GLUT4 IS EXPRESSED IN CIRCULATING LYMPHOCYTES OF DIABETIC PATIENTS. A METHOD TO DETECT EARLY PREDIABETIC STAGES?

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SUMMARY

Type 2 diabetes changes expression of glucose transporters in circulating lymphocytes qualitatively and quantitatively, depending on GLUT isoform. In these blood cells obtained from 25 healthy subjects, as a control group, with no family history of diabetes, matched for age, body mass index and sex, only GLUT1 and GLUT3 were demonstrated. The results showed the surface expression of these glucose transporters in lymphocytes to differ between healthy subjects and diabetic patients. In diabetic patients, 25 in each study group, treated with diet, insulin algorithm of 2 injections and insulin algorithm of 4 injections, differences depended on the mode of therapy. In only one case, for GLUT1 and GLUT3 in lymphocytes of healthy subjects, the difference in the mean fluorescence intensity was not significant (p=0.2). In all other cases, the differences observed between investigated probes were statistically significant (p<0.05). In lymphocytes of diabetic patients, GLUT4 was additionally detected. These results were obtained using two methods: immunocytochemistry and flow cytometry.

INTRODUCTION

Glucose is a major source of energy in most mammalian cells. This hexose is transferred across the plasma membrane by facilitated diffusion along a concentration gradient involving transport proteins called GLUT. In human tissues and cells, 14 isoforms of these proteins have been described: GLUT1-GLUT12, GLUT14 and HMIT. The facilitative transporters exhibit different substrate specificities, kinetic properties and tissue expression profiles. In lymphocytes, expression of GLUT1, GLUT3 and GLUT6 has been detected (1-4). The typical insulin-responsive GLUT transporter isoform was not found in lymphocytes of healthy subjects. GLUT4 was not detected in lymphocytes obtained from patients with type 1 diabetes either (5).

Because GLUT1 and GLUT3 are typically expressed in lymphocytes of healthy subjects, we focused our investigations on the influence of type 2 diabetes and mode of therapy on the expression of GLUT4 in circulating lymphocytes; however, as an additional study, surface expression of GLUT1 and GLUT3 in
lymphocytes of diabetic patients was investigated in comparison with healthy subjects. As an additional control, the effect of lymphocyte incubation in pathologic concentrations of glucose in medium on GLUT4 expression in these blood cells was also assessed.

PATIENTS AND METHODS

Patients

The Medical University of Warsaw Bioethical Commission approved the study. Seventy-five diabetic patients participated in the study after having given an informed consent. Mean age of diabetic patients was 64.6±3.5 years; duration of diabetes 10.1±3.2 years; body mass index (BMI) 24.3±1.3 kg/m²; fasting plasma glucose 6.2±0.7 mmol/L; and HbA1c 6.9±0.6%. Twenty-five patients were treated with diet, 25 patients with insulin algorithm of 2 injections (mean dose 45.68±5.1 IU/24 h) and 25 patients with insulin algorithm of 4 injections (mean dose 65.37±6.0 IU/24 h). Twenty-five healthy subjects with no family history of diabetes, matched for age, BMI and sex were enrolled as a control group. None of these subjects received any medications for 3 months before and during the study.

Isolation of lymphocytes

Five mL of peripheral blood were collected into heparin containing tube. Circulating lymphocytes were separated by centrifugation with Histopaque (Sigma), according to the manufacturer’s instructions.

Culture of lymphocytes

Upon isolation, the lymphocytes obtained from healthy subjects were incubated for 24 h at 36.7 °C in PBS supplemented with 4% fetal bovine serum in different glucose concentrations. The concentrations of glucose in the medium were: 80, 40 and 160 mg/dL as normoglycemic, hypoglycemic and hyperglycemic conditions, respectively.

Immunocytochemistry

Upon isolation from diabetic patients and from healthy subjects, or after incubation, lymphocytes (5x10⁶) were placed onto the slide and 3% H₂O₂ was added (for 10 min at room temperature). Then the cells were washed with PBS, incubated with 2% normal goat serum in blocking solution (1% BSA in PBS) for 30 min at room temperature and washed 3 times with blocking solution. Specimens were incubated for 60 min at room temperature with polyclonal rabbit antisera against C-terminal sequence of GLUT4 (Chemicon, International, CA), diluted 1:200 with blocking solution. After incubation, the cells were washed 3 times with blocking solution and then incubated for 30 min at room temperature, with a second antibody-polyclonal goat anti-rabbit IgG labeled with peroxidase (Chemicon, International, CA), diluted 1:2000. The antigen-antibody complex was visualized using DAB (Gibco) according to the manufacturer’s instructions.

Lymphocytes incubated without the first antibody served as negative control.

Flow cytometry

Surface expression of GLUT1, GLUT3 and GLUT4 isoforms in lymphocytes of healthy subjects and diabetic patients on different modes of therapy was determined after staining with anti-GLUT antisera. For this procedure, 5x10⁵ lymphocytes were washed with 2 mL of buffer for FACS (PBS without Mg²⁺ and Ca²⁺, supplemented with 0.2% BSA and 0.02% sodium azide), centrifuged (4°C, 250g) and resuspended in 100 μL of buffer for FACS. The tubes with the cells were placed on ice. After this procedure, rabbit serum was added for control cell isotyping and 2 μL of the above antisera against investigated GLUTs were added for sample testing. The cells were incubated with antibodies for 30 min on ice and then 2 mL of buffer for FACS were added. The cells were washed again, resuspended in 100 μL of buffer for FACS and incubated for 30 min in the dark and on ice with 5 μL fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulin F (ab’)2.
(Dako). Then 2 mL of buffer for FACS were added, the cells were centrifuged (4 °C, 250 g) and resuspended in 500 μL of buffer for FACS with 1% formaldehyde.

Samples were analyzed using a FACS Calibur Flow Cytometer (Becton-Dickinson, San Jose, CA) equipped with an argon laser (wavelength 488 nm) using CellQuest program. Gates were set on M2. No permeabilization step was required because the study was focused on the GLUTs located on the surface of the cell membrane of the investigated blood cells. Therefore, in the analysis of the expression of investigated GLUT isoforms, we used the value of the mean fluorescence intensity (6,7).

Statistics

Statistical analysis was performed by the Kolmogorov-Smirnov test. A value of D>0.21 was chosen to identify GLUT4 expression in lymphocytes. The mean fluorescence intensity was analyzed using nonparametric Wilcoxon test. In this case, the significance of differences was set at p<0.05.

RESULTS

Immunocytochemistry

Lymphocytes used in this study as a negative control, incubated without the first antibody, remained negative for GLUT4. This result was obtained for investigated blood cells in all cases, both healthy subjects and diabetic patients (data not shown). Lymphocytes obtained from healthy subjects, incubated with anti-GLUT4 antibody, remained negative for GLUT4 using immunocytochemical detection (Fig. 1). Different results were obtained for lymphocytes collected from diabetic patients. In all cases, these circulating blood cells showed positive reaction with antibody against GLUT4 irrespective of the mode of therapy (Fig. 1).
Figure 2. Immunocytochemical detection of GLUT4 in lymphocytes incubated in different glucose concentrations. 
Left panel – positive probe (lymphocytes incubated with both first and second antibodies); right panel – negative 
control (lymphocytes incubated without first antibody) (for details, see text).
Lymphocytes incubated in normoglycemic conditions remained negative for GLUT 4 using immunocytochemical detection. GLUT4 was detected in lymphocytes incubated in hypoglycemic or hyperglycemic medium (Fig. 2). Stronger immunocytochemical reaction was observed in lymphocytes incubated in hyperglycemic conditions.

Flow cytometry

The observations obtained for GLUT4 using immunocytochemistry were confirmed by flow cytometry. In lymphocytes of healthy subjects, negative for GLUT4 using immunocytochemical method, the Kolmogorov-Smirnov test D value was <0.21, suggesting the lack of GLUT4 expression in these lymphocytes. Also, surface expression of this glucose transporter isoform expressed as mean fluorescence intensity was at a very low level (Fig. 3).

<table>
<thead>
<tr>
<th></th>
<th>Healthy subjects</th>
<th>Diet</th>
<th>Diabetic patients Insulin x 2</th>
<th>Insulin x 4</th>
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<td>GLUT1</td>
<td>0.42±0.13</td>
<td>0.38±0.04</td>
<td>0.64±0.18</td>
<td>0.41±0.16</td>
</tr>
<tr>
<td>GLUT3</td>
<td>0.49±0.14</td>
<td>0.28±0.07</td>
<td>0.28±0.09</td>
<td>0.65±0.11</td>
</tr>
<tr>
<td>GLUT4</td>
<td>0.10±0.02</td>
<td>0.53±0.09</td>
<td>0.24±0.09</td>
<td>0.38±0.12</td>
</tr>
</tbody>
</table>

GLUT1 and GLUT3 were detected in all study lymphocytes, yielding Kolmogorov-Smirnov test D values >0.21 (Table 1). On the other hand, type 2 diabetes mellitus and mode of therapy influenced surface expression of GLUT1 and GLUT3 in circulating human lymphocytes. In the lymphocytes of healthy subjects, the value of the mean fluorescence intensity for GLUT3 was higher than for GLUT1; however, these differences did not reach statistical significance (p>0.05). The mode of therapy had a different effect on the glucose transporters investigated (Fig. 3). Very strong stimulation of surface expression of GLUT1 was observed in lymphocytes from diabetic patients treated with diet as a mode of therapy, and lowest in those on insulin algorithm of 2 injections. It is to note that the values of the mean fluorescence intensity were higher in the blood cells obtained from diabetic patients in comparison with healthy subjects, irrespective of the mode of therapy in the former. The differences observed between investigated probes were statistical significant.

The value of the mean fluorescence intensity for GLUT3 was highest in lymphocytes of patients treated with insulin algorithm of 4 injections and lowest in blood cells of patients treated with insulin algorithm of
2 injections. In the latter, the value was lower in comparison with control subjects. The differences between particular values of the mean fluorescence intensity were statistically significant (Fig. 3).

DISCUSSION

Impaired glucose metabolism is a major pathogenic mechanism in glucose homeostasis. Disturbances are also observed in peripheral blood cells. Sustained hypoglycemia affects expression of glucose transporters. For example, granulocyte GLUT4 levels were increased by 73%, which was paralleled by a reduction in GLUT1, and in monocytes GLUT3 was elevated by 134% (5). On the other hand, hyperglycemia and/or hyperinsulinemia characterize diabetes.

In our study, we found nonspecific expression of GLUT4 in peripheral blood lymphocytes. The immunofluorescent detection of this isoform of glucose transporter is notoriously problematic. Therefore we used two independent methods for detection of GLUT4. Using immunocytochemical method, GLUT4 was never detected either in negative control or, more important, in lymphocytes obtained from healthy subjects. This result was confirmed by flow cytometry; the Kolmogorov-Smirnov test D value was <0.21 and the mean fluorescence intensity was at a very low level. On the other hand, in all cases of diabetic patient lymphocytes, irrespective of the mode of therapy, immunocytochemical reaction was positive for GLUT4. Thus, the results obtained by use of the immunocytochemical method suggested the presence of GLUT4 in circulating lymphocytes of diabetic patients. Consistent results were obtained using flow cytometry. In all cases of circulating lymphocytes obtained from diabetic patients, the Kolmogorov-Smirnov test D value for GLUT4 was >0.21 and the mean fluorescence intensity representing surface location of this glucose transporter was manifold higher in comparison with the value recorded for lymphocytes of healthy subjects.

GLUT4 is insulin-dependent glucose transporter. Insulin stimulates translocation of GLUT4 from intracellular compartment to plasma membrane (9). Hyperinsulinemia is a pathologic state observed in diabetic patients. Maybe that is why GLUT4 was expressed in lymphocytes of diabetic patients. Our suggestion was confirmed by the fact that the mean fluorescence intensity depended on insulin dosage. There was another intriguing observation in our study. The study included healthy subjects with no family history of diabetes. All these volunteers had normal level of fasting glucose (mean 90 mg/dL) but in some cases their lymphocytes showed immunoreaction with antisera against GLUT4. This result was obtained using both immunocytochemistry and flow cytometry (10).

Translocation of GLUT4 is also due to glucose; however, this result was obtained for human skeletal muscle (9). Expression of GLUT4 in lymphocytes from diabetic patients treated with diet as a mode of therapy is probably due to prolonged hyperglycemia and/or hyperinsulinemia in these patients. The incubation of lymphocytes for 24 hours in hyperglycemic conditions (180-200 mg of glucose per 100 mL of medium) causes expression of GLUT4 in these blood cells. GLUT4 expression was not found in normoglycemic medium (11). The same result was obtained in our study.

Nonspecific expression of glucose transporters, the same as in case of GLUT4 in lymphocytes of diabetic patients, was also observed in other diseases, especially tumors. For example, GLUT1 is not detected in normal gastric mucosa. GLUT1 protein is detected in 19% of gastric tumors and GLUT1 mRNA in 95% (12). Quantitative and/or qualitative changes in GLUT expression were also observed in human renal carcinoma (13), human brain tumor (14) and human breast cancer (15).

GLUT1 is an isoform of glucose transporter that occurs in lymphocytes. In lymphocytes obtained from diabetic patients, the mean fluorescence intensity was higher in comparison with these blood cells obtained from healthy subjects. It is difficult to explain this result. The study by Calderhead et al. (16) showed that insulin caused an increase in GLUT1 content in
plasma membrane of adipocytes. On the other hand, diabetes has a different influence on GLUT1 expression in different cells (17). Maybe the increase in surface expression of GLUT1 in lymphocytes of diabetic patients is due to the higher blood insulin level in diabetic patients in comparison with healthy subjects. However, this observation requires further investigations.

The same problem is faced trying to explain the results obtained for GLUT3. The results reported by other authors suggest that type 2 diabetes (17,18) or gestational diabetes (19) has no effect on the expression of SLC2A3 gene. The effect of insulin or impaired glucose level in serum on surface expression of GLUT3 in lymphocytes is also unknown. Therefore, as in case of GLUT1, this result needs further investigations.

The results obtained in our laboratory also showed other changes in lymphocytes of diabetic patients, not only in glucose transporters. We found the deoxy-D-glucose uptake by circulating lymphocytes to be impaired in diabetic patients (20). These results need further investigations. Are these disturbances due to changes in glucose transporters? Maybe type 2 diabetes impairs the key enzyme in glucose metabolism?

Lymphocytes may be a valid model system for detection of early stages of impaired glucose tolerance. Furthermore, immunocytochemistry is suitable for this investigation and can be used as an alternative to other diagnostic methods, especially in humans with a family history of diabetes. On the other hand, this hypothesis needs further, more detailed investigations in subjects with impaired glucose tolerance and in newly diagnosed diabetic patients.

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