DOI 10.1007/s10517-020-04929-z

Evaluation of the Effect of Plasma from Patients with Trophic Ulcers on the Function of Dermal Fibroblasts, Mesenchymal Stem Cells, and Endothelial Cells

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Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 2, pp. 98-19, June, 2020
Original article submitted April 23, 2019

We studied the effect of platelet lysate and platelet-poor plasma from patients with trophic ulcers with and without type 2 diabetes mellitus on proliferation, migration, and apoptosis of human dermal fibroblast, mesenchymal stem cells, and endothelial cells. It is shown that plasma obtained from patients with type 2 diabetes mellitus produced inhibitory effects.

Key Words: *plasma; fibroblast; mesenchymal stem cells; endothelial cell line EA.hy 926*

High incidence of trophic ulcers (1-4%), tendency to relapse and expansion of the lesion area, low efficiency of traditional methods of treatment, and high cost of treatment necessitate the search for new methods of therapy of this pathology [6,7]. A promising trend in the treatment of trophic ulcers is the use of cell technologies. We have shown the effectiveness of the use of biomedical cell product in acute myocardial infarction, chronic inflammation in the intestine, and burn skin wound in animals, as well as in age-related macular degeneration in humans [8,9,12,13,15,23]. Dermal fibroblasts have found their application for dermoplasty of trophic ulcers [21]. Encouraging results were also observed after application of platelet-rich autologous plasma due to high content of growth factors [1-5,16]. However, the effect of blood plasma in patients with trophic ulcers with and without diabetes mellitus on the functional properties of cells involved in the regeneration of trophic ulcers has not been studied enough.

Our aim was to study the effect of platelet lysate and platelet-poor plasma from patients with trophic ulcers of different genesis on the proliferation, migration, apoptosis, and cell cycle of human dermal fibroblasts, mesenchymal stem cells, and EA.hy 926 endothelial cells *in vitro*.

MATERIALS AND METHODS

The study was conducted in strict adherence to the principles of the Helsinki Declaration; informed consent was obtained from all participants. The research included 7 patients with trophic ulcers with type 2 diabetes mellitus (group 1), and 7 patients with trophic ulcers of venous etiology (group 2). Platelet lysate (PL) and platelet-poor plasma (PPP) were obtained as described previously [11]. Dermal fibroblast (DF) were derived from skin biopsy specimens (2×1 cm) of donors [29], mesenchymal stem cells (MSC) were isolated from the bone marrow of donors [14]. Cells of the endothelial line EA.hy 926 (kindly provided by Dr. C.J. Edgel, University of Carolina, USA) were cultured as described elsewhere [10].

Proliferation of cells (10^4 per well) in the presence of 10% fetal calf serum (FCS) (control), 10% PL, or 10% of PPP was assessed by inclusion of MTT (5 mg/ml, Sigma) after 72-h culturing in 96-plates (TPP) on a Stat Fax 2100 spectrophotometer. In addition, proliferation and migration of DF were assessed by changes in cellular impedance in real time on an xCEL-

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Ligence System (Roche) as described elsewhere [10]. Cell cycle was studied by staining with propidium iodide (BD) and apoptosis/necrosis was assessed using Annexin V-FITC Apoptosis Detection Kit (BD). The following cell cycle phases were identified: subG0G1 (<2n), G0G1 (2n), G2/M (2n-4n), and S (>4n).

Statistical data processing was performed using Statistica 6.0 (StatSoft, Inc.). The data are present as *M*±*SD*; the significance of differences was estimated using ANOVA; the differences were significant at $p \leq 0.05$.

RESULTS

Fibroblasts are the main producers of extracellular matrix, are involved in repair of skin lesions, and stimulate growth of keratinocytes and blood vessels formation [26]. MSC due to their ability to cytodifferentiation into connective tissue cells, including fibroblasts, as well as due to the ability to produce a wide range of bioactive substances and migration to the area of damage, play a significant role in skin repair/regeneration [25]. The repair/regeneration of damaged tissues proceeds more quickly under conditions of adequate blood supply; in light of this, endothelial cells due to their ability to migrate to the area of vascular endothelium damage and integrate into existing or form new vessels also plays an essential role in skin regeneration [17,22].

According to the results of MTT test, LT and PPP from group 2 patients stimulated DF proliferation in comparison with the control (10% FCS) and LT and PPP from group 1 patients ($p \leq 0.05$; Table 1). LT and PPP from group 1 patients suppressed proliferation of MSC in comparison with the control and LT and PPP from group 2 patients ($p \leq 0.05$). A similar picture was observed for proliferation of EA.hy 926 cells ($p \leq 0.05$).

Thus, the plasma from diabetes mellitus patients with trophic ulcers produced an inhibitory effect on cell proliferation.

It was interesting to study the effect of LT and PPP on proliferation and migration of DF not only at the end point of observation, but in dynamics using xCELLigence system. PPP from patients with trophic ulcers, especially from group 1 patients, suppressed proliferation of DF in comparison with the control, while PL from patients with trophic ulcers, especially group 2 patients, stimulated proliferation of DF in comparison with control ($p \leq 0.05$; Fig. 1, *a*). PPP and PL from patients of both groups (especially PL from group 2 patients) stimulated migration of DF in comparison with the control (Fig, 1, *b*; $p \leq 0.05$).

Thus, according to cell impedance data, the proliferation and migration of DF depended on the source of plasma.

Addition of PL from group 2 patients reduced the number of DF in the G0G1 phase and increased the number of S-phase cells in comparison with the control and PL from group 1 patients ($p \leq 0.05$, Table 2). Whereas PPP from group 2 patients increased the number of DF in the G0G1 phase and reduced the number of S-phase cells in comparison with the control and PL from group 1 patients. In addition, PL from patients with trophic ulcers, especially from group 2 patients, reduced the number of apoptotic and necrotic DF in comparison with the control ($p \leq 0.05$).

As for MSC, PL from group 1 patients reduced the number of cells in G0G1 phase and increased the number of S-phase cells in comparison with the control ($p \leq 0.05$). At the same time, PPP from group 2 patients delayed cells in the G0G1 phase in comparison with the control and PPP from group 1 patients ($p \leq 0.05$). LT from patients of both groups reduced apoptosis and necrosis of MSC in comparison with the control ($p \leq 0.05$). While PPP from group 2 patients increases necrosis of MSC, but reduced the number of apoptotic cells, whereas PPP from group 1 patients reduced necrosis and increased apoptosis of MSC in comparison with the control ($p \leq 0.05$).

PL from group 2 patients reduced the number of EA.hy 926 in the G0G1 phase and increased the proportion of cells in the S phase while in comparison with control, PL from group 1 patients produced an opposite effect ($p \leq 0.05$). PPP from group 2 patients

TABLE 1. Effect of PL and PPP from Patients with Trophic Ulcers against the Background of Diabetes Mellitus (Group 1) and of Venous Etiology (Group 2) on Proliferation of Human DF, MSC, and Endothelial Cells (opt. density units; *M*±*SD*)

Experimental conditions		DF	MSC	EA.hy 926
Control (10% FCS)		0.92±0.14	1.02±0.02	0.83±0.02
Group 1 patients	10% PL	0.45±0.05*+	0.53±0.16*+	0.75±0.03*+
	10% PPP	0.55±0.13*o	0.56±0.18*o	0.75±0.03*o
Group 2 patients	10% PL	1.3±0.43*	1.08±0.11*	0.82±0.02
	10% PPP	1.24±0.41*	1.08±0.2	0.80±0.01*

Note. $p \leq 0.05$ in comparison with *the control, +10% PL from group 2 patients, o10% PPP from group 2 patients.

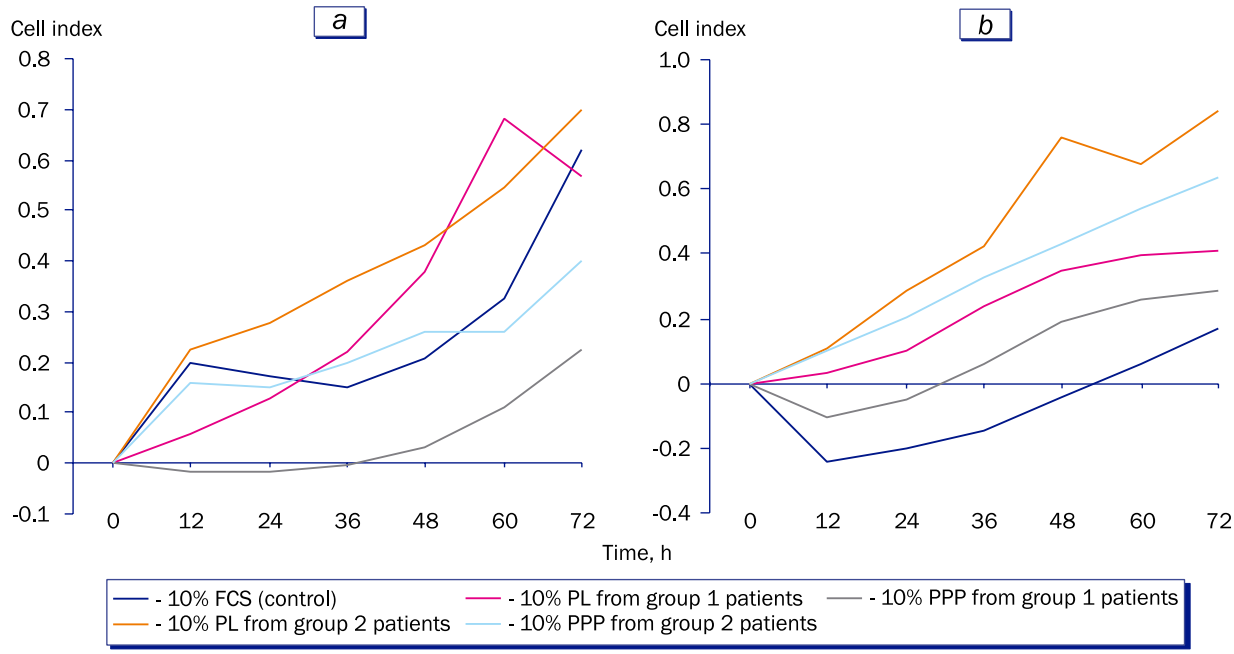

Fig. 1. Effect of plasma on proliferation (*a*) and migration potential (*b*) of human DF assessed by changes in cell impedance in real time.

retards EA.hy 926 in G0G1 phase in comparison with the control and PPP from group 1 patients ($p \leq 0.05$). At the same time, PL from patients of both groups reduced apoptosis and necrosis of EA.hy 926 cells, while PPP from patients of both groups reduces apoptosis and increased necrosis in comparison with control ($p \leq 0.05$).

Thus, PL and PPP from patients with trophic ulcers produced opposite effects on apoptosis/necrosis and the cell cycle of the tested cells.

Our data on the effects of plasma from patients with trophic ulcers with and without diabetes mellitus on proliferation and migration of DF are at controversy with the report of other authors on stimulation of DF proliferation in response to platelet-rich plasma [3,4,19]. This can be explained by the fact that the plasma was obtained from donors and DF were derived from patients. In our study, plasma was obtained from patients and DF were derived from donors.

The efficiency of heparin precipitate of autoplasma in trophic ulcers related to venous pathology and against the background of diabetes mellitus was reported [16]. Stimulation of the synthesis of collagen type I and III by cultured skin fibroblasts from patients with trophic ulcers of venous etiology was observed [4]. The combined use of platelet-rich plasma and platelet-rich fibrin clot in the treatment of patients with venous trophic ulcers accelerated epithelization of the ulcerative skin defect [2]. It is known that human DF obtained from chronic trophic skin ulcers are characterized by reduced proliferative activity and ability to synthesize type I collagen in comparison with fibroblasts obtained from acute wounds [6,23]. The sensitivity of dermal fibroblasts of chronic wounds to growth factors (PDGF, TGF-β1) is reduced [6,27]; on the other hand, impaired functional properties of such cells can be restored *in vitro* under the influence of bFGF, EGF, IL-1β, TNFα [24,27]. In study [3], human DF in the nutrient medium with 10% platelet-rich plasma from donors formed monolayer of high density, while in the presence of 10% FCS, areas of hypo- and hyper-confluence were observed. In the nutrient medium supplemented with 10% platelet-rich plasma, the number of DF was 2-fold higher than in the control (6.5×10^4 and 3.4×10^4 cells per 1 cm^2, respectively; $p \leq 0.05$). MSC are used for treatment of many inflammatory and degenerative processes, including trophic ulcers [8,9,11-13,25]. It was shown that PL from donor stimulated proliferation, migration, and colony-formation of MSC [11]. Endothelial progenitor cells provide postnatal angiogenesis and are the source of endothelial cells, and their administration promotes wound healing and angiogenesis [17,22]. In an experiment on laboratory animals with streptozotocin-induced diabetes and modeled lower limb ischemia, treatment with platelet-rich plasma and PPP increased perfusion, capillary density, and density of mature vessels according to Doppler sonography [18].

Evaluation of the effect of plasma on apoptosis/necrosis and cell cycle showed that platelet-rich plasma after 24 h increased the number of S-phase MSC in

TABLE 2. Effect of PL and PPP from Patients with Trophic Ulcers against the Background of Diabetes Mellitus (Group 1) and of Venous Etiology (Group 2) on Apoptosis/Necrosis and Cell Cycle of DF, MSC, and Endothelial Cells (%; *M*±*SD*)

Experimental conditions		DF	MSC	EA.hy 926
10% FCS (control)	subG0G1	2.0±0.2	4.0±0.1	2.0±0.1
	G0G1	76.0±2.1	84.0±1.1	84.0±1.4
	S	20.0±1.9	11.0±1.7	11.0±0.3
	G2/M	2.0±0.3	1.0±0.1	1.0±0.1
	early apoptosis	0.0±0.1	14.0±0.9	9.0±0.1
	apoptosis	3.0±0.3	22.0±1.5	24.0±1.4
	necrosis	8.0±1.4	10.0±0.3	5.0±0.1
10% PL from group 1 patients	subG0G1	1.0±0.1*	2.0±0.1*	2.0±0.6*
	G0G1	74.0±0.6*	62.0±0.9*	92.0±0.8*
	S	22.0±0.8*	34.0±0.2*	4.0±0.1*
	G2/M	1.0±0.1*	1.0±0.1	2.0±0.3*
	early apoptosis	0±0	14.0±0.1	0.0±0.2*+
	apoptosis	2.0±0.1*	4.0±0.1*	0.0±0.2*
	necrosis	4.0±0.1*	2.0±0.1*	2.0±0.4*
10% PPP from group 1 patients	subG0G1	4.0±0.2*	2.0±0.1*	3.0±0.1
	G0G1	53.0±0.4*	84.0±1.4	83.0±1.8
	S	43.0±0.4*	11.0±0.3	4.0±0.1
	G2/M	2.0±1.1	1.0±0.1	1.0±0.1
	early apoptosis	13.0±0.2*	9.0±0.1*	1.0±0.1
	apoptosis	4.0±0.1*	24.0±1.4*	1.0±0.1
	necrosis	0±0*	5.0±0.1*	11.0±0.1
10% PL from group 2 patients	subG0G1	7.0±0.1*+	7.0±0.1*+	3.0±0.1*+
	G0G1	64.0±0.4*+	61.0±0.4*	63.0±1.1*+
	S	24.0±0.5*+	30.0±0.1*+	32.0±0.7*+
	G2/M	2.0±0.1**	1.0±0.1	2.0±0.1*
	early apoptosis	1.0±0.1*+	14.0±0.2	0±0*
	apoptosis	1.0±0.1*+	7.0±0.1*+	1.0±0.1*+
	necrosis	4.0±0.1*	4.0±0.1*+	2.0±0.1*
10% PPP from group 2 patients				
	subG0G1	1.0±0.1*o	0.0±0.1*o	1.0±0.1*o
	G0G1	86.0±0.1*o	90.0±0.4*o	83.0±0.1*o
	S	13.0±0.4*o	9.0±0.4*o	1.0±0.1*o
	G2/M	0±0*	1.0±0.1	0±0*o
	early apoptosis	7.0±0.1*o	2.0±0.2*o	1.0±0.2*
	apoptosis	4.0±0.1*	10.0±0.2*o	2.0±0.2o
	necrosis	0±0*	15.0±0.2*o	9.0±0.3*o

comparison with FCS and did not significantly change the proportion of G0G1- and G2/M-phase cells. Moreover, the authors showed that platelet-rich plasma reduced apoptosis of MSC, but after 48 h, no significant differences between the FCS and platelet-rich plasma by their effect on apoptosis and cell cycle were observed [30]. Authors think that platelet-rich plasma activates DNA replication and suppresses apoptosis at the early stage, which increased the proliferative potential of MSC. Platelet-rich plasma has been shown to increase viability, proliferation, migration, and the proportion of G0G1- and S-phase MSC from the adipose

tissue and inhibit caspase-3 activity [20]. Platelet-rich plasma also promoted the production of IL-6, IL-8, IL-10, IFNγ, and VEGF by mature adipocytes.

Thus, our experiments showed that plasma from patients with trophic ulcers against the background of diabetes mellitus produced an inhibitory effect on functional activity of human DF.

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