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SHORT COMMUNICATION

Sample transport and/or storage can cause falsely low HbA1c levels in blood cells measured by enzymatic assay

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Abstract
Although HbA1c measurement by enzymatic assay (EA-HbA1c) is widely used in health-screening settings in Japan, recent studies have suggested lower EA-HbA1c levels as compared with HbA1c levels measured by high-performance liquid chromatography (HPLC-HbA1c). Hypothesizing that falsely low levels of EA-HbA1c are attributable to hemolysis caused by sample transport and/or storage, we measured EA-HbA1c in blood cells and whole blood after sample transport and compared them with HPLC-HbA1c levels. Blood samples were collected from ten non-diabetic individuals into sodium fluoride-containing blood collection tubes and immediately measured for EA-HbA1c in blood cells. After transport, the blood samples were again subjected to measurement of EA-HbA1c levels in blood cells and whole blood the following day. These EA-HbA1c levels were compared with HPLC-HbA1c levels. EA-HbA1c levels in blood cells measured immediately after sample collection did not significantly differ from HPLC-HbA1c levels. Transported blood samples showed hemolysis and significantly lower EA-HbA1c levels in blood cells, as compared with HPLC-HbA1c levels, whereas no significant difference was observed between EA-HbA1c levels in whole blood and HPLC-HbA1c levels. Transported blood samples showed hemolysis and falsely low EA-HbA1c levels in blood cells. Hemolysis caused by sample transport and/or storage might be responsible for the falsely low EA-HbA1c levels. This should be kept in mind, because falsely low HbA1c levels may lead to a false-negative diagnosis of diabetes.

Keywords HbA1c · Hemolysis · Sodium fluoride · Enzymatic assay

Introduction

HbA1c is widely used as a therapeutic target marker and as a diagnostic marker for diabetes. This has led to an increasing frequency of HbA1c measurement in the current health-screening settings throughout Japan. The available methods for measuring HbA1c include high-performance liquid chromatography (HPLC), affinity chromatography, immunoassay, and enzymatic assay (EA) [1]. Among these methods, EA is the most widely used in health-screening settings, as it is less expensive and can process a larger number of samples more quickly than the other methods. Otabe et al. showed that HbA1c levels measured by EA (EA-HbA1c) are lower than HbA1c levels measured by HPLC (HPLC-HbA1c) [2]. Recently, we also reported that HbA1c measured by EA during health checkups are lower than those measured by HPLC assay [3]. HPLC-HbA1c was measured on-site, while EA-HbA1c in blood cells was measured off-site after transport of the same sample in both studies.

We hypothesized that the falsely low level of EA-HbA1c was attributable to hemolysis caused by sample transport and/or storage. To verify this hypothesis, we collected blood samples from non-diabetic individuals into sodium fluoride-containing blood collection tubes for glucose measurement, transported them, and measured EA-HbA1c levels using CinQ HbA1c, a kit capable of measuring HbA1c levels in both whole blood and blood cells using an enzymatic assay.

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Subjects and methods

Subjects

Ten non-diabetic individuals (8 men and 2 women, age 33.7 ± 4.4 years, and casual plasma glucose level 95.5 ± 12.0 mg/dL) were included. The current study was performed in accordance with the Declaration of Helsinki, and was approved by the ethics committee of Hakuhokai Central Hospital (date of approval; July 25, 2018, approval no.: H30-3).

Laboratory methods

Blood samples were collected into two sodium fluoride-containing blood collection tubes for glucose measurement (Venoject II, Terumo Co., Tokyo, Japan). One was immediately centrifuged and subjected to HbA1c measurement in blood cells by EA (CinQ HbA1c, Arkray, Inc., Kyoto, Japan) [4]. The other sample in a test tube stand was transferred to Arkray Inc. by courier in a refrigerated state, and HPLC-HbA1c and EA-HbA1c were measured the day following blood sample collection. For measurement of HPLC-HbA1c as a control, the HA-8190 system (fast mode; Arkray, Inc., Kyoto, Japan) was used to measure HbA1c in whole blood. For measurement of EA-HbA1c, samples were centrifuged at 800*g* for 5 min and HbA1c in blood cells was measured. The samples were then agitated and HbA1c was measured in whole blood.

Statistical analyses

Results are expressed as mean ± SD. For statistical analyses, the paired Student's *t* test was used to compare two parameters. To analyze correlations between two parameters, Pearson's correlation coefficient was performed with the StatView computer program (Version 5.0 for Windows, Abacus Concepts, Berkeley, CA). *P* values < 0.05 were considered to indicate a statistically significant difference.

Results

EA-HbA1c levels measured immediately after blood sample collection (5.40 ± 0.16%) did not differ significantly from HPLC-HbA1c levels (5.40 ± 0.19%; Fig. 1a). All samples showed hemolysis the day after blood collection, with a plasma hemoglobin level of 331 (289–373) [median (interquartile range)] mg/dL. EA-HbA1c levels in blood cells (5.21 ± 0.19%) were significantly lower than HPLC-HbA1c levels and EA-HbA1c levels measured immediately after blood sample collection. In contrast, EA-HbA1c levels in whole blood (5.40 ± 0.18%) were significantly higher than EA-HbA1c levels in blood cells and did not differ significantly from HPLC-HbA1c levels or EA-HbA1c levels measured immediately after blood sample collection. EA-HbA1c levels in blood cells measured after transport showed a significant correlation with HPLC-HbA1c levels ($R = 0.980$, $P < 0.001$), although the regression line for the two variables ($y = 0.939x + 0.14$) was shifted below the $y = x$ line (Fig. 1b).

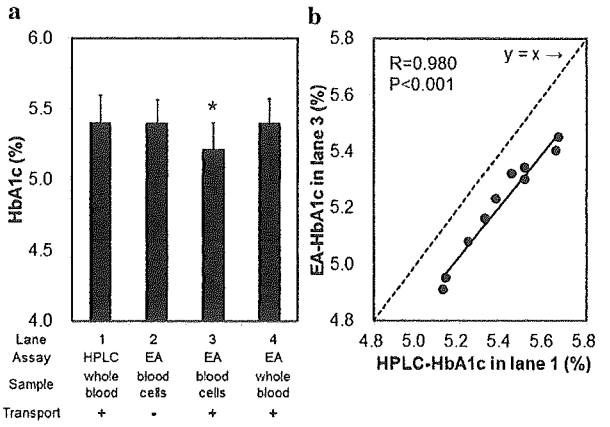

Fig. 1 Comparison of EA-HbA1c and HPLC-HbA1c levels measured under different conditions. **a** HbA1c levels in ten non-diabetic individuals were measured using different methods under different conditions. Lane 1: Collected blood samples were transported and HbA1c levels were measured in whole blood by HPLC the following day. Lane 2: Collected blood samples were immediately subjected to measurement of HbA1c levels in blood cells by EA. Lane 3: Collected blood samples were transported and HbA1c levels in blood cells were measured by EA the following day. Lane 4: Collected blood samples were transported and HbA1c levels were measured in whole blood by EA the following day. * $P < 0.001$ vs. lane 1, lane 2, and lane 4. **b** Post-transport EA-HbA1c levels in blood cells (**a**, lane 3) and HPLC-HbA1c levels (**a**, lane 1) in ten non-diabetic individuals were plotted to calculate a regression line. The dotted line represents $y = x$. *HPLC* high-performance liquid chromatography, *EA* enzymatic assay

Discussion

EA-HbA1c levels were accurately measured on-site immediately after sample collection, whereas EA-HbA1c levels in blood cells measured off-site after sample transport were falsely low. This means that diabetes screening based only on HbA1c-measured off-site in health-screening settings may underestimate diabetes. The present findings are important, given the increasing frequency of EA-HbA1c measurement in current health-screening settings.

The fact that there was no significant difference between EA-HbA1c levels measured immediately after blood sample collection and HPLC-HbA1c levels demonstrates the

accuracy of EA-HbA1c measurements. In contrast, EA-HbA1c levels in blood cells measured after sample transport were about 0.2% lower than HPLC-HbA1c levels. It has been documented that sodium fluoride-containing blood collection tubes for glucose measurement tend to cause hemolysis due to increased osmotic pressure [5]. This finding may decrease HbA1c values through hemolysis even if the sample is merely stored. However, the low level of HbA1c in health-screening settings suggests that sample transport further enhances hemolysis. Such hemolysis is more likely to occur in older red blood cells (RBCs) [5]. Moreover, because older RBCs contain more HbA1c than younger RBCs [6], measuring hemolyzed samples for HbA1c in blood cells, which mainly reflect HbA1c contained in younger RBCs, is likely to produce lower values. In contrast, EA-HbA1c in whole blood, with measurements conducted in homogeneous samples, appeared not to be affected by hemolysis.

In the current health-screening system, many examinees have their blood samples collected at nearby health-screening facilities and the collected blood samples are transported to testing laboratories, which conduct laboratory tests. Blood collection tubes for glucose measurement are commonly used to collect blood samples for measurement of plasma glucose and HbA1c. At testing laboratories, samples are centrifuged to obtain the plasma fraction for measuring the glucose concentration. Then, the precipitated fraction is used to measure HbA1c in blood cells. One of the reasons for measuring HbA1c in the blood cells likely involves saving the labor of mixing the sample after measuring plasma glucose to measure HbA1c in whole blood. Furthermore, among the three kits for HbA1c measurement by enzymatic assay available in Japan, only Arkray's CinQ HbA1c kit can measure HbA1c in both blood cells and whole blood. The other two kits can only measure HbA1c in blood cells, not in whole blood. Therefore, in most health-screening settings, blood samples collected into blood collection tubes for glucose measurement are sent to outside testing laboratories for measurement of EA-HbA1c in blood cells. Our data suggest that this transport and/or storage process causes hemolysis of blood samples, leading to falsely low levels of EA-HbA1c in blood cells [3]. With the urgent need to modify the current system, further studies are warranted to address these issues based on the present findings and develop a method of accurately measuring HbA1c. Although there were only ten subjects in this study, it is considered to be statistically correct results, because there is a significant difference. However, it is desirable to verify the present findings using many subjects in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human rights and informed consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent or a substitute for it was obtained from all patients prior to their participation in this study.

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