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Serum collected from rats with myocardial infarction increases extracellular matrix accumulation by myofibroblasts isolated from myocardial infarction scar

Lucyna Piera[1], Jacek Szymański[2], Tomasz Kun[3], Anna Krzymińska[1], Marlena Juszczak[4], Jacek Drobnik[1*]

Abstract

The effect on extracellular matrix content is believed to be an average of several serum derived compounds acting in opposition. The aim of the study is to determine whether whole serum of rats with myocardial infarction may modify the accumulation of extracellular matrix in cultures of myofibroblasts isolated from the myocardial infarction scar. A second aim is to determine whether the tested serum can also degranulate the mast cells. Serum was collected from rats with sham myocardial infarction, rats with myocardial infarction induced by coronary artery ligation and control animals. The experiments were carried out on myocardial infarction scar myofibroblasts or mast cells from the peritoneal cavity. The cultures were divided into three groups containing eight cultures each: one treated with serum from control rats, from animals after sham operation or from those after myocardial infarction. In all groups, the serum was used at concentrations of 10%, 20% or 30%. The total collagen content (Woesner method) glycosaminoglycan level (Farandale method), cell proliferation (BrdU), histamine secretion from mast cells (spectrofluorymetry), β1 integrin and α-smooth muscle actin expression (flow cytometry) were evaluated. Isolated cells were α-smooth muscle actin positive and identified as myofibroblasts. Serum derived from rats with myocardial infarction increased collagen and glycosaminoglycan content in the cultures and modified myofibroblast proliferation in a concentration-dependent manner. The serum also results in an imbalance between collagen and glycosaminoglycan levels. The content of β1 integrin was not influenced by myocardial infarction serum. The serum of rats with myocardial infarction is involved in regulation of collagen and glycosaminoglycan content in myofibroblast cultures, as well as the modification of their proliferation. These changes were not accompanied with integrin β1 density variations. The serum of the myocardial infarction rats did not influence the mast cell degranulation.

Keywords: Collagen; Glycosaminoglycans; Healing; Integrins; Myofibroblast

Introduction

The nature of the extracellular matrix determines the mechanical properties of the intact heart and the myocardial infarction (MI) scar (1). Some complications of myocardial infarction such as aneurysm formation, rupture or expansion of myocardial infarction are caused by a weakening of the mechanical strength of the scar. This effect is mainly related to impairment of the quantity and quality of the extracellular matrix (2). The molecules of extracellular matrix are bound by integrins (3). These adhesive molecules within the cell membrane connect the extracellular matrix with the cytoskeleton (4). Integrin signaling influences the shape of cells and regulates cell migration, proliferation (5) and cytokine secretion (6).

The healing of myocardial infarction consists of several interdependent processes working in accord (7). Repair is governed by both local (mediators of inflammation, growth factors) and systemic regulatory factors (immune and neuro-humoral stimuli). Myofibroblasts are responsible for extracellular matrix synthesis, and mast cells are known to be involved in the regulation of extracellular matrix deposition. The mast cells participate in the control of the healing process of superficial wounds, as well as collagen deposition within the granulation tissue of a wound (8). Histamine, the mast cell mediator, increases the metabolic activity of wound fibroblasts and augments the secretion of transforming

[1] Department of Pathophysiology, Medical University of Lodz, ul. Żeligowskiego 7/9, 90-752, Lodz, Poland
[2] Central Scientific Laboratory, Medical University of Lodz, ul. Mazowiecka 6/8, 92-215, Lodz, Poland
[3] Department of Behavioral Pathophysiology, Medical University of Lodz, ul. Żeligowskiego 7/9, 90-752, Lodz, Poland
[4] Department of Pathophysiology and Experimental Neuroendocrinology, Medical University of Lodz, ul. Żeligowskiego 7/9, 90-752, Lodz, Poland

Corresponding author: Dr Jacek Drobnik, Department of Pathophysiology, Medical University of Lodz, ul. Żeligowskiego 7/9, Łodz, Łodz Voivodeship 90-752, Poland
E-mail: drob@op.pl

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growth factor β1, a potent profibrotic compound (9). Frangogiannis et al., (10) report the accumulation of mast cells within the myocardial infarction scar, especially in the area of collagen deposition. This observation supports the hypothesis that mast cells mediate the regulation of collagen accumulation within the myocardial infarction scar.

Compounds derived from serum of myocardial infarction affected subjects have been found to have an contradictory effect on extracellular matrix accumulation (11-17). The aim of the present study is to determine whether whole serum of rats with myocardial infarction, acting directly on myofibroblasts, may regulate the accumulation of collagen or glycosaminoglycans (GAG) and have an influence on β1 integrin count. The β1 integrin subunit is the common part of integrins binding to collagen (α1β1, α2β1, α11β1 and α12β1) and could be used as the marker showing the changes of the density of these receptors. This experiment allow to assess whether changes of collagen level are accompanied with variations of β1 integrin density. In addition, the study examines whether the serum may modify mast cell degranulation, thus having an indirect influence on extracellular matrix deposition within the myofibroblast culture.

Materials and Methods

Animals and experimental design

The experiments were approved by the Local Commission of Ethics in Lodz (permission number is 5/ŁB 533/2011). All animal experiments were conducted according to the humane guidelines specified in Polish animal protection legislation dated January 15, 2015.

Forty-four male *Wistar* rats weighing 290 g + 30 g were housed with free access to commercial food pellets (LSM, Bacutil, Poland), and tap water *ad libitum*. The rats were kept under a 12-hour light / dark cycle; at 7:00 AM, the light was turned on. Twenty-eight days after induction of myocardial infarction, the rats were euthanized. The moderate and large myocardial infarction scars were aseptically removed and used for cell culture preparation.

The animals were divided into three groups containing 12 rats each: intact control, rats with sham infarction and animals with myocardial infarction. Blood was collected from the heart under aseptic conditions and centrifuged (3000 rpm). Samples from different rats were mixed altogether and then serum was stored at -70°C.

Left coronary artery ligation

Occlusion of the left coronary artery was induced by ligation according to Seyle´s method with some modifications (18). The animals were anesthetized with pentobarbital (50 mg/kg b.w.) (Sandoz, Rotkreuz, Switzerland) and then ventilated with positive pressure through a tube inserted into the trachea and attached to a small-animal respirator (Kent Scientifific Corporation, Torrington, CT, USA). The left coronary artery was ligated with nylon suture 6/0 (Aesculap, Tuttlingen, Germany) after opening the pericardium. The ligation was performed in the middle of an imaginary line between the left margin of the pulmonary cone and the closest point of insertion of the left auricular appendage. On the 28th day of the experiment, the heart was removed, and the left ventricle, right ventricle, scar and septum were separated and weighed. The measurements of the surface area were performed planimetrically. Rats with moderate and large infarctions were used for the study. A ratio of the scar surface area to free wall area on the left ventricle higher than 30% was assumed as medium or large myocardial infarction.

Cell culture

The scars were removed aseptically and were incubated in a 0.1% collagenase solution (Sigma, St. Louis, MO, USA) (37°C, 5% CO_2) for three hours. Later, the tissues were centrifuged (1000 rpm, 5 min) and the cell pellets were washed and plated on Petri dishes (Nest Scientific, Rahway, NJ, USA). The adherent cells were cultured at 37°C, under a humidified atmosphere of 5% CO_2 and 95% air. The new cell culture was set up at an initial cell density of 8 x $10^3/cm^2$ in six well plates (TPP, Trasadingen, Switzerland). The numbers of all cells and necrotic cells stained with trypan blue at room temperature (5 min) were counted in the Bürker chamber. All experiments were carried out on cells at the second passage. The isolated cells had been identified as myofibroblasts by electron microscopy. An earlier study found the myofibroblasts to have a typical structure containing caveolae and fibers distributed parallel to the cell membrane (18). Positive staining of smooth muscle actin confirmed the identification of cells as myofibroblasts (Fig. 1). The cultures were divided into three groups: group 1 treated with serum of intact control rats, group 2 administered with serum of animals with sham operation and group 3 treated with serum of rats with myocardial infarction. Each group contained three subgroups treated with rat serum at concentrations of 10%, 20% or 30% in culture medium.

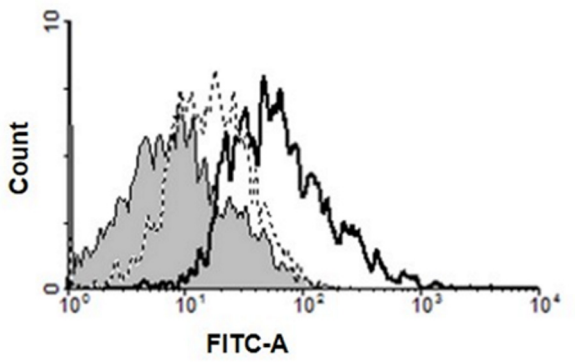


Figure 1. FACS profile showing the expression of smooth muscle actin within myofibroblasts from MI scar (right plot) comparing with isotypic control (plot in the middle) and autofluorescence (left plot).

Isolation of mast cell

Eight rats were euthanatized by exposure to a carbon dioxide and oxygen mixture. Following exsanguination, HEPES buffer (pH 6.9) (Sigma, St. Louis, MO, USA) was injected to the peritoneal cavity and the abdominal walls were massaged for 90 s. After opening the abdominal cavity, fluid containing a mixture of peritoneal cells was collected and purified by centrifugation at a density gradient of percoll (Sigma, St. Louis, MO, USA). The cellular suspension obtained after centrifugation contained mast cells. Units of 5 x 10^3 mast cells were added to the tubes and then incubated for 30 min in a water bath (37°C) with the test sera from controls, sham operated rats or animals with myocardial infarction (at concentrations of 10%, 20% or 30%). Samples incubated with buffer served as negative controls. Histamine concentration was measured in the sediments and supernatants of cells by spectrofluorometry, based on condensation of histamine with ortho-phthalic aldehyde (OPA) (Sigma, St. Louis, MO, USA). The percentage of histamine released from mast cells with regard to total histamine content within the cultures (in both supernatants and sediments) expressed the degree of mast cell degranulation.

Determination of collagen

Woessner' method was used for total collagen evaluation (19). The cells were dried at 60°C. Then samples were assayed for hydroxyproline by hydrolysis with 6 N HCl at 100°C for 24 h. Hydrolysates were neutralized by 5 N NaOH. For further analysis, samples of 0.2 ml were taken and diluted with redistilled water to a final volume of 1.0 ml. Chloramine T (POCH S.A., Gliwice, Poland) in citrate buffer was applied to oxidize hydroxyproline to pyrrole, then samples were shaken for 5 min and incubated at RT for 20 min. To remove the excess of chloramine T, 0.5 ml of perchloric acid (Merck, Darmstadt, Germany) (3.15 M) was added. After five minutes, the samples were treated with 0.5 ml of 20% p-dimethylaminobenzaldehyde (Sigma, St. Louis, MO, USA) and incubated in a 60°C water bath for 20 min. The optical density was measured at 560 nm on a spectrophotometer.

Determination of glycosaminoglycans

Glycosaminoglycans estimation was performed using the 1,9-dimethylmethylene blue (DMMB) assay according to Farndale et al. (20). The samples were dried to constant weight at 60°C. The dry sample was added to the mixture composed of 0.75M NaOH and 50 mM natrium borate (Merck, Darmstadt, Germany) and incubated for 1 h in a water bath at 73°C. After incubation, the pH was neutralized with 6N HCl to approximately 7.0 and then 72µl of 100% TCA (POCH S.A., Gliwice, Poland) was added to each sample to precipitate proteins. After centrifugation (6000 rpm, 30 min), 6.0 ml of 99,9% ethanol was added to the supernatant. To precipitate glycosaminoglycans, the samples were put into a refrigerator (-20°C) overnight and then centrifuged (12 500 rpm, 30 min). The precipitate containing GAG was resolved in distilled water. Finally, 1.2 ml of DMMB reagent was added to 50µl of the sample and the absorbance was measured at 525nm on a spectrophotometer. DMMB reagent is composed of 51 mM of DMMB (Sigma-Aldrich Co., St. Louis, MO, USA), 45 mM glycine (Sigma, St. Louis, MO, USA), and 41 mM NaCl adjusted to pH 3.0 with 1M HCl.

Flow cytometry

To assess the expression of β1 integrins on myofibroblasts, the flow cytometry experiments were performed. After fixation at 4˚C during 30 min (Fixation Buffer, BD Biosciences, Franklin Lakes, NJ, USA) the cells were washed and centrifuged (1000 rpm, 5 min), stained at a temperature of 4°C – 8°C for 30 min in Stain Buffer (BD Biosciences, Franklin Lakes, NJ, USA) and then the samples were washed. The cells were centrifuged for 5 min at 1000 rpm and treated with the following antibodies: Arm Hamster IgG isotype control conjugated with PE (cat. no. 12-4888-81; eBioscience, San Diego, CA, USA), anti-mouse/rat CD29 conjugated with PE (cat. no. 12-0291-82; eBioscience, San Diego, CA, USA). For actin expression, the cells were fixed, permeabilized with Perm Buffer (Biosciences, Franklin Lakes, NJ, USA), and then stained with the following antibodies: Alpha-Smooth Muscle Actin Antibody conjugated with FITC (cat. no. NBP-2-34522F; 1:500, Novus Biologicals, Littleton, CO, USA) and mouse IgG2a Kappa Light Chain Isotype Control conjugated with FITC (cat. no. NBP1-43955; 1:500; Novus Biologicals, Littleton, CO, USA). Based on preliminary results, the sera at a concentration of 10% were tested in final experiments. The FACS Canto II analytical flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) was used for analysis. Results were analysed by Kaluza Analysis 1.5a software (Beckman Coulter).

Statistical analysis

The Kruskal-Wallis test was used for statistical analysis. Differences between the groups were evaluated by multiple comparisons of mean ranks (Dunn's test). The minimal level of significance was $P<0.05$. Statistical calculations were performed using Statistica 13 software (StatSoft Poland).

Results

Identification of myofibroblasts

The isolated cells had various shape: longitudinal, fusiform, spindle and stellate. Moreover, the flow cytometry experiments showed that the cells isolated from the myocardial infarction scar were α smooth muscle actin positive (Fig. 1). The observations suggest that isolated cells are identified as myofibroblasts. These cells could be distinguished from fibroblasts that are α smooth muscle actin negative (18, 19).

Collagen accumulation

The cultures treated by serum from intact rats or from sham-operated animals were found to have similar collagen content. This observation concerns all concentrations of serum applied to the cell cultures. However, the level of collagen in cultures treated with serum from rats with myocardial infarction applied at concentrations of 10% and 20% was greater than cultures treated with the serum of intact control rats or

animals with sham operation. In cultures treated with 30% of serum from animals with myocardial infarction, no significant collagen augmentation was observed (Fig. 2A) compared with intact controls and sham myocardial infarction.

Glycosaminoglycan content

No significant difference in glycosaminoglycan level in myofibroblast culture was found following treatment with 10% or 20% serum taken from rats with myocardial infarction compared to treatment with serum taken from intact control rats or animals with sham myocardial infarction. A higher collagen/glycosaminoglycan ratio was observed in cultures treated with 10% or 20% serum from MI rats compared to intact control or sham operated groups (Table 1). Moreover, no significant difference in glycosaminoglycans level was observed between cultures treated with the serum of rats with sham myocardial infarction and those treated with serum from intact control animals. It was found that 30% serum from the myocardial infarction rats increased the level of glycosaminoglycans in cell cultures (Fig. 2B) to a greater degree than the serum from the intact control and sham operated rats.

Myofibroblast proliferation

Accelerated proliferation of myofibroblasts was observed in cultures treated with 20% serum from rats with myocardial infarction compared to cells treated with the serum of sham operated animals. (Fig. 2C). However, reduced cell proliferation was observed in myofibroblasts treated with 20% serum from sham operated rats compared with serum from intact animals. In contrast, 30% serum derived from rats with myocardial infarction inhibited proliferation of myofibroblasts compared to cells treated with serum of sham myocardial infarction; however, serum from the sham group increased cell proliferation to a greater degree than serum derived from intact controls (Fig. 2C). Hence, the serum from the myocardial infarction rats normalized the effects of sham operated groups.

Histamine release from mast cells

Sera from rats with sham operation or myocardial infarction induced greater histamine release from the mast cells than serum from controls. In addition, serum from myocardial in-

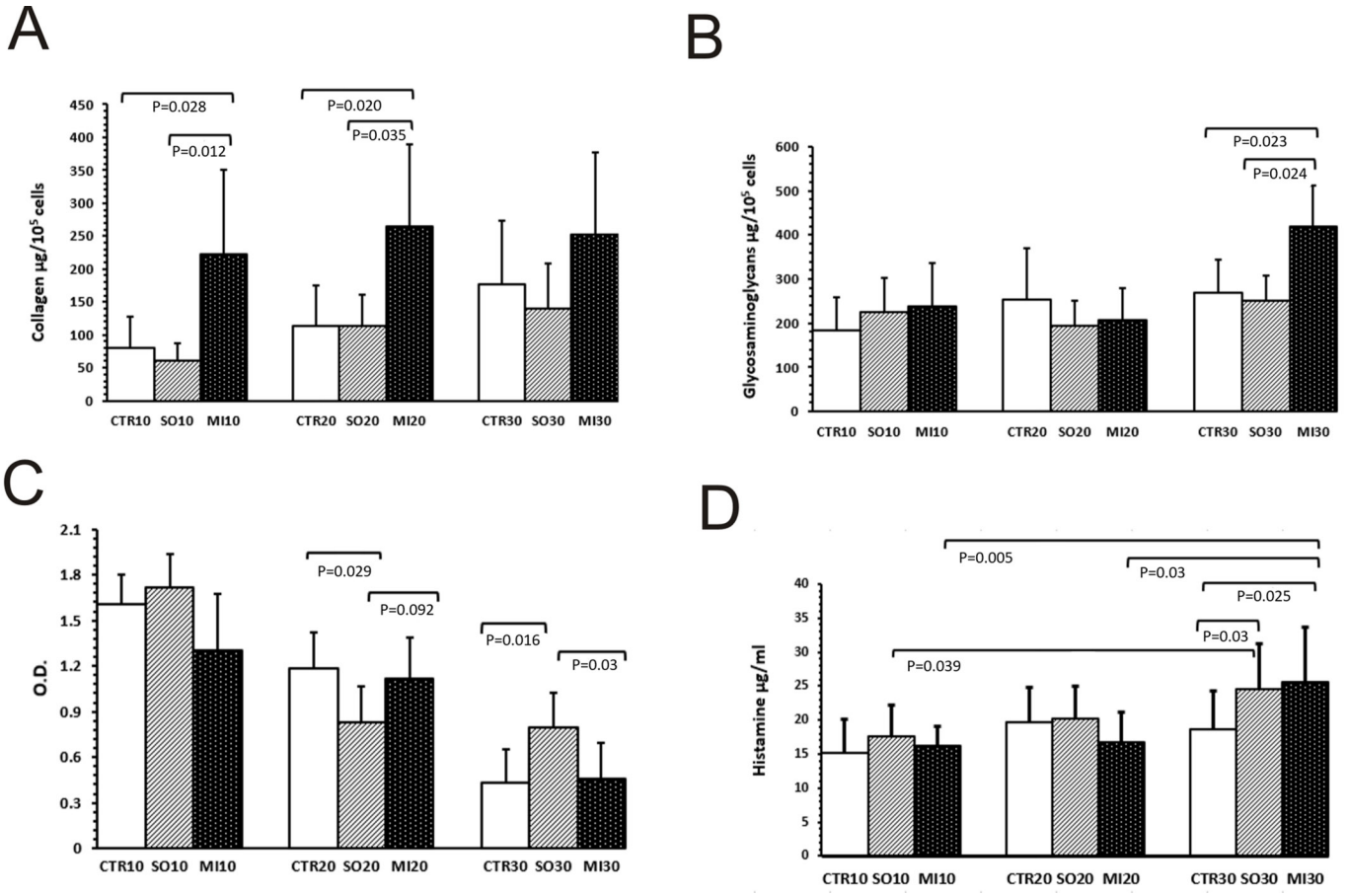


Figure 2. Total collagen (A) or glycosaminoglycans (B) content (µg/10^5 cells) in cultures of myofibroblasts isolated from the myocardial infarction scar treated with 10%, 20% or 30% serum derived from intact control group (CTR), rats with sham myocardial infarction (SO) or animals with induced myocardial infarction (MI). Effects of different concentration serum of intact, sham-operated and MI rats on myofibroblasts proliferation (C). Effects of different concentration serum of intact, sham-operated and MI rats on the histamine release by the mast cell (D). Each value expresses the mean of eight samples + standard deviation (SD).

Table 1. Collagen to glycosaminoglycans (collagen/GAG) ratio in myofibroblast cultures treated with 10%, 20% or 30% serum derived from intact control group (CTR), rats with sham myocardial infarction (SO) or animals with induced myocardial infarction (MI).

	CTR 10	SO 10	MI 10	CTR 20	SO 20	MI 20	CTR 30	SO 30	MI 30
collagen/ GAG	0.430	0.273	0.928	0.447	0.586	1.274	0.657	0.557	0.603

farction rats induced higher release of histamine from the mast cells at 30% compared to when it was administered as 10% or 20%. Similarly, serum from animals treated with sham operation provoked greater histamine secretion when administered at a concentration of 30% than 10% (Fig. 2D).

Integrin β1 expression

The present study confirmed integrin β1 expression on the surface of myofibroblasts derived from myocardial infarction scar (Fig. 3A). No difference was found between the cultures treated with the three types of serum regarding integrin β1 subunit density on the myofibroblast cell membrane (Fig. 3B).

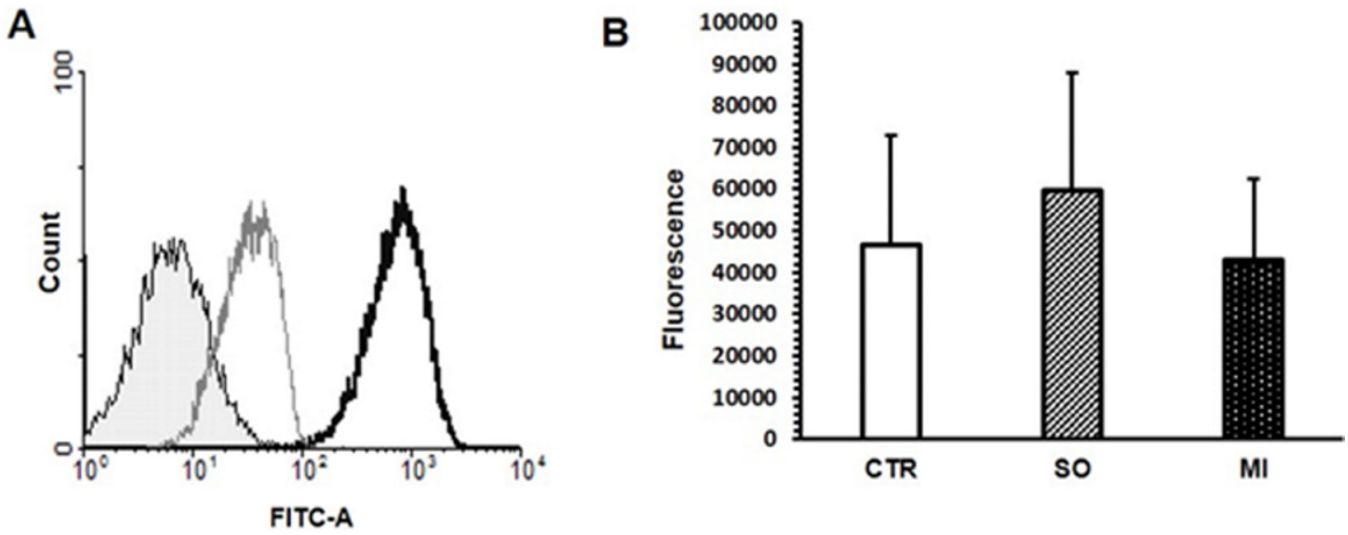


Figure 3. FACS profile (A) showing the expression of β1 integrin on myofibroblasts from MI scar (right plot) comparing with isotypic control (plot in the middle) and autofluorescence (left plot). Density of β1 integrin on myofibroblasts (B) within the cells treated with 10% serum derived from intact control group (CTR), rats with sham myocardial infarction (SO) or animals with induced myocardial infarction (MI). Each value expresses the mean of eight samples + standard deviation (SD).

Discussion

The healing of myocardial infarction results in the deposition of an extracellular matrix within the scar and determines its mechanical properties. Collagen level is an essential determinant of scar strength and stiffness (21). Both type I and type III collagens are synthesized in the infarcted area in the rat heart (22). Earlier data has shown excessive collagen accumulation by scar tissue in rats with induced myocardial infarction (23). However, the present study sheds light on the regulatory mechanism responsible for augmentation of collagen deposition within the myocardial infarction scar. Our findings clearly show that the direct action of serum derived from rats suffering from myocardial infarction augments the accumulation of collagen within myofibroblasts cultured from a myocardial infarction scar.

Heart failure caused by myocardial infarction results in the induction of an endocrine response characterized by the release of profibrotic renin, angiotensin, aldosterone (24), endothelin (25) and catecholamines (26), as well as various antifibrotic compounds such as atrial natriuretic peptide (27). Of these, noradrenalin augments collagen type I expression in heart fibroblasts from neonatal rats (11). Angiotensin II increases the synthesis of collagen type I and III in heart fibroblasts (12, 13). Moreover, both aldosterone(14) and endothelin (2) are known to increase fibrosis of the heart. In contrast, atrial natriuretic peptide (15) and adenosine (16) inhibited collagen synthesis by heart fibroblasts. Several cytokines are released by the ischemic segment of the myocardium, including tumor necrosis factor-α (TNF- α), transforming growth factor-β1 (TGF- β1), and interleukin-4 (IL-4). TGF-β1 increases the synthesis of collagen,

and proteoglycans but TNF-α and IL-4 inhibit collagen synthesis (17).

Our present data shows that antifibrotic compounds in serum of rats with myocardial infarction are outweighed by those increasing collagen accumulation. Moreover, serum plays an important role in the development of the extracellular matrix in scar myofibroblast culture and may be involved in the formation of a balanced myocardial infarction scar.

Collagen-binding integrins are involved in regulation of collagen metabolism within heart fibroblasts (6). The integrin β1 subunit is the common part of collagen receptors comprising different integrins (α1β1, α2β1, α10β1 and α11β1) binding to collagen. None of the tested sera changed the density of integrin β1. Contrary to collagen, the results suggest that integrin β1 counts on the myofibroblast surface are not regulated by the sera of rats with myocardial infarction. However, our results confirm β1 integrin expression in myofibroblasts derived from myocardial infarction scar. Similarly, our previous paper confirmed the expression of α2β1integrin in fibroblasts from human heart tissue (6).

Serum from rats with myocardial infarction induces glycosaminoglycan augmentation within the scar myofibroblast culture by acting directly on the myofibroblasts. However, this was only observed for the highest serum concentration. The two lower concentrations of serum obtained from rats with myocardial infarction, modified the collagen to glycosaminoglycan ratio (Table 1) by augmenting collagen content while maintaining a stable glycosaminoglycan level (Table 1). Glycosaminoglycans are bound to collagen via electrostatic interactions (28). Both proteoglycans and glycosaminoglycans control the kinetics of collagen fibrillogenesis (29). The addition of decorin to polymerizing collagen increased the tensile strength of fibers (30). The proteoglycans also inhibit metalloproteinases (31). It is therefore possible that the disproportion between collagen and glycosaminoglycans may be responsible for modification of collagen metabolism. The present results are supported by our earlier studies showing an increased level of glycosaminoglycans within the scar of myocardial infarction rats. Peak of glycosaminoglycan content was observed on the sixth week after ligation of the coronary artery, and later decreased on the twelfth week after induction of myocardial infarction. A similar phenomenon was found in the myocardium remote to infarction (32). Therefore, the present study sheds light on the mechanism of effects observed in vivo and indicates that serum from rats with myocardial infarction may increase glycosaminoglycan content within the scar of myocardial infarction by acting directly on the myofibroblasts.

The contradictory effects observed for the different concentrations of sera (20% and 30%) from sham-operated rats on myofibroblast proliferation could be caused by factors released to the blood due to experimental stress (surgery or thoracic wound repair). Earlier studies have also noted that different concentrations of inflammatory mediators have opposite effects (33). The effect on cell proliferation is believed to be an average of several compounds acting in opposition. Thus, Saito et al., (34) showed inhibition of fibroblast growth by cortisol or serum collected from the patient after gastrointestinal surgery. In addition, serum obtained from patients with inflammatory disease (ocular graft versus host disease) was found to increase the proliferation of fibroblasts (35). In the present study, the serum of rats with myocardial infarction reversed the effect of sham operation and normalized myofibroblast proliferation. Thus, noradrenalin (36, 37) or aldosterone (38) stimulates greater proliferation of fibroblasts in the heart. Furthermore, proliferation of cardiac fibroblasts was dependent on reactive oxygen species levels (39). In contrast, B-type natriuretic peptide inhibits the proliferation of cardiac fibroblasts (40).

Histamine secreted by the mast cells augments collagen content in cultures of myofibroblasts derived from myocardial infarction scar tissue (19). The serum of rats with myocardial infarction did not modify the histamine release. These results clearly suggest that the compounds released in rats with myocardial infarction do not participate in the regulation of mast cell degranulation. Sera from sham-operated animals and rats with myocardial infarction increased histamine release from the mast cells in culture compared with control rats. Therefore, the observed effect is more closely linked with the effects of thoracic chest surgery and wound repair in both sham-operated animals and rats with myocardial infarction. Some cytokines (41) are known to be involved in mast cell activation.

The present study reports the effect of whole serum on extracellular matrix deposition in scar myofibroblast cultures. The serum is composed of various antifibrotic and profibrotic compounds; however, it was no possible to precisely identify the main compounds involved in scar development, and this is a limitation of the present study. This should be addressed in further studies.

Conclusion

This study shows that serum obtained from rats with myocardial infarction is involved in the regulation of extracellular matrix accumulation within scar myofibroblast culture. This effect acts by elevated collagen and glycosaminoglycan accumulation within the cultures of scar derived myofibroblasts, as well as modification of myofibroblast proliferation. On the other hand, treatment results in an imbalance between collagen and glycosaminoglycan levels. However, the serum is unable to modify histamine secretion from mast cells or expression of β1 integrin on myofibroblasts. Our findings indicate that compounds derived from serum of rats with myocardial infarction may play an important role in myocardial infarction scar development. Although the serum contains factors with contradictory effects on collagen accumulation, the profibrotic effect dominates.

Competing Interests

The authors declare that they have no competing interests.

Ethical Compliance

The experiments were approved by the Local Commission of Ethics in Lodz (permission number is 5/ŁB 533/2011).

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