 and 10 days after injury, when compared with the control group. **Conclusion**: The topical use of FN in duodenal sutured wounds in rats enhances healing by stimulating the appearence of fibroblasts into the wound site and development of granulation tissue. This acceleration of the repair process may have an important application in the healing of duodenal wounds.

KEY WORDS - Fibronectin. Duodenum. Wound healing. Imunohistochemistry.

Introduction

Fibronectins (FN), an important group of high molecular weight glycoproteins, are present, in an insoluble form, in extracellular matrix and basal membranes and, in the soluble form, in plasma and other fluids[1,2,3]. They are dimers of two similar peptides bound by disulphide bridges at the carboxy-terminal site and each subunit of this protein possesses high-affinity binding sites with cell-surface receptors through tetrapeptide Arg-Gly-Asp-Ser (RGDS), and also with a series of molecules, such as collagen, heparin, fibrin, actin, heparan sulfate, hyaluronic acid, DNA, gangliosides and tropomyosin[4,5,6]. This features allows their participation in many cellular functions, such as, maintenance of cell morphology, cellular adhesion, opsonization, phagocytosis and wound healing[8,9].

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The living organism when suffering a cellular aggression responds immediately with a complex reaction from the vascularized connective tissue, causing an inflammatory process whose biochemical mechanisms produce migration, proliferation and cellular differentiation by an interaction between the cells and the extracellular matrix components, specially hyaluronic acid and FN, allowing the healing of the damaged tissue[7,10]. FN acts in wound healing making easier the migration of macrophages, monocytes and immune cells to the injured area and allowing platelet adhesion to the injured cells of the veins[9].

Studies carried out about wound healing after topical application of FN in skin wounds in rats[7], in the corneal epithelial wound in rabbits[11] and periodontal surgical wound in patients[12] suggest a beneficial FN action in the healing process. The purpose of this experimental study was to evaluate the duodenal wound healing after the topical application of FN in the suture of duodenum injuries and to observe the presence of this protein in the whole duodenal wall, in the duodenal injured areas and in the plasma of Wistar rats.

Methods

Thirty adult Wistar rats "Rattus norgegicus albinus", weighing 280±23g, were used in this study. They were divided into two groups - control and test, with 15 rats each, subdivided into 3 subgroups of 5 animals.

Surgical procedures - The rats were anesthetised with sodium pentobarbital 20 mg/kg intraperitoneal. After a 5cm medium laparotomy, a 1.5cm longitudinal lesion was done in the duodenum, and sutured with polypropylene 6-0. It was injected in the sutured wound, one milliliter of a 0,9 % saline solution in the control group and twenty microliters of 1% fibronectin in the test group. After two minutes, in order to assure complete distribution of FN in the sutured duodenum, the abdomen was closed with nylon 4-0.

The rats were observed after surgery in individual cages with water and food *ad libitum*, for 5 days (subgroups C_1 and T_1), 7 days (subgroups C_2 and T_2) and 10 days (subgroups C_3 and T_3). After that time, they were killed with an anesthetic overdose. A five-mililiter of blood sample with EDTA and two fragments of the duodenum, were collected from each animal - one frgment from the sutured area and another from the intact region distant five centimeters from the lesion.

Procedures with the biopsy fragments - The fragments were maintained for 8 hours in an ethanol fixative solution, and submitted to a routine histological process, using the automatic tissue processor (OMA CM69), being this material embedded in paraffin. The three-mM wax-embedded duodenum sections were stained with hematoxylin-eosin (HE), and Masson trichromic in order to examine the histological aspect of the whole duodenal wall and the wound healing process.

Immunolabelling - Identification of cellular FN through immunohistochemical reaction: the three-mM sections settled on silane (Sigmaâ) pre-coated slides were dewaxed and rehydrated. Endogenous peroxidase activity was inhibited by incubation in two sequential baths of 15 min each with hydrogen peroxide. Slides were placed in PBS 7,4 and sequentially incubated in a moist chamber in a refrigerator with the anti-fibronectin primary antibody "Rabbit Anti-Human Fibronectin" (Dakoâ) diluted 1:1000, overnight. A secondary antibody biotinylated "Biotinylated Swine Anti-rabbit Immunoglobulins" (Dakoâ) diluted 1:500 was added and incubated for 30 min in a moist chamber at room temperature.

After the wash with PBS, a Streptavidin Biotinylated Horseradish Peroxidase Complex (Dako) diluted 1:100 was applied to the sections for 30 min. The reaction of the enzyme activity was developed through the chromogenic substrate with diaminebenzidine and hydrogen peroxide. All sections were counterstained for 30 sec with Harris's hematoxylin, dehydrated and mounted in balsam. This immunohistochemical reaction was classified by grades (1-4) after microscopic examination according to the intensity of FN on the layers of duodenum structures (villosities, crypts, *muscularis mucosae and muscular propria*), especially in the injured areas.

Identification of plasmatic fibronectin through the Western Blotting method - In order to study the plasmatic fibronectin, an electrophoresis in polyacrylamide gel (SDS-PAGE) was done[13]. Samples were reduced with 2% (v/v) 2-mercaptoethanol and an apparent molecular mass was stimated using the following proteins as standard: bovine serum albumin (66 kDa), ovalbumin (45kDa), carbonic anhydrase (30 kDa), lysozyme (14,4 kDa) and fibronectin 1%. Gels were either directly stained with Coomassie blue or subjected to the electrotransfer of the fragments to nitrocellulose sheets.

After the transfer of proteins, the nitrocellulose sheets were incubated with the anti-fibronectin primary antibody "Rabbit Anti-Human Fibronectin" (Dakoâ) diluted 1:1000 and a blocking buffer, overnight. Next, the nitrocellulose sheets were incubated with a secondary antibody biotinylated "Biotinylated Swine Anti-rabbit Immunoglobulins" (Dakoâ) diluted 1:500 for 6 h and washed, then, with distilled water. A "Streptavidin Biotinylated Horseradish Peroxidase" complex diluted 1:500 was added to them and incubated for 30 min and the enzyme activity was developed through the chromogenic substrate with diaminebenzidine and hydrogen peroxide.

Microscopic examination - The microscopic examination was performed with a magnification of 200X. The quantitative analysis was done using a digital system. The microscopic slides were examined by an *Olimpus* optical microscope. The images were captured by a Sony camera and digitalized in the *Software Image Pro-plus* 3.0 (*Media Cybernetics - LP, USA*). The images were measured in pixels and processed in a multimedia system. The colagen fibers, inflammatory cells, fibroblasts and necrosis areas were quantitated using density units. In this study, the variables necrosis, abscess, acute and chronic inflammation, mucosal regeneration, granulation tissue and collagen were evaluated.

Statistical analysis - The data were analyzed by the Tukey's method and the Student t-test, considering the differences statistically significant when $p<0,05$.

Results

All the animals survived without complications. In the control subgroup C^1 the histopathologic exams revealed the scores 24789.8±3867.6 and in test subgroup T^1, observed by five days, the score was 39111.8±7716.8. The difference was significant, as shown in table 1.

TABLE 1 - Distribution of histopathologic density of inflammatory elements in the subgroups observed for five days.

Animals Nº	Subgroup C^1	Subgroup T^1
1	20714	28217
2	30342	46908
3	22549	46107
4	23290	38211
5	27054	36116
Mean±sd	24789.8±3867.6	39111.8±7716.8

The difference is significant (p=0,007).

The mean score in the rats with seven days of observation was 27166.4±4163..2 in the C^2 and 42259.0±5621.5 in T^2, as observed in table 2. In the subgroups observed for 10 days the scores were 37417.6±5056.0 to C^3 and 50942.2±7160.8 to T^3 (Table 3). The differences were significant when compared C^1 -T^1, C^2-T^2, e C^3 - T^3 ($p<0,05$).

TABLE 2 - Histopathologic mean density on the subgroups C^2 and T^2, observed during seven days.

Animals Nº	Subgroup C^2	Subgroup T^2
1	32978	37976
2	24071	36590
3	26112	40308
4	22909	47411
5	29762	49010
Mean±sd	27166.4±4163.2	42259±5621.5

The difference is significant (p=0.01).

TABLE 3 - Histopathologic mean density of inflammatory elements in the subgroups observed for ten days.

Animals Nº	Subgroup C^3	Subgroup T^3
1	46022	44914
2	36810	45840
3	36590	62010
4	32977	54155
5	34689	47792
Mean±sd	37417.6±5056.03	50942.2±7160.8

The difference is significant (p=0,04).

When considered the scores refered to the fibronectin expression in the duodenal sutured tissue, the total score in the control group was 175, corresponding to the mean 11,6±3,09. In the test group the total score was 215, and the mean 14,3±1,76. The difference was significant ($p<0,05$), meaning that the fibronectin stayed in the healing tissue was observed in a higher level in the test group then in the control one. So, the data permited to infer that there was a linear association between the level of fibronectin and the histopathologic scores in the healing tissues. The *Western blotting* method showed that the plasmatic fibronectin expression increased with the days of healing.

Discussion

There have been reports indicating that fibronectin is associated with different stages of wound healing and that the topical application of a fibronectin solution has increased the rate of healing in rabbits' corneal injury models[11], in rats' skin wounds[7] and in the treatment of periodontal surgery in patients with severe periodontitis[12]. Its functions in blood coagulation[15,16], in opsonization[9,14], in the fibroblasts, monocytes and macrophages chemotaxis[6] and during the synthesis, maturation and stability of the collagen[17], justify the speculation of these protein effects as a topical agent on the wound healing.

Cheng et al[7] using three procedures of topical application of FN in skin injuries on rats have shown that the acceleration of the healing process was the same after a daily application for two days, two daily applications for twelve days and with only one application immediately after the injury. In this experimental study on Wistar rats, we did only one application of FN. The duodenum has been chosen as the target of the injury not only for the peculiar characteristics that this organ presents from the anatomical and physiological viewpoint and surgical facilities[18,19] but also considering the fact that we have not found in literature studies about topical application of FN in duodenal injuries.

In the present study we observed the presence of FN on the whole duodenum wall, especially in the villosities, crypts, *muscularis mucosae,* and muscular itself and in the duodenal injury areas. Pearlstein et al[3] showed that the cells of the epithelial crypts in the small bowel of rats synthesized FN *in vitro* and found this protein in the basal membrane of the intestine, suggesting its synthesis *in vivo.*

To observe the duodenal wound healing after topical FN application, scores were obtained after the microscopic examination of the histopathological findings and of the FN distribution in the duodenal injury areas in the control and test groups. It was possible to see the increasing expression of this protein along the healing period, especially in the test group, showing a good absorption of the exogenous FN applied in the injury area. Longaker et al[20] after comparing fetal and adult's injury extracellular matrix, observed that, among the extracellular matrix components, FN was deposited later in the adult's injury as compared to fetal injury.

Our data showed that the average effect of the treatments in the sutured areas (saline solution 0,9% and FN solution 1%) was not the same for the two groups. So, it was accepted the hypothesis that the duodenum wound healing after the use of the FN exhibited a better histological aspect and the difference was significant ($p<0,05$). These data coincide with the findings of Cheng et al[7] and Nishida et al[11], who studied the effects of this protein in the healing of cornea and skin injuries, respectively.

During the initial period of the healing, the most important cellular phenomena are monocytes and fibroblast migration in the injured tissue and the real debridment and cleaning process done by the macrophages through phagocytosis. At this phase, exogenous FN can be the source of extra chemotaxis, which results in an increase of fibroblast and monocytes population in the injured tissue at the same time, as the macrophages and the fibroblasts use the exogenous FN, that is incorporated in the fibrin in the injured tissue to accelerate its mobility[7,9].

Analysis by *Western blotting* showed that the plasmatic FN expression, like the cell surface FN, had increased with the days of healing. The plasmatic FN is very similar to the cellular FN in its chemical, biological and immunologic properties. However they differ in the carbohydrates and in the ability to agglutinate erythrocytes and restore the normal morphology of the transformed cells. Some studies observed that in rats there are differences between the cellular and the plasmatic FN structures regarding the number of amino acids in some segments[21,22].

Conclusion

1 - The fibronectin (FN) was expressed in the wall of normal and injuried duodenum of rats;

2 - During the duodenal wound healing, the FN expression (plasmatic or cellular surface) increased with the healing evolution;

3 - The topical use of exogenous FN in the duodenal sutured wounds favored and enhanced the healing process, when compared with controls.

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RESUMO – A fibronectina (FN), um componente da grande família das glicoproteínas do plasma e da matriz extracelular, desempenha um importante papel na cicatrização das feridas. **Objetivo:** Avaliar os efeitos da fibronectina na cicatrização de lesões duodenais suturadas, e estabelecer correlação dos parâmetros de cicatrização com os níveis tissulares e séricos da substância. **Métodos:** Foi realizado estudo experimental com 30 ratos Wistar adultos divididos em dois grupos. No grupo de controle (n=15) uma lesão duodenal suturada foi tratada com aplicação tópica de 1ml de solução 0,9% e no grupo teste (n=15) a lesão duodenal foi tratada com FN 1%. A cicatrização da lesão foi estudada após cinco, sete e dez dias de observação. O estudo histopatológico foi realizado nas colorações hematoxilina-eosina, tricrômico de Masson e reação imunohistoquímica para FN. Um sistema digital de graduação histológica foi usado para obtenção de escores para cada grupo. **Resultados:** A FN esteve expressada em todas as camadas estudadas do duodeno. Os níveis celular e plasmático da FN aumentaram linearmente com a evolução do processo cicatricial. No grupo teste a FN contribuiu para melhorar a cicatrização das lesões nos três intervalos do estudo, quando se fez a comparação com o grupo de controle. **Conclusão**: O uso tópico de FN em feridas duodenais suturadas de ratos melhora a cicatrização mediante o incremento no aparecimento de fibroblastos, colágeno e tecido de granulação. Esta aceleração na cicatrização pode significar um importante papel na consolidação dessas lesões.

DESCRITORES - Fibronectina. Duodeno. Cicatrização. Imunohistoquímica.

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