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Application of a bridging ELISA for detection of anti-erythropoietin binding antibodies and a cell-based bioassay for neutralizing antibodies in human sera

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ABSTRACT

Although erythropoietin (EPO)-related pure red-cell aplasia (PRCA) is a rare disorder, attention still needs to be paid because underline mechanism of EPO immunogenicity is various and controversial. Among several assay systems for screening of anti-EPO binding antibodies (Abs), we adopted and setup the bridging ELISA using streptavidin-coated plate. To test their neutralizing activities, cell-based neutralizing (NT) bioassay was setup. When we analyzed serum samples by using these two assays, we found two positive results in the two samples. In the sample 1, 411.9 ng/ml of anti-EPO Abs were found and neutralizing activity of 36.2% at 1:5 serum dilution was detected. In the sample 2, 40.5 ng/ml of anti-EPO Abs were found and neutralizing activity of 96.7% was detected. Our results indicate that the higher anti-EPO antibody (Ab) level in a serum does not always lead to the stronger neutralizing activity. This report gives crucial consideration to the needs of establishing clear criteria to link various assay parameters with the onset of PRCA and its progression.

1. Introduction

Human erythropoietin (EPO) is a glycoprotein that stimulates proliferation and differentiation of erythroid progenitor cells in the bone marrow. Patients with chronic kidney disease (CKD) suffer from anemia caused by reduced production of EPO in the kidney. Recombinant human EPO protein has been used successfully to treat anemia associated with CKD [1]. Recently, attention has been paid to the development of several side effects by EPO therapy including EPO-related pure red-cell aplasia (PRCA), in some patients with CKD. PRCA is a rare disorder of erythropoiesis that leads to a severe anemia due to an extremely decreased production of red blood cells [2,3]. EPO-related PRCA is thought to be caused by the production of EPO neutralizing antibodies (Abs) that eliminate the biological activity of EPO as well as endogenous EPO in patients undergoing therapy [4]. The clinical significance of Abs against EPO has been emphasized by a recent increase in PRCA cases [3,5–7].

Several assays are currently used to screen anti-EPO Abs, including enzyme-linked immunosorbent assay (ELISA) [8,9], radioimmunoprecipitation (RIP) [3,10], surface plasmon resonance (SPR) [11] and bioassays measuring EPO neutralizing Abs. However, there has been no standard assay established to compare data from different laboratories.

Anti-EPO Abs are detectable by a conventional or a bridging ELISA. Variations of these assays have been developed to improve sensitivity and specificity [12]. Conventional ELISA detects serum Abs that bind to EPO proteins coated on plastic plates by using enzyme-conjugated secondary Abs and its substrates. To reduce nonspecific background, an optimization is needed in conventional ELISA. In a bridging ELISA, a bridge is formed between EPO immobilized on plastic wells and enzyme-labeled EPO via anti-EPO Abs. A bridging ELISA reduces background readings due to fewer amplification steps in the procedure and the requirement for two specific binding events for the target EPO, which increases a specificity of the assay. This method is rapid, easy, inexpensive, high throughput, and highly specific. However, this method can give nonspecific matrix effects and may fail to detect low affinity Abs. Recently the

Abbreviations: EPO, erythropoietin; PRCA, pure red-cell aplasia; Ab, antibody; Abs, antibodies; NT, neutralizing; CKD, chronic kidney disease.

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new double antigen bridging ELISA was developed [8,13,14]. Anti-EPO Abs in CKD patients' sera bind via one antigen binding site to biotinylated-EPO immobilized to streptavidin-coated microtiter plates and via second site to digoxigenylated-EPO. The amount of bound anti-EPO Abs is determined by an anti-digoxigenin Ab conjugated to peroxidase. This assay was very sensitive (a lower limit of detection is 1 ng/ml), specific (no backgrounds in normal serum samples) and suitable for screening large numbers of samples.

Although the appearance of neutralizing anti-EPO Abs are important diagnostic aspect in Ab-mediated PRCA, RIP, ELISA, and SPR assays may be only used to test anti-EPO binding Abs. To detect and characterize neutralizing activity of anti-EPO Abs, neutralizing (NT) bioassay is required. Primary erythroid cells or certain cell lines are stimulated to form colonies or proliferate in the presence of recombinant EPO. Activity of recombinant EPO could be inhibited by a patient serum sample if this contains neutralizing Abs against EPO. Because primary cells grow slowly and finitely, immortalized or cancer cell lines have been utilized as alternative EPO-responder cells. These cell lines include UT-7 cell (human erythroleukemia cell line) [2] and IL-3-dependent murine hematopoietic cell line 32D [15]. It has been reported that proliferation of UT-7 cells expressing a large number of EPO receptors was inhibited by PRCA patients' serum [2]. IL-3-dependent murine hematopoietic cell line 32D was transfected by human EPO receptor gene to express EPO receptors on their cell surface. Therefore, this cell line is able to respond to human EPO as well as murine IL-3. Swanson et al. found anti-EPO Abs from the CKD patients' sera and checked their neutralizing activity with bioassay using murine 32D cell line [15]. Although a neutralizing activity of serum samples can be determined by NT bioassay, it has some hurdles to overcome. Each laboratory uses different EPO-responder cell lines, sensitivity of bioassay is not so high and scale-up and automation is rather difficult.

In this paper, we set up a bridging ELISA for detection of anti-EPO binding Abs and a cell-based NT bioassay to test neutralizing activity of these EPO binding Abs in human sera. We applied these assays to screen serum samples, isolated two anti-EPO positive samples, and examined their neutralizing activity. We have found that the lower level of anti-EPO Ab shows the higher neutralizing activity. This was most likely due to the more neutralizing anti-EPO Abs despite the lesser total amount of Abs in one sample. The follow-up studies are required to link this result to clinical data.

2. Materials and methods

2.1. Biotinylation of erythropoietin (EPO)

Biotin was labeled to recombinant EPO with EZ-Link® Sulfo-NHS-LC-Biotinylation Kit (Pierce) according to the manufacturer's protocol. In brief, 10 mM Sulfo-NHS-LC-Biotin was dissolved in PBS and mixed with 1 mg of EPO for 2 h on ice. A Zeba™ desalt spin column was used to exchange buffer and remove excess biotin reagent. The level of biotin incorporation was measured by HABA assay. Protein concentration was determined by DC Protein Assay (Biorad).

2.2. Digoxigenylation of EPO

Digoxigenin-3-O-methylcarbonyl-aminocaproic acid-N-hydroxysuccinimide ester (DIG, Roche) was labeled to EPO according to the manufacturer's protocol. In brief, 5 mg of DIG was dissolved in DMSO and incubated with 0.5 mg of EPO dissolved in PBS, pH 8.5, for 2 h at room temperature (RT). A Microcon® Centrifugal Filter Units (Millipore) was used to exchange buffer and remove excess DIG reagent. Protein concentration was determined by DC Protein Assay (Biorad).

2.3. Preparation of standards and serum samples for a bridging ELISA

Mouse monoclonal anti-EPO antibody (Ab) (Epo1, abcam) was used as standards for ELISA. 160, 80, 40, 20, 10, 5, and 0 ng/ml of standards were prepared by mixing Epo1 monoclonal antibody (mAb) of each concentration with normal human serum (1:5 dilution) in PBSAT (phosphate buffered saline, 1% BSA, 0.05% Tween-20). Serum samples were prepared with 1:5 dilutions in PBSAT.

2.4. Bridging ELISA

Streptavidin-coated 96-well microplates or 8-well strips (Pierce) were washed twice with PBST (phosphate buffered saline, 0.05% Tween-20). After washing, 1 μg/ml of biotinylated-EPO (BIO-EPO) was added to each well and incubated for 1 h at RT. After incubation, plates were washed three times in a microplate washer (Molecular Devices) or manually. Standards mentioned above were mixed with an equal volume of 40 ng/ml of digoxigenylated-EPO (DIG-EPO). This mixture was added to wells and incubated for 2 h at RT. Also 1:5 diluted serum samples were mixed with an equal volume of 40 ng/ml of DIG-EPO in PBSAT and this mixture was added to wells and incubated for 2 h at RT. After incubation, plates were washed three times and anti-DIG Fab-HRP conjugate (Roche) diluted in PBSAT was added to each well and then incubated for 1 h. After washing, ABTS solution (Roche) was added to each well and incubated. Optical density was measured at 405 nm (reference wavelength: 492 nm) by Versamax microplate reader (Molecular Devices).

2.5. Bioassay

Human acute myeloid leukemia cell line, UT-7 (DSMZ, Germany) was maintained in alpha-MEM (Hyclone) supplemented with 20% FBS (Gibco) and 1 IU/ml of EPO in a humidified CO_2 incubator for more than 6 months. UT-7 cells were washed three times with alpha-MEM and added to each triplicate well at 10^4 cells in EPO free culture medium for 1 day. Two fold dilutions from 20 IU/ml of EPO, 20 ng/ml of GM-CSF (R&D systems), or 20 ng/ml of BSA (Calbiochem) were mixed with equal volumes of UT-7 cells in wells and incubated for 3 days at 37 °C in a CO_2 incubator. After incubation, EZ-Cytox cell viability assay kit (Daeil Lab Service, Korea) was used to measure cell proliferation. 15 μl of kit reagent was added to each well and incubated for 1–6 h in CO_2 incubator. After incubation, optical density was measured at 450 nm (reference wavelength: 690 nm).

2.6. Preparation of positive controls and serum samples for neutralizing (NT) bioassay

Rabbit polyclonal anti-EPO Ab (H-162, Santa Cruz Biotechnology) was used as positive controls of NT bioassay. 40, 20, 10, 5, 2.5, 1.25, 0.63, and 0.31 ng/ml of samples were prepared by dissolving H-162 Ab in the UT-7 medium. Serially diluted serum samples (ranging from 1:1.25 to 1:80 dilutions) were prepared in the same media.

2.7. NT bioassay

UT-7 cells were washed three times with PBS and incubated in EPO free UT-7 growth medium for 1 day. Following day, 10^4 cells were seeded in each triplicate well. 0.8 IU/ml of EPO and various concentrations of standards or serum samples were mixed and incubated for 1 h at 37 °C. After incubation, these mixtures were added to the cells to reach a final concentration of 0.2 IU/ml

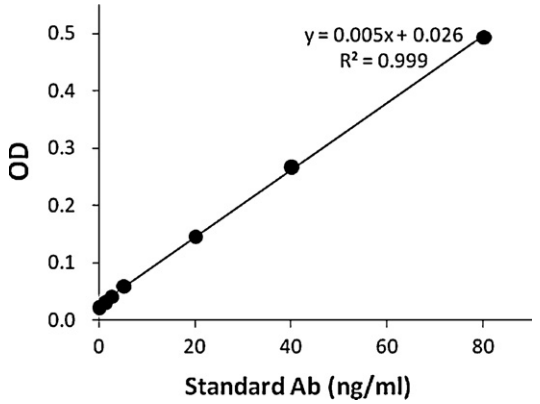

Fig. 1. Standard curve of the anti-EPO bridging ELISA. Mouse monoclonal anti-EPO Ab was used as standards. Several dilutions of standards spiked with human serum (1:5 dilution) were mixed with DIG-EPO and added to the streptavidin-coated microplate well pre-immobilized with BIO-EPO and incubated for 2 h at RT. Then, anti-DIG Fab-HRP was added to each well and incubated for 1 h at RT. ABTS solution was added and absorbance was measured at 405 nm with 490 nm as reference wavelength. Absorbance values of standards were subtracted by the value of the blank of the ABTS-substrate only.

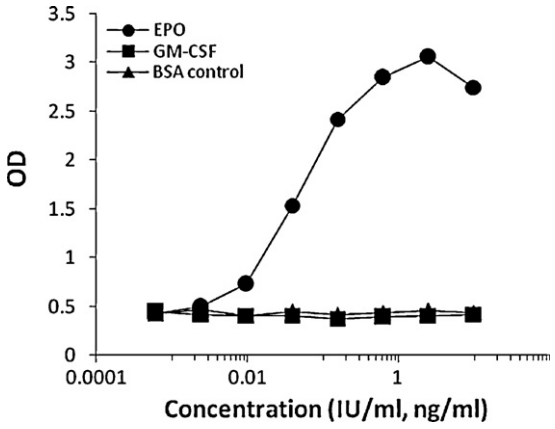

Fig. 2. Proliferative responses of UT-7 cell line to serially diluted recombinant EPO, GM-CSF, or BSA. Cells were plated at 10^4 per well and incubated in the presence of increasing concentrations of EPO, GM-CSF, or BSA. After incubation for 3 days, tetrazolium salt (WST-1) was added to measure cell proliferation. Absorbance was measured at 450 nm. Each value represents the mean of triplicate samples.

EPO and incubated for 3 days at 37 °C in CO_2 incubator. After incubation, EZ-Cytox cell viability assay kit was used to measure cell proliferation. 15 μl of kit reagent was added to each well and incubated for 1–6 h in CO_2 incubator. After incubation, optical density was measured at 450 nm (reference wavelength: 690 nm).

3. Results

3.1. Setting up a bridging ELISA for screening of anti-EPO binding Abs in the serum samples

Anti-EPO bridging ELISA utilizes the divalent EPO binding domain on each Ab that forms a bridge between two differently labeled antigens at optimal concentration. This assay has been shown to have very low backgrounds compared to the non-bridging conventional ELISA [16] (data not shown). A bridging ELISA used in this study was based on the streptavidin/biotin-coated ELISA [8] with little modification. For example, reference material was mouse monoclonal anti-EPO Ab instead of affinity purified rabbit polyclonal Abs. In addition, EPO was biotinylated only through amino group with a biotin-to-EPO ratio of 15.6.

Standard curve (Fig. 1) was very similar (data not shown) to that of anti-EPO Ab assay kit (kindly provided by Dr. Anton Haselbeck, Roche Pharma Research). The final concentrations from 1.25 to 80 ng/ml of standards were established. The lower limit of detection (LLD) was 0.3 ng/ml (mean of blank + 3 SD) and the lower limit of quantitation (LLQ) was 3.2 ng/ml (mean of blank + 6 SD). Intra-assay precision for normal sample serum was 6.9% CV (n = 10) and inter-assay precision of EPO Ab positive human serum was 11.3% CV (n = 5).

Negative reference group was chosen from healthy donors and CKD samples (n = 143) to determine cut-off point. The 99th percentile of negative reference group was determined at 13.5 ng/ml. Three negative sera ranging from 0 to 20 ng/ml (data not shown) were shown to contain no EPO binding Abs in the following two repetitive assays and showed no EPO neutralizing activity in the NT bioassay. All serum samples with over 0 ng/ml of concentration in the first assay were analyzed repeatedly and sometimes confirmed by a conventional ELISA.

3.2. Setting up a NT bioassay for detection of anti-EPO neutralizing Abs in the serum samples

To test the neutralizing activity of anti-EPO binding Ab in the positive serum samples, cell-based NT bioassay was setup. EPO diluted serially from 10 IU/ml proliferated UT-7 cells in a dose-dependent and saturated manner (Fig. 2). Cell proliferation was activated only under the presence of EPO not in the case of GM-CSF and BSA controls. To establish EPO-dependent subline of UT-7 (UT-7/EPO), we cultured UT-7 cells in the presence of Epo (1 U/mL) for more than 6 months [17]. The measurement of cell proliferation was performed by non-radioactive and spectrophotometric assay (WST-1). Cell starvation for 1 day and incubation for 3 days were required for obtaining high resolution data for our assay (data not shown). Intra-assay precision for normal sample serum was 10.5% CV (n = 9) and inter-assay precision of % neutralization of positive control (10 ng/ml) was 2.1% CV (n = 8). Reference to this proliferation data, 0.2 IU/ml of EPO was chosen for a single concentration used in the following NT bioassay.

0.2 IU/ml of EPO was completely neutralized by 10 ng/ml of positive control (rabbit polyclonal anti-EPO Ab) and activity was decreased proportionally to the dilutions (Fig. 4). Normal control sera were multiply diluted and their neutralization background was tested to determine the cut-off point (Fig. 3). As all tested values of dilutions were positioned under the 25% neutralization and maximum % was 20.8%, 25% was determined as assay threshold for the detection of EPO neutralizing positive serum. Similarly, 25% assay cut-off was previously reported in the ^{3}H-thymidine uptake neutralizing assay using UT-7/EPO cell line [18]. When routine analysis was performed, multiple dilutions of one or two normal controls were always included and activity above 25% was not observed.

3.3. Application of a bridging ELISA and NT bioassay to distinguish anti-EPO binding Ab with neutralizing Ab in the serum sample

Serum samples from EPO-resistant cases were screened to test if they had anti-EPO binding Abs or not with a bridging ELISA. Among them, two serum samples were identified for containing anti-EPO binding Abs (Fig. 4A). Concentration of sample 1 was 411.9 ng/ml and sample 2 was 40.5 ng/ml. These concentration values were well above the 99th percentile of 13.5 ng/ml. Normal serum control did not contain any anti-EPO Ab.

To compare the binding pattern of serum samples 1 and 2 in detail, multiple dilutions were prepared and performed the bridg-

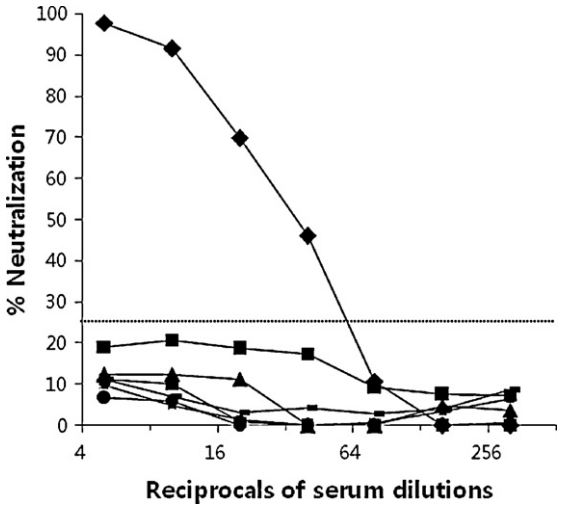

Fig. 3. A multiple dilution approach to determine the cut-off value of NT bioassay. Rabbit polyclonal anti-EPO Ab was used for a positive control at 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625 ng/ml (closed diamond). Normal human serum samples ($n = 6$) were tested at 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320. These samples were incubated with 0.2 IU/ml of EPO and incubated for 1 h at 37 °C. These mixtures were added to 10^4 UT-7 cells and incubated for 3 days and cell proliferative assay was performed as described in Fig. 2. Final results were determined as percentage neutralization with the following formula: % neutralization = [(OD of EPO alone − OD of EPO with diluted serum sample)/(OD of EPO alone − OD without EPO)] × 100. Mean OD values of triplicate samples are shown. The horizontal line represents the assay cut-off point.

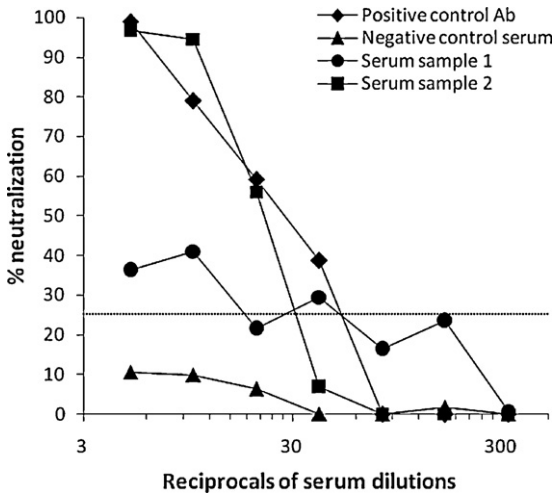

Fig. 5. Comparison of neutralizing activity between serum samples 1 and 2. Neutralizing activities of multiple dilutions of serum 1 and 2 were examined by NT bioassay. Mean values of triplicate samples are shown and minus values were changed to zero. Rabbit polyclonal anti-EPO Ab was used for a positive control at 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625 ng/ml. For details of procedure see Fig. 3.

ing ELISA. Dose dependency and specificity were indicated at both samples. Serum sample 1 contains significantly higher Ab titers than serum 2 through the range from 1:5 to 1:20 dilution (Fig. 4B).

After finding of two anti-EPO positives, to test the neutralizing activities at every possible dilution, NT bioassay was performed as described in Figs. 2 and 4. To date, an association between the level of Ab and neutralizing activity has not been demonstrated. It was just reported that serum samples with a high Ab level (>1000 ng/ml) are associated with a complete neutralizing activity [13]. Because the Ab levels of our samples were below 1000 ng/ml, we speculated that the serum sample with higher level of Ab would show the stronger neutralizing activity. However, unexpectedly, serum sample 2 possessed much stronger neutralizing activity than sample 1 (Fig. 5). This tendency was maintained clearly to 1:40 dilution. Neutralizing pattern of sample 2 was quite similar to positive control Ab and exhibits almost complete neutralization activity at 1:5 dilution. Every dilution of samples 1 and 2 showed higher neutralizing activity than normal control serum. In conclusion, serum sample 1 containing the much higher anti-EPO Abs (411.9 ng/ml) than sample 2 (40.5 ng/ml) showed the much less potent neutralizing activity against EPO (36.2%) than sample 2 (96.7%) at 1:5 dilution (Fig. 5).

4. Discussion

Ab-associated PRCA is a rare adverse event from EPO administration to CKD patients. However, new cases of Ab-associated PRCA have still deposited in FDA safety databases [19]. Considering unreported PRCA, undetected PRCA, and non-developed PRCA despite the presence of Ab throughout the world, it is still important to be

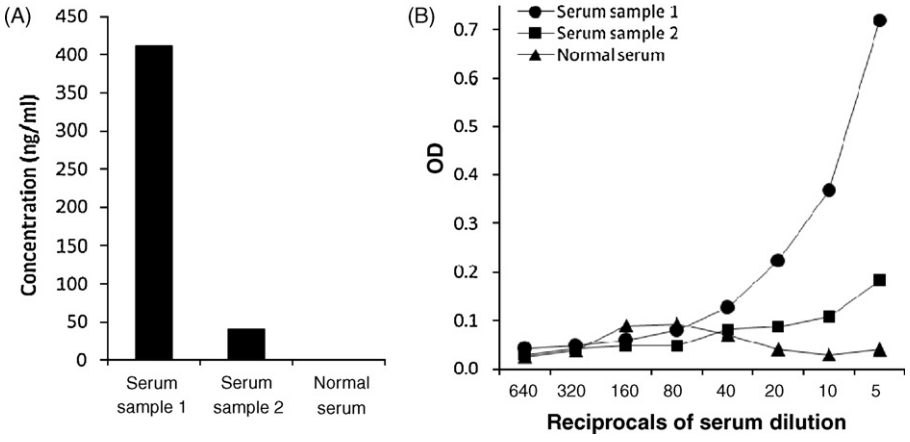

Fig. 4. Identification of serum samples 1 and 2 containing anti-EPO Abs and comparison of dose-dependent binding to EPO. BIO-EPO was added to streptavidin-coated microplate and incubated for 1 h at RT. After incubation, anti-EPO mAb standards or a final dilution of 1:10 test serum were mixed with DIG-EPO and added to the microplate well and incubated for 2 h at RT. Then, anti-DIG Fab-HRP was added to each well and incubated for 1 h at RT. ABTS solution was added and absorbance was measured at 405 nm with 490 nm as reference wavelength. To determine the anti-EPO Ab concentration in the serum, standard curve was prepared using absorbance value of standards. The calculated concentration was multiplied by the dilution factor (A). Dose-dependent binding of serially diluted serum 1 and 2 to EPO was tested by a bridging ELISA. BIO-EPO was added to streptavidin-coated microplate and incubated for 1 h at RT. After incubation, anti-EPO mAb standards or serially diluted serum 1 and 2 were mixed with DIG-EPO and added to the microplate well and incubated for 2 h at RT. Then, anti-DIG Fab-HRP was added to each well and incubated for 1 h at RT. ABTS solution was added and absorbance was measured at 405 nm with 490 nm as reference wavelength. Mean values of duplicate measurements are shown (B).

aware of the EPO-induced anti-EPO Abs. Due to the lack of a universal standardized assays as well as unavailability of the present Ab assays to clinicians, many cases seem to be neglected sometimes. In this study, we have chosen and setup two high throughput assays for measuring and characterizing anti-EPO Abs and tried to apply to the clinical serum samples in Korea. They can also be valuable to assess and regulate the immunogenicity issue of biosimilar EPO products and modified EPO preparations newly appearing in the market.

An assay for the analysis of anti-EPO binding Ab in this study was the bridging ELISA, which is sensitive, specific, and convenient for managing large number of serum samples [8]. Compared to a conventional ELISA, a bridging ELISA is more specific and quantitative [12]. Streptavidin/biotin-coated bridging ELISA is more sensitive than the directly coated bridging ELISA [14]. These advantages of streptavidin/biotin-coated bridging ELISA over conventional and directly coated bridging ELISAs were also observed in our studies (data not shown). Additionally, streptavidin/biotin-coated bridging ELISA is comparable to RIPA and closely correlated with each other in quantitative terms [13]. We also found an anti-EPO positive case by a bridging ELISA confirmed by RIPA (data not shown).

Demonstrating neutralizing activity of anti-EPO Ab in a serum is important in diagnosis of Ab-mediated PRCA. An assay for the analysis of anti-EPO neutralizing Ab in this study was the UT-7/EPO cell line-based bioassay, which has been validated [18]. Instead of ^{3}H-thymidine as a marker for cell proliferation, we used non-radioactive and spectrophotometric assay. Usage of cell line provides some advantages over primary bone marrow cells for EPO NT bioassay including greater reproducibility and consistent cell growth characteristics and EPO responsiveness [12]. We used serially diluted serum samples for obtaining semi-quantitative results.

By a bridging ELISA, two serum samples were identified as anti-EPO positives. By NT bioassay, these two samples were also proved to possess neutralizing activities. However, discrepancy existed between the level of anti-EPO binding Abs and the neutralizing activities. Namely, the higher Ab level did not lead to the stronger neutralizing activity. Similarly, Gross et al. reported that some cases showed an unchanged or even increased neutralizing activity despite falling levels of anti-EPO Abs [13]. As described in several epitope mapping studies of mouse monoclonal Abs on EPO [20–22], most probably there exist neutralizing epitopes on EPO. Based on this data, we postulated if neutralizing Abs are major population in serum, the EPO-induced cell proliferation would be inhibited in proportion to them. However, if non-neutralizing Abs are major population even total level of anti-EPO Abs is relatively high, neutralizing activity could be lower than expected. Sample 2 was diagnosed for hypocellular marrow with relative erythroid depression via bone marrow examination and sample 1 was not subject to the biopsy yet. Clinical follow-up studies with these two samples are under trial and will provide some indications about relevance of assay data with clinical outcome.

5. Conclusions

A consensus has not been reached on which assay is the most meaningful for screening and predicting onset of Ab-mediated PRCA [12]. Thus, between a bridging ELISA and a NT bioassay, which assay is more clinically relevant is not well-defined. And clear criteria of Ab level and neutralizing activity inducing PRCA is also not established. Gross et al. discussed that samples with a low Ab level need to determine the neutralizing activity. When non-neutralizing Abs are diagnosed, continuation of EPO therapy seems possible, while neutralizing Abs are detected, EPO therapy should be discontinued [13]. To this point, it would be helpful to develop an ELISA assay for quantitation of neutralizing Ab rather than the total binding Ab in matching with the results of NT bioassay. Additionally, we suggest cancer cell line-based NT bioassay for a suitable and shortcut screening method for large group samples by skipping an Ab assay such as ELISA, RIP, or SPR.

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