Methodological and Preanalytical Evaluation of a RAGE Immunoassay

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Abstract. Background: Soluble receptor of advanced glycation end products (sRAGE) is a promising biomarker for the prognosis and the monitoring of cancer and of acute diseases such as trauma and sepsis. Materials and Methods: We investigated the methodological characteristics of an ELISA for sRAGE (R&D Diagnostics) including intra- and inter-assay imprecision, dilution linearity and differences in various serum and plasma materials. Furthermore, the influence of various preanalytical factors such as time and storage temperature before and after centrifugation prior to definite deep freezing, as well as multiple freeze-thaw cycles, were tested. By the use of sera from 30 healthy individuals, a reference range and the dependency on patient characteristics was established. Results: Intra-assay imprecision (coefficients of variation (CV): 6.0-11.5%) and inter-assay imprecision (5.9-7.8%) were in an acceptable range of manual assays. Linearity testing yielded satisfying results with dilution recoveries of 99-131%. Results of serum, EDTA-plasma (recovery of 85.9-114.7%), and heparin-plasma samples (88-102%) were quite comparable, while results from citrate-plasma were slightly lower (78-96%). There was no influence of the time to centrifugation after 6 and 24 hours (recoveries 87-102%) at storage temperatures of 4˚C and 25˚C. Similarly, results were the same when samples were kept at 4˚C and 25˚C after centrifugation for up to 7 days (recoveries 88-109%). Repeated freeze-thawing of samples did not affect the results obtained for the RAGE protein (recoveries 92-104%). The median value of healthy individuals was 1.10 ng/ml, with 90% limits of 0.52 to 1.49 ng/ml. Conclusion: sRAGE ELISA is a very robust and safe assay which produces reliable quantitative results for sera and plasma measurements.

RAGE is a type I transmembrane protein belonging to the immunoglobulin superfamily and is composed of three immunoglobulin-like domains, a transmembrane domain and a cytoplasmic domain (1). Originally known as a receptor for advanced glycation endproducts (AGE) it has been shown to be a multiligand receptor also for high mobility group box 1 (HMGB1), S100 family members, DNA, RNA, serum amyloids, immunoglobulin light chains and possibly of further as yet unknown ligands. Through these various interactions, it plays an important role in chronic diseases such as diabetes and amyloidosis, as well as in tumors of different kinds, and inflammatory conditions such as sepsis and trauma (2-4).

In addition to the membrane-bound forms of RAGE, which are difficult to examine under clinical conditions, soluble forms (sRAGE) have been found to be measurable markers in venous blood samples. Two different mechanisms are considered in order to explain the origin of sRAGE: alternative splicing of premRNA resulting in endogenous secretory RAGE (esRAGE) and sRAGE deriving from proteolytic cleavage of the membrane-bound receptor, so-called cleaved or cRAGE (5, 6). With its functions as a membrane-associated receptor this molecule is important in cell activation, inflammation and cell migration. Its function as a soluble receptor may be that of a decoy receptor and the measured quantity of sRAGE may correlate with its expression on cell surfaces.

Some articles have already reported a potential use of sRAGE as a serum marker for monitoring a wide range of acute and chronic diseases, so far focusing on diabetes and its related complications (7-9).

In order to be able to perform reliable clinical studies on this subject, preanalytical examination of methodological performance is a crucial issue. Brown and Fraser recently presented some promising data on the analytical performance
of an enzyme-linked immunosorbent assay (ELISA). The stability was tested up to 24 hours after centrifugation of blood samples and the biological variation was evaluated, coming to the conclusion that quantitative analysis of sRAGE shows adequate performance characteristics and may be most useful in disease monitoring (10).

Here we extend those stability investigations to a longer time interval before and after centrifugation and to different temperature conditions. This would be particularly important if samples are shipped from non-hospital facilities by postal services or over the weekend to a laboratory. Furthermore the influence of multiple freeze-thaw cycles has been shown to be relevant for samples used in research facilities and the comparison of different serum and plasma sample types is important for the comparability of clinical and research data.

Materials and Methods

Methodological characteristics of the RAGE immunoassay. In order to analyze the methodological performance of the RAGE immunoassay (R&D Diagnostics, Abingdon, UK), the intra- and inter-assay imprecisions were tested in three different sera samples with different RAGE concentrations under five and eleven repetitions, respectively. The dilution linearity was assessed at four serial two-step dilutions (1:1, 1:2, 1:4, 1:8) using two sera samples with high RAGE concentrations.

Concerning different materials, serum and EDTA-plasma from seven patients, and heparin- and citrate-plasma from four of the original seven patients were tested.

Evaluation of preanalytical potentially influencing factors. Sera of six volunteers were exposed to several potentially influencing factors such as prolonged time of transportation, storage at different temperatures after centrifugation and several freeze-thaw cycles.

In the first experiment, blood samples that were collected into serum separator tubes (Sarstedt GmbH, Nümbrecht, Germany), were stored at 4°C (refrigerator temperature) and 25°C (room temperature) for 0 h, 6 h, and 24 h prior to centrifugation at 3000 g for 15 min. Finally, the serum was aliquoted and deep-frozen at −80°C immediately.

In a second experiment, blood samples were centrifuged 15-30 min after venous puncture at the same conditions (3000 g for 15 min). Then they were aliquoted and were kept for 0 h, 6 h, 24 h, 48 h and 7 days at different temperatures (4°C and 25°C) before freezing at −80°C.

Finally, samples were thawed and refrozen at −80°C once, twice and three times in order to assess the influence of multiple thawings.

Samples were analyzed in batches containing all samples from a single patient in one immunoassay run, in order to minimize interassay analytical errors.

RAGE assay. The RAGE assay was performed according the instructions of the manufacturer (R&D Diagnostics, Abingdon, UK). The assay is based on a quantitative sandwich enzyme immunoassay technique. The microplates were pre-coated with monoclonal antibodies specific for the extracellular domain of RAGE. In each well, 50 μl of standards, controls or samples were added to 100 μl of assay diluent, covered by an adhesive strip, and were left to incubate for two hours at room temperature in order to enable the binding of any RAGE molecules to the fixed antibodies. RAGE-antibody complexes were cleared from unbound substances by a four-fold washing step, using 400 μl of washing buffer in each step. The RAGE conjugate (200 μl), consisting of polyclonal antibodies also specific for the extracellular domain of RAGE was then added and was left to incubate for two hours at room temperature. After a further washing procedure, 200 μl of substrate solution was added to each well. The plate was then incubated for 30 min at room temperature, protected from light by an aluminium foil cover, and was stored in a dark cupboard. Finally, 50 μl of stop solution were added to each well and the intensity of the resulting color was measured photometrically within 30 min at a wavelength of 450 nm, without reference filter. Final concentration values in ng/ml were calculated by use of the standard curve material provided by the manufacturer (R&D Diagnostics, Abingdon, UK).

Statistics. For all comparisons, the means (or medians) and the ranges were calculated. The coefficients of variation were assessed for imprecision experiments. Recoveries were calculated for the dilution, the material and the stability in the experiments. In healthy individuals, the median and the 90th percentiles of RAGE values were established.

Results

The intra-assay imprecision tested in five repeats of three serum samples was 6.0% in the low concentration RAGE sample (0.12 ng/ml), and 5.6% and 11.5% in the samples with higher RAGE concentrations (0.88 and 1.07 ng/ml), respectively. The inter-assay imprecision was assessed by performance of eleven repeats of two serum samples (1.38 and 1.67 ng/ml respectively) at 5.9% and 7.8%.

The dilution linearity of the two sera with high RAGE concentrations (undiluted values of 0.88 and 1.01 ng/ml) yielded satisfying results, with a mean dilution recovery of 117% (range=99.1-131%).

The measurement of RAGE in serum and EDTA-plasma samples revealed similar results, with a mean recovery of 95.4% (range=85.9-116%). RAGE values in heparin-plasma were also comparable, with a mean recovery of 97.0% (range=88.3-102%), whereas results from citrate-plasma were generally slightly lower, with a mean recovery of 88.1% (range=77.9-96.1%).

Prolonged storage of blood samples before centrifugation at room temperature (25°C) did not result in major deviations on the concentrations of RAGE, with mean recoveries of 97.7% after 6 h (range=87.0-111%) and of 93.6% after 24 h (range=86.1-103%).

The storage of serum samples after centrifugation at 4°C and 25°C for a time period of up to 7 days also did not affect RAGE levels. The mean recovery after 6 h was 99.4% (range=90.3-111%) at 4°C and 100.5% (88.5-112%) at 25°C; after 24 h, it was 99.5% (range=88.3-112%) at 4°C and 98.8% (87.8-115%) at 25°C; after 48 h it was 100.6% (range=97.9-106%) at 4°C and 96.1% (91.4-106%) at 25°C;
and after 7 days, it was 104.9% (range=102-108%) at 4°C and 100% (88.7-109%) at 25°C, respectively (Figure 1).

Finally, repeated freeze-thaw cycles did not lead to different RAGE results, yielding a mean recovery of 98.5% (range=92.6-104%) after two freeze-thaw cycles and of 98.4% (range=89.9-104%) after three freeze-thaw cycles. The measurement of RAGE in sera of 30 healthy individuals showed a wide range of values, with a median of 1.10 ng/ml and 90% limits of 0.52 to 1.49 ng/ml.

Discussion

With the involvement of sRAGE in numerous acute, chronic and neoplastic diseases rapidly becoming a greater subject of interest in clinical studies, knowledge on analytical performance characteristics of sRAGE ELISA is crucial for the correct interpretation of the measured values and the study of results in general.

While Brown and Fraser have already reported some promising data on the analytical performance of a RAGE ELISA for serum determinations (10), we can confirm their results concerning the good intra- and inter-assay imprecision, which were in the acceptable range for manual assays (coefficients of variation <15%), as well as a satisfying dilution linearity in the present study. An important finding was the very good comparability of RAGE results obtained in serum, EDTA- and heparin-plasma, while the RAGE levels in citrate-plasma were somewhat lower.

Concerning the stability of RAGE when blood samples were exposed to adverse preanalytical conditions, our data confirmed the robustness of the analyte and the assay. While Brown and Fraser have already described high stability of sRAGE in serum and plasma after centrifugation for up to 24 h, we extended the time of investigation considerably to seven days and found remarkably stable results for storage of serum samples after centrifugation at both 4°C and 25°C, that is, for storage conditions in a refrigerator or at room temperature. This finding is highly relevant, as the transport of samples from external sites sometimes takes days or may be delayed during the weekend. Furthermore, it shows that results for RAGE of multicentric studies are comparable even if samples are stored after centrifugation in a different manner.

Figure 1. Median and individual courses of receptor for advanced glycation end product (RAGE) levels during different preanalytical conditions. Storage for different times at 25°C before centrifugation (A), at 25°C after centrifugation (B), at 4°C after centrifugation (C), and after different freeze-thaw cycles (D).
A further preanalytical feature that is critical for some biomarker levels, such as nucleosomes (11), is delayed centrifugation. Therefore, we investigated the storage of blood samples at room temperature up to 24 h prior to centrifugation and found very stable RAGE results. This point is relevant as the preanalytic standards are not always respected in diagnostic studies.

An issue of special importance is the repeated freezing and thawing, if serum material is limited in diagnostic studies, if repetition of the measurement is necessary, or if the cold chain is broken for any reason. However, for RAGE, there was no significant influence of several freeze-thaw cycles on the obtained marker levels.

In line with the observations of Brown and Fraser (10), we found a broad range of normal serum sRAGE levels in healthy individuals. This is noteworthy and shows the high variation of this marker, which may be attributed to metabolic differences that are not necessarily linked to any pathology. However, it also shows that RAGE may be less efficient as a screening marker for any disease but may be more useful for intra-individual monitoring or for risk assessment for a given pathology.

Conclusion

RAGE ELISA was shown to be a robust assay that may be applied to different blood materials for producing reliable, safe results under usual and difficult preanalytic conditions and could very well be established in routine practice.

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References