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ROLE OF ADAPTATION TO CHRONIC HYPOXIA IN THE DEVELOPMENT OF DYSIMUNIC DISORDERS IN DIABETIC FOOT ULCER

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ABSTRACT

The purpose of the study is to establish the relationship between the immune-mediated DFU in patients with T2DM and the degree of HIF-2-dependent adaptation to chronic hypoxia.

Materials and Methods: We conducted a comparative analysis of patients' clinical characteristics, laboratory markers of infection and individual parameters of adaptive immunity in patients of the main group with neuropathic and neuroischemic forms of T2DM (subgroup 1, n=51, ulcer area less than 6 cm^2; subgroup 2, n=28, ulcer area more than 6 cm^2), as well as in patients of the comparison group (n=44) with similar forms of T2DM. It was found that the nature of the response to hypoxia, mediated by HIF-2α, played an important role in regulating the activity of the adaptive immune response and the inflammatory-reparative process in patients with T2DM and destructed foot tissue.

Results: The presence of foot wounds in patients with T2DM indicates a disruption of the HIF-2α-dependent adaptive response, which is accompanied by activation of inflammatory mediators and a decrease in IgG-dependent mechanisms. In patients with T2DM, increased HIF-2α expression in combination with increased production of immunoglobulins, is obviously a protective mechanism that prevents defects of foot tissue.

Conclusion: The clinical and laboratory use of hypoxia biomarkers is promising for predicting the risk of infectious and destructive complications in patients with T2DM.

Keywords: diabetic foot ulcer, inflammation, adaptive immunity, HIF-2α.

INTRODUCTION

Chronic wounds caused by type 2 diabetes mellitus (T2DM) are a global public health problem and are expected to affect 439 million adults worldwide by 2030 [1]. The problem is of social significance due to the frequency of chronic non-healing wounds in patients with diabetes (an indication for limb amputation in 90% of cases) and the high proportion patients becoming disabled after the disease. Currently, diabetic foot

ulcer (DFU) still poses a major challenge to physicians because its treatment is complex, associated with a high risk of infection, recurrence, limb amputation, and even death. Therefore, a comprehensive understanding of the pathogenesis of DFU is of great importance [2]. Hyperglycemia caused by T2DM leads to the formation of a pathogenetic triad in tissues: inflammation, endothelial dysfunction and hypercoagulation, disrupting the healing processes of diabetic wounds. Several endothelial, inflammatory and procoagulant biomarkers, such as VWF, IL-6, TNF-α, D-dimer and PAI-1, have been studied in diabetic patients with microvascular and macrovascular complications [3]. However, many questions remain about the pathogenetic mechanisms, initiating the development of DFU and their progression. It has been established that in fact, in 80% of patients with diabetes mellitus, chronic wounds are associated with capillary dysfunction and hypoperfusion of lower limb tissues [4]. This suggests tissue hypoxia to be considered the cornerstone of the DFU pathogenesis. New information about hypoxia-inducible factors (HIFs) appearing in the literature confirms their possible role in the course of morphogenetic processes in the injured limb [5]. Thus, endothelial HIF-2 is considered as a therapeutic target, which, when pharmacologically activated, can protect tissues from the long-term consequences of alteration by suppressing inflammation and helping tissue repair [6].

The hypothesis about the involvement of immune system dysfunction in infectious complications in DFU was further developed [7]. The state of the immune system in patients with diabetes was studied, but the results were contradictory [8, 9]. Against the background of chronic hyperglycemia (mainly more than 10 mmol/l), in vivo and in vitro studies described changes in several stages of phagocytosis [10, 11], including oxidative burst [12], abnormalities of individual subpopulations of lymphocytes [13], as well as immunoglobulins [14]. There is evidence that one of the key regulators of immunometabolism in controlling the phenotype and function of immune-competent cells are hypoxia-inducible transcription factors – HIFs, designed to compensate for the effects of hypoxia by activating oxygen-independent pathways [15]. HIF activation also increases glycolysis in adaptive immune cells. HIF-mediated glycolysis is associated with the differentiation and development of T and B cells. In T cells, HIF-dependent induction of glycolysis has been associated with the activation and differentiation of various T cell subtypes, as well as effects on T cell cytotoxicity and memory. In B cells, HIF-dependent changes in glycolysis are associated with B cell development, IgG class switching, and IL-10 production [16].

A few studies have demonstrated changes in the humoral component of the adaptive immune system in T2DM in the form of increased levels of total IgA and IgG in the serum of patients with cardiovascular complications [17], with DFU compared with the control group of diabetics (without infectious complications) [18]. There are also significant differences in IgG subclasses, in particular increased levels of IgG1 and IgG3, indicating inflammatory activation of the immune system [19]. Thus, it can be assumed that in patients with infectious complications of DFU, there is a relationship between the degree of chronic tissue hypoxia and the immunoreactivity of the humoral immune system. However, no specific relationship in the HIF-2/IgG system has been described in patients with chronically infected DFU.

Purpose of the study: to establish the relationship between the immune-mediated development of DFU in patients with T2DM and the degree of HIF-2-dependent adaptation to chronic hypoxia.

MATERIALS AND METHODS

The study group included 79 patients with type 2 diabetes mellitus (neuropathic and neuro-ischemic forms) aged from 30 to 70 years (average age 52.3±15.7 years) with infected DFU. The comparison group consisted of 44 patients of comparable age, gender and type of diabetes, but without a history of diabetic foot ulcers.

Patients of the main group were divided into subgroups with similar depth and area of the foot lesion using the modified PEDIS classification (International Working group on the Diabetic Foot. International Consensus on the Diabetic Foot with supplements. Edition on CD.-Amsterdam.-2003), according to which the subgroup 1 included patients with a wound area less than 6 cm^2 (E1, n=51); the subgroup 2 included those with a wound area of more than 6 cm^2 (E2, n=28). Changes in the blood supply to the lower limb (P) in patients of groups 1 and 2 varied slightly and corresponded to stages 1-2 of diabetic micro-macroangiopathy without critical ischemia (P1-2). The prevalence of the infectious process (I) corresponded to stages I2-3. Sensitivity disorders in nerve endings (S) in the majority of the examined patients of the main group were of a similar nature and corresponded to stage S1-2.

Exclusion criteria: critical limb ischemia (ankle-brachial Doppler index <0.6) or severe arterial stenosis or occlusion requiring endovascular/surgical revascularization), signs of acute infection, treatment with corticosteroids or other immunosuppressive drugs, systemic immune dysfunction or malnutrition. Before inclusion in the study, each patient signed an informed consent form.

All examined patients underwent blood glucose level (spectrophotometrically; Abbott Architect, USA) and glycosylated hemoglobin tests (HbA1c: high-performance liquid chromatography; Tosoh G8, Japan) at the admission. Laboratory tests for infection markers included CRP (determined by the turbidimetric method;

Abbott Architect, USA) and the number of blood cells (by spectrophotometry; SYSMEX, Japan). Serum levels of immunoglobulins were also measured (IgM and IgG using immunoturbidimetry; Abbott Architect, USA).

Patients' clinical characteristics, laboratory markers of infection, and individual parameters of adaptive immunity were compared between study groups and subgroups. Data analysis was carried out using the standard package of statistical methods of the Statistica 10.0 program. Descriptive data were presented as means ± standard deviation; differences between all study groups were determined using t-test, Mann-Whitney test and multiple comparisons using Kruskal-Wallis test. Differences were considered statistically significant at $p<0.05$. Spearman's rank correlation coefficient was used to determine significant correlation between the assessed data.

RESULTS AND DISCUSSION

In patients with DFU, the median ulcer duration was 8.5 months (range from 2 to 60 months) and the median ulcer area was 1.4 cm^2 (range from 0.08 to 25.2 cm^2). The clinical characteristics of the examined patients of the main group (subgroups 1 and 2) and the comparison group are presented in Table 1.

Table 1.Characteristics of the examined patients

Parameters	Patients with DFU		Patients without DFU (n=44)
	Wound area less than 6 cm^2 (n=51)	Wound area more than 6 cm^2 (n=28)	
Age (years): - min-max (Me) - M±m	$31-80 (50,7) 49,81±9,88	33-78 (53,2) 50,73±10,41	30-69 (49,1) 48,95±8,46
Gender, n (%) - male - female	36 (70,6) 15 (29,4)	15 (53,6) 13 (46,4)	33 (75,0) 11 (25,0)
Duration of diabetes (years): - min-max (Me) - M±m	3-33 16,87±7,85	5-29 17,40±9,06	2-20 10,95±7,11
Insulin therapy, n (%): - No - Yes	16 (31,4) 35 (68,6)	10 (35,7) 18 (64,3)	25 (56,8) 19 (43,2)
Use of oral antidiabetic drugs, n (%): - No - Yes	35 (68,6) 16 (31,4)	16 (57,1) 12 (42,9)	19 (43,2) 25 (56,8)
Ulcer localization, n (%): - toe - metatarsal bone - midfoot, hindfoot	10 (19,6) 24 (47,1) 17 (33,3)	8 (28,6) 13 (46,4) 7 (25,0)	0 0 0
Ulcer depth, n (%): - superficial - deep	33 (64,7) 18 (35,3)	16 (57,1) 12 (42,9)	0 0

The average age of patients in both studied groups did not differ significantly. Male patients prevailed over female patients, with gender differences being most pronounced in patients with T2DM without DFU. Patients of the main group (with DFU) were characterized by a longer duration of the disease compared to patients without DFU (by 54% and 59%, respectively, $p=0.01$). Also, the proportion of patients receiving insulin therapy was approximately twice as high in the main group, while the ratio of patients with and without insulin therapy in the comparison group turned out to be approximately equal and amounted to

56.8/43.2. The opposite pattern was seen in the use of oral antidiabetic drugs. The predominant localization of the ulcerative defect, often superficial, was the metatarsal bone in patients with DFU of both subgroups.

A comparative analysis of laboratory parameters in patients of the subgroup 1 (with chronic superficial DFU, E1) and the comparison group revealed an increase in the serum level of C-reactive protein by 35.65% (p=0.01), IgG by 39.80 (p=0.03), glucose by 21.10% (p=0.008) and HbA1 by 10.59% (p=0.02). At the same time, the relative number of $CD19^+$ lymphocytes in the blood was 2.2 times lower than that in the comparison group (p=0.0007) against the background of non-significant differences in the absolute number of $CD19^+$ lymphocytes (lower by 12.30%) and the level of IgM (by 3.41%). The content of HIF-2α in patients of the subgroup 1 was significantly lower than that in the comparison group (by 32.43%, p=0.05). Table 2.

Table 2. Comparative characteristics of inflammation and humoral immunity parameters between the examined groups of patients

	Main group (n=79)		
Indicators	Subgroup 1, n=51	Subgroup 2, n=28	Comparison group
Serum glucose, mmol/l	9,87±3,56 *p=0,08*	11,04±5,23 *p=0,007* p_{1-2}=*0,05*	8,15±1,48
HbA1, %	6,89±1,63 *p=0,09*	8,13±17 *p=0,004* p_{1-2}=*0,009*	6,23±1,11
C-reactive protein, mg/l	2,93±1,35 *p=0,01*	11,14±9,67 *p=0,03* p_{1-2}=*0,0001*	2,16±0,88
Relative number of $CD19^+$ lymphocytes, %	10,84±4,19 *p=0,0007*	9,92±3,87 *p=0,0005* p_{1-2}=*0,06*	23,77±2,02
Absolute number of $CD19^+$ lymphocytes, cells/μl	264±108 *p=0,01*	232±99 *p=0,006* p_{1-2}=*0,04*	301±117
IgM, g/l	0,91±0,37 *p=0,12*	1,30±0,69 *p=0,02* p_{1-2}=*0,001*	0,88±0,61
IgG, g/l	10,16±1,58 *p=0,03*	7,94±1,04 *p=0,009* p_{1-2}=*0,027*	13,19±2,05
HIF-2α, μg/l	0,25±0,10 *p=0,05*	0,13±0,06 *p=0,003* p_{1-2}=*0,0009*	0,37±0,15

Note: Data are presented as means ± SD; p - level of significance between patients with DFU and the comparison group (diabetics without DFU), p_{1-2} - level of significance between the subgroups 1 and 2 (patients with DFU)

Subgroup 2 (with chronic superficial DFU, E2) had an increase in the serum level of C-reactive protein by 5.16 times (p=0.0003), in glucose by 35.46% (p=0.007) and HbA1 by 33.40% (p=0.004), while the IgG content was lower by 39.80 (p=0.009) compared to patients without DFU. At the same time, the relative number of $CD19^+$ lymphocytes in the blood was lower by 58.3% (p=0.0005) than that in the comparison group against the background of a lower absolute number of $CD19^+$ lymphocytes (less by 22.92%) and IgM level (by 3.41%). The content of HIF-2α in patients of the subgroup 1 was 2.85 times lower than in the comparison group (p = 0.0003).

In general, the deficiency in the increase of the serum level of immunoglobulins in the subgroups 1 and 2 was less pronounced compared to the deficiency in the absolute number of $CD19^+$ lymphocytes (by 12.3% and 22.9%, respectively). The maximum levels of IgM and CRP in the blood serum occurred in the subgroup 2, while IgG maximum level was in patients in the comparison group and was accompanied by a relatively low concentration of CRP. In patients of the subgroups 1 and 2, the IgG level did not reach that of the comparison group by 22.9% (p=0.03) and 39.8% (p=0.0009), respectively, which indicated possible immune dysfunction in the presence of an infectious inflammatory-destructive process in the area of tissue defect.

The dissonance of the absolute and relative numbers of $CD19^+$ lymphocytes in patients with DFU of the subgroups 1 and 2 was noteworthy – the degree of decrease in the absolute number of these cells compared to the same parameter for the relative number of these lymphocytes was 4.4 and 2.5 times less, respectively. [20] found a similar pattern too and attributed lower serum lymphocyte levels to previously described immune dysfunction at the lymphoid stem cell level or other factors including stress [21], age [22], and possibly diabetes mellitus alone.

Changes in the humoral part of the adaptive immune system in the form of a decrease in the serum level of immunoglobulins of both classes (M and G) do not contradict the deficiency described in the literature caused by long-term persistence of a bacterial infection. However, taking into account the data [23] on selective deficiency of the IgG2 subclass and its ability to opsonize bacteria, the predominant overproduction of IgG and CRP playing an important role in the inflammatory-reparative process, in patients of the comparison group, becomes clear [24]. Patients of the subgroup 1 did not show a sharp increase in the level of CRP, despite the presence of infectious complications and tissue defects, but a slight decrease in the level of IgG in the blood (by 22.9% compared to the group without DFU, p=0.03) and increased CRP (by 35.65%, p=0.01 compared to the group without DFU) demonstrate a disruption in the relationship between the antigen-stimulated immune response and inflammation, which may be due to diabetes mellitus. A more pronounced imbalance between CRP and IgG was found in patients of the subgroup 2, where the increase in the first was 5.16 times compared to the comparison group, and the decrease in the level of serum IgG reached 39.8% (p=0.009) compared to the comparison group.

Based on the above data, we could assume that in patients with infected chronic DFU, the reactivity of the humoral immune response is reduced, which, probably, in combination with weakened innate immunity [25] and a decrease in the absolute and relative number of $CD19^+$ lymphocytes, contributes to the chronicity of tissue destruction. One of the protective mechanisms in this case may be cellular adaptation to O_2 deficiency, performed with the help of the hypoxia-inducible transcription factor-2 (HIF-2). However, analysis of the serum level of HIF-2α in patients of the main group showed its low values in both subgroups, which were lower than those in the comparison group (by 32.43% and 64.86%, respectively), and the indicator in the subgroup 2 was 48% lower than that in the subgroup 1). That is, this mechanism can be implemented only in patients with T2DM without DFU.

To clarify the relationship between the content of HIF-2α and indicators of inflammation and humoral immunity in the examined patients, we carried out a correlation analysis, the results are shown in Table 3.

Table 3. Correlation coefficients between indicators of inflammation and adaptive immunity in the examined patients

Indicators	Main group (n=79)		Comparison group
	Subgroup 1, n=51	Subgroup 2, n=28	
Glucose - HIF-2α	-0,78	-0,86	0,57
CRP - HIF-2α	-0,65	-0,71	0,49
ANL - HIF-2α	0,44	0,53	0,27
RNL - HIF-2α	-0,41	-0,59	-0,32
IgM - HIF-2α	-0,50	-0,45	-0,11
IgG - HIF-2α	0,69	0,79	0,28

Noteworthy was the presence of an inverse relationship between most of the studied indicators, except for the absolute number of $CD19^+$ lymphocytes and HIF-2α (and only in patients of the subgroup 2 the

correlation coefficient was of medium strength), as well as between IgG and HIF-2α. A strong negative correlation occurred in patients with DFU of the main group between the level of glucose and HIF-2α, CRP and HIF-2α, and a positive correlation of similar strength was between IgG and HIF-2α, which suggests the existence of a relationship between the degree of inflammation and the secretory activity of the effector cells of humoral immunity, on the one hand, and the expression of the transcription factor HIF-2α. On the contrary, in patients in the comparison group (without DFU), the correlation coefficients in the above pairs were positive, although with different degrees of connection.

Inflammation, endothelial dysfunction and hypercoagulation correlate with each other, and play an important role in the development of vascular complications in patients with diabetes due to a decrease in oxygen (O_2) supply. The central mediators of cellular adaptation to tissue O_2 deficiency are hypoxia-inducible transcription factors - HIF-1 and HIF-2, which modulate intracellular energy metabolism, angiogenesis, erythropoiesis, apoptosis and cell proliferation [15, 26]. In addition to systemic hypoxia caused by the metabolic effects of hyperglycemia and insulin resistance, hypoxic areas can also form in the tissues of the lower limbs due to pathologic walls of microvasculature blood vessels, microthrombosis and infection with pathogenic bacteria, viruses, fungi and protozoa [27]. Other factors also contribute to the formation of a tissue hypoxic environment: increased O_2 consumption by inflammatory resident cells and infiltrating immune cells, as well as changes in the diffusion capacity of the intercellular matrix. High glucose and the local inflammatory environment of the wound can lead to a structural and functional deficiency of endothelial progenitor cells, thereby suppressing the expression of vascular endothelial growth factor (VEGF), which ultimately prevents the formation of new blood vessels and the formation of granulation tissue. In this scenario, the hypoxic microenvironment can initiate protective mechanisms by HIF-1 and HIF-2, reducing cell death and pathogenicity of microorganisms by increasing the expression of VEGF [28]. However, the results of our study demonstrated a decrease in the body's protective potential during the formation of foot wounds in patients with T2DM and DFU in the form of a decrease in the expression of HIF-2α and associated inflammatory and immune reactions. Considering that the regulator of HIF-2α activity is the expression density and catalytic activity of PHD2, a decrease in the hydroxylation properties of which ensures the stability of HIF-1 [29] (and of HIF-2 too, in conditions of chronic hypoxia), it can be assumed that PHD2 maintains high activity, aimed at eliminating metabolic carbohydrate-fat dysfunction [30] or the possibility of modulating the expression of the intracellular effector pathway HIF-2α by other metabolic regulators. Moreover, when assessing the degree of inflammatory and immune reactions in patients with DFU, it should be taken into account that PHD2 plays the role not only of a "HIF regulator" in the cascade of oxygen-recognition signals, but also induces HIF-independent antihypoxic protective reactions, modulates various cellular responses to hypoxia and preconditioning stimuli [31,32] and can implement compensatory mechanisms. Judging by the literature data and the presence of tissue defects in patients of subgroups 1 and 2, the activity of PHD2 is most likely reduced, and the expression of HIF-2α is blocked by non-canonical mechanisms. On the contrary, in patients without DFU, HIF-2α activity was higher, which served as an inducer of protective mechanisms aimed at maintaining tissue homeostasis in the lower limbs.

CONCLUSION

Thus, we have established a relationship between the immune-mediated development of DFU in patients with T2DM and the degree of HIF-2-dependent adaptation to chronic hypoxia. In the presence of foot wounds in patients with T2DM, the HIF-2α-dependent adaptation response is impaired, which is accompanied by activation of inflammatory mediators and a decrease in IgG-dependent mechanisms. In patients in the comparison group, an increase in the expression of HIF-2α in combination with an increase in the production of immunoglobulins is obviously a protective mechanism that prevents the development of foot tissue defects in patients with T2DM.

The clinical and laboratory use of hypoxia biomarkers is promising for predicting the risk of infectious and destructive complications in patients with diabetes and for their monitoring.

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