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ARTICLE

Phosphorylation of Protein Kinase Akt by Mtorc2 in Peripheral Blood Mononuclear Cells of Patients with Cancer and Diabetes.

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ABSTRACT

Akt/mTOR/p70S6K1 signaling pathway plays an important role in the pathogenesis of cancer and diabetes. Macrophages and lymphocytes are involved in the pathogenesis of diabetes, diabetic atherosclerosis, formation of insulin resistance as well as immune response to cancer and tumor maintenance. The aim of the study was to determine the Akt activation by mTORC2 in peripheral blood mononuclear cell (PBMC) of patients with type 2 diabetes and cancer. The following groups were studied: control group, patients with type 2 diabetes, cancer patients and patients with both cancer and diabetes. The amounts of phospho-Akt (p-S473) and phospho-p70S6K1 (p-T389) were determined using ELISA kits. The amount of phosphorylated Akt significantly increases in PBMC of patients with cancer. There was no effect in PBMC from patients with type 2 diabetes and significant decrease in the amount of phospho-Akt in PBMC of the patients group both with cancer and diabetes. p70S6K1 activation was observed in PBMC of the groups 2 and 3 patients. Thus, chronic diseases such as type 2 diabetes and cancer can affect the signaling mechanisms in blood cells. The state of Akt phosphorylation in leukocytes can indicate the activity of mTORC1 and its substrates, which may be important for the evaluation of the pathological process and the efficacy of the drugs.

1. Introduction

Protein kinase Akt (v-act murine thymoma viral oncogene homolog) plays a key role in regulation of cell growth, homeostasis, survival, proliferation and metabolism [1]. Akt is activated by PDK1 via T308 phosphorylation in the T-loop of the catalytic domain and by rapamycin-insensitive mTORC2 through S473 phosphorylation in the hydrophobic region on the C-tail. Akt enhances insulin-dependent translocation of GLUT-4 and glucose transport, and activates downstream protein kinases mTORC1 and p70S6K that control protein synthesis and biogenesis of ribosomes. Dysregulation of the PI3K/Akt/mTOR/p70S6K signaling lead to severe diseases such as cancer, obesity and type 2 diabetes (T2D).

The peripheral blood mononuclear cell (PBMC) include several types of cells that play a significant role in the development of pathological conditions such as diabe-

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tes and cancer [2-4]. The pathway PI3K/Akt is involved in the activation of macrophages and lymphocytes, secretion of cytokines, initiation of inflammatory processes and immune surveillance failure [5].

The serine/threonine kinase mTOR forms two different signaling complexes, mTORC1 and mTORC2, by binding several proteins. mLST8, DEPTOR, and Tti1/Tel2 complex are contained in both mTORC1 and mTORC2. RAPTOR and PRAS40 are specific to mTORC1, and RICTOR, mSin1 and PROCTOR 1/2 are specific to mTORC2. These kinase complexes interact with specific substrates and initiate various signaling events that modulate cellular functions. mTORC1 controls the main cellular anabolic processes, linking them to the availability of nutrients; mTORC2 phosphorylates and activates Akt, controlling cellular metabolism, survival and organization of the cytoskeleton. The actions of mTORC1, mTORC2 and Akt are closely intertwined in some contexts. Thus, in growing and proliferating cells, Akt is a critical activator of mTORC1, and activated mTORC1 mediates by feedback inhibition of mTORC2 and Akt. Therefore, mTORC1, mTORC2 and Akt constitute a key metabolic signaling network that coordinates many of the metabolic processes in growing, proliferating cells and metabolic tissues [6, 7].

The aim of the work was to determine the activation of Akt, the main effector kinase of PI3K/Akt/mTORC/p70S6K cascade, in PBMC of patients with T2D and cancer.

2. Materials and methods

The study was conducted in the diabetology department of the Institute. All patients signed informed consent to conduct further diagnostic and research study. Immediately after collection, the blood was layered on histopaque 1077 (Sigma, USA), centrifuged at 500 g (RT) for 15 min in the 15 ml conical Falcon™ tubes, the PBMC collected were washed in PBS and frozen at -80 °C until use. For determination of phospho-Akt1/2/3 (p-S473) and phospho-p70S6K1 (p-T389) amounts ELISA kits 85-86046 and 85-86053 respectively (Invitrogen, USA) were used. The studies were carried out in triplets. The cells were lysed in the extraction buffer with inhibitors of proteases and phosphatases from the kits. The protein concentration in the lysate was determined using BCA protein assay kit (Novagen, USA). The measurements were carried out on a microplate reader (Bio-tek Instruments, USA) at a wavelength of 450 nm.

The OD values of samples obtained are located on the calibration curve satisfactorily coinciding with a theoretical line that indicates no scattering of the data.

The results of the study are presented as M ± SD, n = 6 - 15. To compare the data groups One-Way ANOVA and Student's *t*-test (with statistical module of Origin 7.0 software) were used. Values of $P \leq 0.05$ were considered as significant.

The following groups were investigated: 1 – control group (n = 6) – healthy people, representative by age; 2 – patients with T2D (n = 12); 3 – cancer patients (n = 15); 4 – patients with both cancer and T2D (n = 7). Patients with T2D used combined treatment with insulin and metformin. Patients with diabetes (groups 2 and 4) have HbA1c level – 7.4-9.2%. The patients of groups 3 and 4 have uterine, breast and bowel cancers. All examined patients belonged to the Caucasian race, age was in range from 46 to 72. Gender and BMI characteristics of the patients are shown in the Table 1. The patients and a control group were selected with close age and body mass index.

3. Results and Its Discussion

The PBMC include monocytes/macrophages and lymphocytes (T-cell, B-cell, NK) involved in the processes of cellular and humoral immunity. PI3K/Akt/mTOR is a signaling cascade that largely determines the functioning of these blood cells in diabetes and malignant neoplasm [2-4, 8].

As shown in Figure 1"2", the content of Akt phosphorylated by mTORC2 does not differ from the control in the group of patients with diabetes.

Table 1. Gender and BMI characteristics of the patients

	I group		II group		III group		IV group	
	Men (n=3)	**Women (n=3)**	**Men (n=6)**	**Women (n=6)**	**Men (n=5)**	**Women (n=10)**	**Men (n=3)**	**Women (n=4)**
Age (years)	57.0±1.70	60.0±3.71	60.17±2.94	57.50±1.17	60.4±5.8	59.2±2.19	61.3±2.12	55.5±1.9
BMI (кg/m2)	27.39±2.37	30.22±1.13	32.44±1.25	32.12±1.49	30.75±1.46	32.47±0.93	32.8±1.6	29.2±1.8

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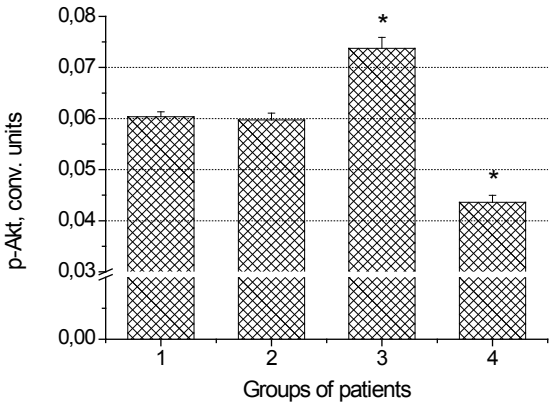

Figure1. Amount of phospho-Akt1/2/3 (p-S473) in peripheral blood mononuclear cell of patients with cancer and diabetes

PBMC cells were isolated from the blood, lysed in the extraction buffer with inhibitors of proteases and phosphatases and phospho-Akt1/2/3 amounts was determined using of ELISA kits.

All patients belonged to the Caucasian race with age in range from 46 to 72. The patients and a control group were selected with close age and body mass index.

1 - control (n = 6); 2 - patients with type 2 diabetes (n = 12); 3 - cancer patients (n = 15); 4 patients with both diabetes and cancer (n = 7). To compare the data groups One-Way ANOVA and Student's *t*-test were used. M ± SD, * - the difference from the control group is significant, P <0,05.

It is known that in tissues of patients with T2D, as a result of prolonged exposure to high doses of insulin, the activity of Akt and its downstream kinases, mTORC1 and p70S6K, is enhanced, resulting in phosphorylation of key adapter protein IRS-1 (S307 and other amino acid residues), its degradation, impaired insulin signaling and, consequently, insulin resistance [9]. The level of phosphorylated Akt in PBMC of patients with T2D is probably determined by the ratio of metformin and insulin effects. Metformin activates the AMPK and inhibits mTORC1 activity, but improves insulin signaling. Insulin activates the signaling cascade of PI3K/Akt/mTORC1 and inhibits the activation of AMPK by metformin [10]. The final result of the interaction of these drugs and the signaling mechanisms induced is the state of Akt activity.

At the same time, in PBMC of patients with T2D we observed 143.4±18.9% (M±SD) enhancement of p70S6K1 phosphorylation (p-T389), indicating the activity of the PI3K/Akt/mTORC1/p70S6K1 pathway. Obviously, mTORC2 does not participate in the activation of Akt in this context.

It has been shown earlier that PBMC of patients with systemic insulin resistance express matrix metalloproteinase 9, hypoxia-induced factor 1α, split AMPKα, IRS-1 phosphorylated on S312, Akt phosphorylated on T308, and TLR4 [11, 12]. TLR4 activation in macrophages caused increased expression of scavenger receptors via mTORC2/Akt/mTORC1 signaling cascade which accelerates oxLDL uptake and foam cell formation – key event in the pathogenesis of atherosclerosis [13].

In PBMC of cancer patients enhancement of Akt phosphorylation (p-S473) was observed (Figure 1.3). The phosphorylation level of p70S6K is also increased to 120.6±9.17 %. Thus, mTORC2 may contribute in activation of mTORC1 and its downstream targets.

It is well established that the PI3K/Akt pathway is frequently dysregulated in cancer [14]. Unlike mTORC1, the regulation of mTORC2 and its functional contribution to cancerogenesis remain poorly understood. Recent studies demonstrate that an intact mTORC2 is required for the activation of Akt *in vitro* and *in vivo* [15]. mTORC2 combines the effects of extracellular growth factors and survival signals with pathways controlling cell growth and proliferation. mTORC2 binds extracellular signals (such as growth and cytokine factors) with mTORC1 activation, cell proliferation and survival through direct phosphorylation of the protein kinase Akt on Ser473 in its hydrophobic C-tail that is required for its maximum activation [16]. Both complexes control each other, since the Akt regulates the phosphorylation of PRAS40, which disinhibits the activity of mTORC1, while p70S6K regulates Sin1 to modulate the activity of mTORC2 [17].

It has been shown that loss of mTORC2 in macrophages suppresses tumor growth. The mTORC2-mediated pathway comprising PI3K and Akt, is important for the accented glucose metabolism, promotes the activation of M2 macrophages (alternatively activated macrophages) and involves M-CSF as an upstream activator. M2 macrophages function in constructive processes, such as wound healing and tissue repair, and disable the activation of the damaging immune system by the secretion of anti-inflammatory cytokines such as IL-10 [18]. It is also known that macrophage M2 polarization – a key pro-tumoral phenomenon [19]. mTORC2 activity was found to be elevated in glioma cell lines and primary tumors as compared with normal brain tissue. Xenograft studies also supported a role for increased mTORC2 activity in tumorigenesis and enhanced tumor growth [20].

There was estimated a strong link between diabetes (especially T2D) and cancer. Hyperinsulinemia enhances the expression of insulin and IGF receptors that causes a cumulative mitogenic effect. Hyperglycemia provides cancer cells with excess of glucose [21]. Therefore somewhat

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unexpected was the decrease in the amount of phospho-Akt (Figure1"4") in the PBMC of patients group both with cancer and diabetes. The level of phosphorylation of p70S6K is also reduced to 89.7 ± 6.27% of the control level and more than 30% compared to the cancer group (Figure 1"3"). Consequently, in the PBMC of group 4 patients the activity of Akt, mTORC1 and p70S6K is significantly suppressed, as compared to the group of cancer patients.

Probable explanation for such inhibition may be competition for common signaling mechanisms. It is also possible the participation of tumor suppressors, such as TRIB3 (Tribbles pseudokinase 3), which suppress activation of Akt by mTORC2 in tumors [22]. The involvement of IKKα, a subunit of the IKK complex that controls NF-κB activation, is also not excluded. IKKα regulates the mTORC2 kinase activity directed to Akt on S473 and Akt-mediated phosphorylation of FOXO3a and GSK-3β, but not other Akt-associated targets such as TSC2 and PRAS40, which control the mTORC1/p70S6K activity [23]. TLR4 expression in macrophages mentioned above could activate NF-κB signaling [24].

It should be noted that we did not observe a significant difference in the activity of both kinases in the PBMC between patients with different types of cancer within groups 3 and 4. We also did not observe gender differences.

4. Conclusion

Thus, chronic diseases such as type 2 diabetes and cancer may have a systemic effect on signaling mechanisms in different tissues of the body, including blood cells. The state of Akt phosphorylation in PBMC can indicate the activity of mTORC1 and its substrates, which may be important for the evaluation of the pathological process and the efficacy of the drugs.

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