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FIBRONECTIN-DEPENDENT MECHANISMS INVOLVED IN FIBROPLASTIC PROCESS ACTIVATION IN AEROBIC PURULENT WOUND UNDER ARMENICUM PASTE TOPICAL TREATMENT

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ABSTRACT

Topical characteristics of fibronectin localization in wound soft tissues in the presence of topical application of Armenicum paste were studied on an experimentally induced model of an aerobic purulent wound.

Comprehensive research methods (morphological, morphometric and fluorescent-microscopy) made it possible to establish that Armenicum can have a highly favorable effect on the course of regional inflammatory process in soft tissues of a purulent wound. At relatively early stages of the wound (3rd and 5th day of observation), due to the application of Armenicum paste both in outer and deep wound areas "dense" deposits of fibronectin-positive material in extracellular matrix are observed, mostly in the locations of newly-formed collagenous fibrils and in perivascular areas of microvessels. It is very noteworthy that most of the inflammatory infiltrate cells (leukocytes and macrophages) and separate fibroblasts contained fibronectin-positive material. The latter fact furnishes support for direct and/or mediated effect of the drug Armenicum on targeted stimulation processes in situ by the phagocytic type cells of fibronectin.

The obtained results of immunomorphological analysis were correlated with those of morphological studies. Thus, signs of noticeable phagocytic type cells activation and "dense" fibronectin deposits in interterritorial matrix were most prominent in those areas of the septic wound where reparative-proliferative processes were very clearly marked.

KEYWORDS: *aerobic wound, reparatory-proliferative process, fibronectin, Armenicum drug*

INTRODUCTION

The problem of wound infection has been highly urgent in modern medicine. The republic is located in a seismically dangerous zone. Compression and burn wounds occupy a special place among complications caused by earthquakes [*Porfiryev BN, 1989; Mikhailov A et al., 1991; Mukha A, Stepanov V, 1989; Aoiki N et al., 2007; Gevorkian AG et al., 2011; Menzel CL et al., 2011*].

In addition, especially frequent have been the cases of aerobic, anaerobic and mixed wounds with various infection genesis, which are very difficult to treat due to microbial "metamorphosis" and microorganisms' tolerance to medication, especially antibiotics. To this end, clinical testing of new therapeutic anti-inflammatory and immunomodulatory drugs justifies that scientific-methodological approach which should be employed by clinicians in their everyday practice.

Over the last 30 years, issues associated with identifying the role of fibronectin in the activation of fibroplastic processes, as well as while studying pathogenesis aspects of an inflammatory wound process have been effectively worked out. We favor dwelling on some biological effects of fibronectin brought about strictly in the connective tissue [*Clark RA et al., 1983; Baluda VN, 1984; Litvinov RI, 1984; Kuznik BI et al., 1989; Mkrtchyan LN, Zilfyan AV, 2011; Zilfyan AV et al., 2012*], i.e. its involvement in the formation and differentiation of the con-

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nective tissue. Thus, fibronectin produced in situ, i.e. in the connective tissue (macrophages, leukocytes, and fibroblastic cells act as source of synthesis) is involved in production of extracellular matrix building blocks: glucosamine glycosaminoglycans (hyaluronic and chondroitin acids), collagen, laminin etc. That is why, fibronectin does double service at the early stages of development of extracellular connective tissue structures: as a stimulant for synthesis of necessary connective tissue components and also as an area for fibroblasts' attachment to extracellular substrates fibrinogen and fibronectin itself and/or its precursors.

On the other hand, the same fibronectin is involved in the inhibition of reactions responsible for excessive collagen formation, especially due to connective tissue pathology that accounts for impaired balanced synthesis of fibrous structures. Thus, fibronectin acts as one of the universal factors depending on a situation facilitating balanced synthesis of non-cellular structures of the connective tissue.

The objective of this study was to investigate the role of fibronectin in reparatory proliferative processes in a purulent wound under the treatment with topical Armenicum (ARM) paste.

MATERIALS AND METHODS

The trials involved 120 albino rats weighing 130-150 gram. The animals were divided into two groups: control and test. The model of a purulent wound, proposed by Hovhannisyan SS et al (1987), was induced in the animals of both groups.

The test group's wound surface was treated with ARM paste, diluted in 3 *ml* saline on the basis of 150 *mg* per 100 *g* of animal's weight. The drug was applied three times (1 *ml* per application) in 4 hour intervals during the first day after reproduction of purulent wound model. The animals of both groups were removed from the trial on the 3rd, 5th and 9th day of the regional inflammatory process. The trial protocol was approved by the YSMU research ethics committee.

Upon fixing the specimens in Carnoy's solution and their processing in increasing ethanol concentrations, wound tissue samples were taken and embedded in paraffin. Paraffin sections were stained using common morphological methods: hematoxilin-eosin, toluidine blue, and Van Gieson's stain. The specimens were examined using a Micros three-lens light microscope (Austria).

Five to seven μm thick frozen sections were prepared from newly-frozen wound tissue samples and exposed to immunofluorescence analysis to identify fibronectin. The indirect Coombs test was performed using rabbit serum at the first stage of specimens processing against fibronectin "Sigma" (USA), which, according to the company's recommendations, selectively reacts not only with human, but also rat, mouse, goat, pig, chicken and buffalo tissues. Working serum dilution of 1:50 in 0.01 *M* phosphate buffered saline 7.4 *pH* was used.

The second stage of the technique involved the use of FITC-labeled serum against rabbit IgG (Sigma) in 1:20 working serum solution in 0.01 *M* phosphate buffered saline 7.4 *pH*.

Necessary (negative) controls allowing to rule out nonspecific feature of fluorescence were set. To this end, at the second stage of the indirect Coombs test, instead of FITC-labeled serum against rabbit IgG, the specimens were processed with respective FITC serums against human, rat and mouse IgG (Sigma). In order to rule out autofluorescence, the specimens from the wound were examined microscopically either without processing them with both serums or after processing them with only rabbit serum against fibronectin.

All the stages of specimen processing were carried out in stringent observance of the enzyme-linked immunosorbent assay protocol presented in the manual of "Sorin Company", USA.

Planimetric study was performed using histiostereometric net proposed by G.G. Avtandilov. Five cryostat sections were obtained from each wound tissue at various levels of the test material. As indicated above, the indirect immunofluorescence test was run to detect fibronectin in soft tissues of the wound. Fields of specific fluorescence in four minor squares accidentally coinciding with 25 points in each square were counted in each test specimen. After specific fluorescence sites in four squares of the specimen were counted, a mean value that was later included in the variation series, was calculated. In each subgroup, wound soft tissues from 16 animals were subjected to quantitative immunofluorescence analysis. The specimens were examined with "Boeco", Germany trinocular fluorescent microscope with histiostereometric net, installed in the

eyepiece. Statistical analysis was based on Student's test using SSAT software package.

RESULTS AND DISCUSSION

Immunomorphological analysis results revealed that on the 3rd day of the trial in the control group animals only solitary sites of the purulent wound soft tissues contained fibronectin-positive material. Thus, it was possible to detect specific fluorescence only in outer parts of the purulent wound in the dystrophic and decayed linearly-oriented collagen fibers. It was also possible to observe the presence of fibronectin in the cytoplasm of solitary leukocytes and macrophages (Fig. 1a,b). The sites of specific fibronectin fluorescence were observed much more often in the animals of the control group on the 5th day of the trial. The presence of fibronectin-positive material was found both in outer and deep parts of the connective tissue segment of the wound. In the latter case, the presence of fibronectin via specific homogenous and fine-granular fluorescence was detected as well on the surface of solitary linearly-oriented collagen fasciculi.

The condition of the control group animals' purulent wound on the 8th day of the trial was the same as on the 5th day.

As early as the 3rd day of regional inflammatory process the animals of the test group developed extensive nidi of fibronectin deposits in soft tissues of the purulent wound. Fibronectin was detected both in outer and deeper parts of the purulent wound. Thus, specific fluorescence was detected along mul-

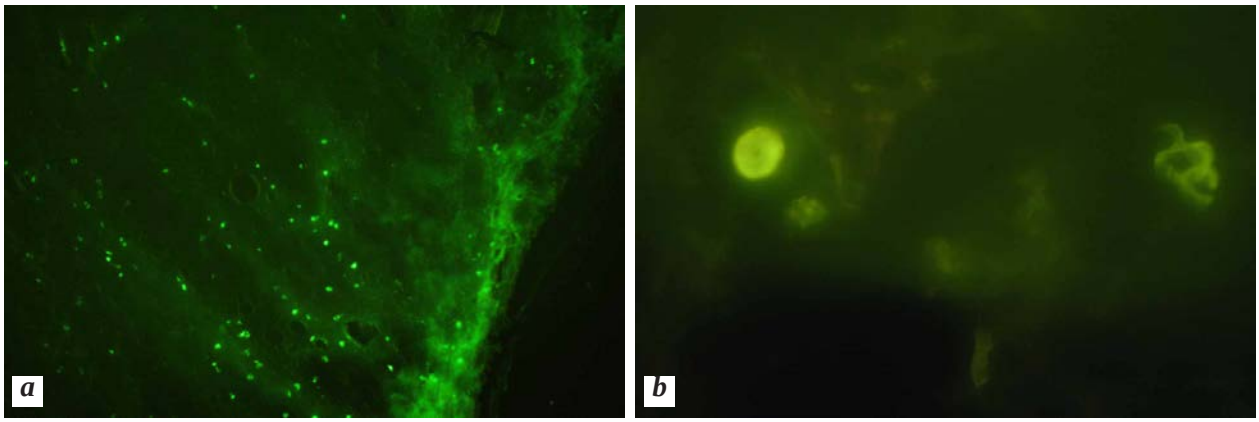

FIGURE 1. Presence of fibronectin in the purulent wound of control group animals on the 3rd day of observation Fluorescent microscopy.
***a)** Specific fluorescence in inflammatory infiltrate cells in the outer parts of the wound. Ob. 10, oc. 10.*
***b)** Specimen specification a) Presence of fibronectin-positive material in the cytoplasm of solitary leukocytes. Ob. 40, oc. 10.*

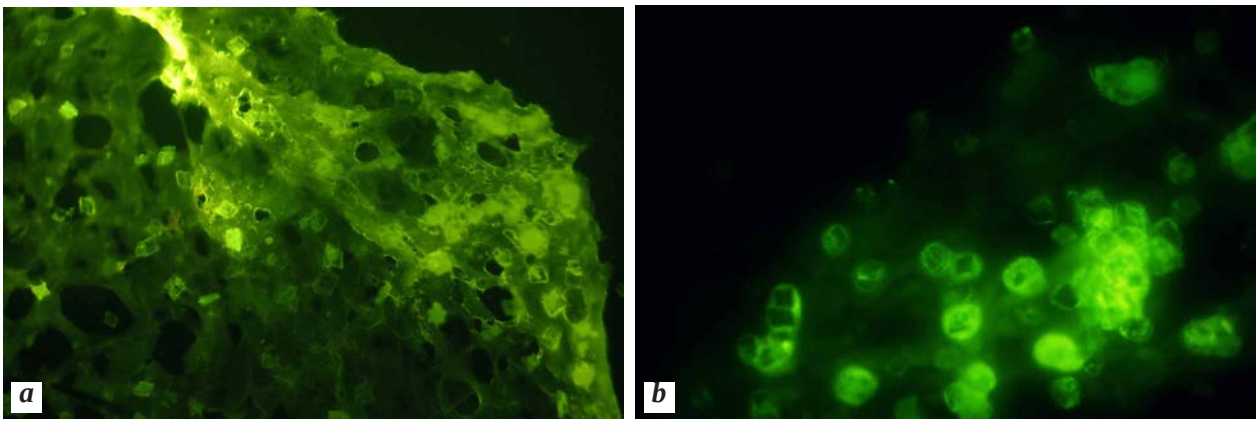

FIGURE 2. Presence of fibronectin in the purulent wound of test group animals on the 3rd day of observation Fluorescent microscopy.
***a)** Presence of fibronectin-positive material in multiple inflammatory infiltrate cells. Specific fluorescence along structurally intact fibrous structures. Ob. 10, oc. 10.*
***b)** Specimen specification a) Fine-granular and/or homogenous fluorescence in the cytoplasm of macrophages and leukocytes. Ob. 40, oc. 10.*

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tiple structurally intact collagen fibers, and also in the dystrophic and decayed sites of cellular and non-cellular components of the connective tissue (Fig. 2a). Especially noteworthy is the fact that specific fluorescence was observed in the inflammatory infiltrate cells – mostly in macrophages and leukocytes. Fibronectin-positive material in their cytoplasm was detected as fine-granular and/or homogenous specific fluorescence (Fig. 2b).

On the 5th day of the trial, the test group animals still had extensive nidi of fibronectin-positive material deposits in the wound soft tissues in the wound's connective tissue segment. Unlike the previous observation period of the test group animals, on the 5th day of the trial, nidi of fibronectin-positive material deposits were located not only in the dystrophic and decayed sites of connective tissue structures, but also along and on the surface of newly-formed collagen fibrils, distinguished by compact mutual orientation and the ability to congregate in fasciculi. At the same time, the amount of macrophage and leukocyte cells still containing fibronectin-positive material decreased considerably.

On the 8th day of the trial, only solitary nidi of specific fibronectin fluorescence, located mostly along solitary newly-formed collagen fibers, were detected in soft tissues of the wound of the test group animals. It should be noted that in the given observation period the highly low content of fibronectin-positive material in the test group animals was determined in the presence of final differentiation of granulation tissue into porous unformed one, i.e. when the test group animals recovered from the wound defect by way of secondary intention (substitution).

TABLE

Areal values of specific fibronectin fluorescence in aerobic wound soft tissue in the presence of topical application of Armenicum paste

All groups (n=20)	Fibronectin values at different stages of regional inflammatory process		
	Day 3	Day 5	Day 8
Control	10.07±0.61	17.36±0.62	40.5±1.72 P_2<0.0005
Test	16.26±0.89 P_1<0.0005	52.2±1.83 P_1<0.0005	25.96±1.21 P_1<0.0005 P_3<0.0005

NOTES:

P_1 – in relation of the values of test groups to corresponding values of control groups;

P_2 – in relation of control group value on the 8th day of trial to corresponding control group value on the 5th day;

P_3 – in relation of test group value on the 8th day to corresponding test group value on the 5th day.

The results of fibronectin-targeted quantitative fluorescent microscopic analysis of soft tissues from purulent wound are presented in the table.

As is presented in the table, the control group animals' highest values of fibronectin in purulent wound soft tissues were determined at relatively late stages of the regional inflammatory process, i.e. on the 8th day of the trial. Thus, given value exceeded those of the control group animals on the 3rd and 5th days of observation by 4.0 and 2.3 times respectively.

The test group animals under the treatment with Armenicum paste exhibited a somewhat different picture. The highest fibronectin values were determined at a relatively early stage of the regional inflammatory process, i.e. on the 5th day. Thus, the value of the test group animals exceeded those on the 3rd and 8th days of observation by 3.2 and 2 times respectively.

The results of immunofluorescent analysis testify in favor of the fact that topical application of Armenicum paste induces processes responsible for fibronectin synthesis in situ much earlier than in the control group animals. Exactly in this observation period (on the 5th day of trial) the highest fibronectin values were detected. All of the abovementioned suggests that application of Armenicum paste at the early stages of a regional inflammatory process results leads to "dense" fibronectin deposits in the extracellular matrix, which, to a certain degree, determines the intensified process of collagen formation. That is exactly why one of the mechanisms of relatively early maturation of granulation tissue into porous unformed connective tissue, in our view, is fibronectin localized in the interterritorial matrix. The present immunomorphological analysis also demonstrated that a possible source of intensified fibronectin synthesis in test group animals was fibroblastic and phagocytic cells (macrophages and leukocytes) localized *in situ*, whose levels in the observation period rose considerably. Marked increase in fibronectin content is also observed in the mentioned cells. It is subsequently deposited on the surface of newly-formed fibrous structures, thus inducing early activation of fibroblastic processes in a purulent wound.

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