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Biosimilar recombinant human erythropoietin induces the production of neutralizing antibodies

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Recombinant human erythropoietin (r-HuEpo) has been used for the treatment of renal anemia. With the loss of its patent protection, there has been an upsurge of more affordable biosimilar agents, increasing patient access to treatment for these conditions. The complexity of the manufacturing process for these recombinant proteins, however, can result in altered properties that may significantly affect patient safety. As it is not known whether various r-HuEpo products can be safely interchanged, we studied 30 patients with chronic kidney disease treated by subcutaneous injection with biosimilar r-HuEpo and who developed a sudden loss of efficacy. Sera from 23 of these patients were positive for r-HuEpo-neutralizing antibodies, and their bone marrow biopsies indicated pure red-cell aplasia, indicating the loss of erythroblasts. Sera and bone marrow biopsies from the remaining seven patients were negative for anti-r-HuEpo antibodies and red-cell aplasia, respectively. The cause for r-HuEpo hyporesponsiveness was occult gastrointestinal bleeding. Thus, subcutaneous injection of biosimilar r-HuEpo can cause adverse immunological effects. A large, long-term, pharmacovigilance study is necessary to monitor and ensure patient safety for these agents.

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EDITOR'S NOTE:
Biosimilar is a term applied to subsequent versions of biopharmaceutical products that have been approved by the regulatory authorities of a given country. The pathway for approval is thus specific for that country, and because of regulatory differences, the biosimilar classification may not apply in other countries.

Recombinant human erythropoietin (r-HuEpo) was the first biotherapeutic medicinal product derived from recombinant DNA technology for the treatment of anemia in patients with chronic kidney disease (CKD). Although r-HuEpo raises hemoglobin (Hb) levels in CKD and improves morbidity associated with anemia in CKD patients, the adverse immunological effect of innovative r-HuEpo administered subcutaneously can result in anti-r-HuEpo-associated pure red-cell aplasia (PRCA) in some patients.[1–5] With the expiration of patent protection for the innovative r-HuEpo, many so-called 'similar' biological r-HuEpos became available and were licensed as 'biosimilar r-HuEpos'.[6] These biosimilar r-HuEpos are more affordable, allowing patients with CKD easy access to treatment for renal anemia worldwide. Aside from the complexities of the biopharmaceutical manufacturing process, variations in host cells, vector, DNA sequence, or the purification and glycosylation processes can result in alterations from the reference innovative r-HuEpo product. Such alterations may cause impurities or aggregation of the protein that may augment the immune response and can have major biological impact.[7] Because there are few preclinical and clinical comparisons between each biosimilar r-HuEpo and its reference product, therefore it is unknown whether r-HuEpo products can be interchangeable or substituted. This continues to be a dilemma for many clinicians in treating anemia in CKD patients.

As for Thailand, the first innovative r-HuEpo-α form was licensed in 1990 and the innovative r-HuEpo-β was approved for use in 1998. The first biosimilar r-HuEpo-α form became available in 1997 for the treatment of anemia of CKD. Since then, there has been an explosion of biosimilar r-HuEpo products in the Thai drug market. Under the generic drug paradigm of the Thai Food and Drug Administration, 14 biosimilar r-HuEpos were licensed by 1 January 2009. These products came from various countries such as Argentina, China, South Korea, and India. The number of cases using biosimilar r-HuEpos have increased enormously because of their more affordable prices. With their usage, adverse effects of the less than identical therapeutic agents have started to increase. Many clinicians in Thailand were starting to see an increase in PRCA cases which raised an important issue whether the immunogenicity of biosimilar therapeutic agents were indeed equivalent to the innovative r-HuEpo.

As we are the only center to assay anti-r-HuEpo in Thailand, we decided to conduct a pilot study to investigate whether there were presences of anti-r-HuEpo antibody in CKD patients who received subcutaneous biosimilar r-HuEpo injections for the treatment of renal anemia and developed loss of efficacy (LOE).

RESULTS

Patients

A total of 30 CKD patients who received subcutaneous injections of biosimilar r-HuEpo had LOE during 2008. All patients had not been treated with any innovative r-HuEpo agents before using biosimilar r-HuEpo products. Table 1 shows the patients' characteristics, issues related to the use of biosimilar r-HuEpo, and laboratory results. There were no statistically significant differences in gender, age, CKD status, dialysis modality, CKD etiology, intact parathyroid hormone levels, r-HuEpo doses, and Hb levels before LOE between anti-r-HuEpo-positive and -negative patients. However, there were statistically significant differences in duration of r-HuEpo exposure, Hb levels at time of LOE, and counts of reticulocytes between the anti-r-HuEpo-positive and -negative patients (Table 1). The iron studies, serum folate, and B_{12} level were adequate and comparable in both groups. The mean duration of biosimilar r-HuEpo exposure in antibody-positive patients were significantly shorter than antibody-negative patients ($P = 0.001$). The antibody-positive patients had more severe anemia and lower counts of reticulocytes when compared with the antibody-negative patients. The mean age of negative control was 54.1 ± 13.9 years. All negative control cases had Hb levels between 10 and 12 g/dl with mean r-HuEpo doses of 120 ± 37 unit/kg/week. The mean age of pure negative control was 32.3 ± 5.9 years. All pure negative control cases had Hb levels more than 12 g/dl.

Anti-r-HuEpo antibody

The mean duration of developing LOE and anti-r-HuEpo testing were 1.5 ± 0.5 months. By radioimmunoprecipitation assay, anti-r-HuEpo was detected in 23 patients. Samples with a total counts per minute (c.p.m.) of more than 0.9% at 1:20 dilution was considered positive for anti-r-HuEpo. From the

Table 1 | Patients' characteristics and issues related to the use of biosimilar r-HuEpo and laboratory results

	Anti-r-HuEpo positive	Anti-r-HuEpo negative	*P*
Numbers of patients (cases)	23	7	—
Gender, male/female (case/case)	13/10	3/4	0.526
Age, years ± s.d.	61.1 ± 21.4	52.8 ± 4.8	0.784
CKD status, cases (%)			0.647
Predialysis	8 (34.8)	2 (28.6)	
Hemodialysis	14 (60.9)	4 (57.1)	
Peritoneal dialysis	1 (4.3)	1 (14.3)	
Etiology of CKD, cases (%)			0.393
Diabetic nephropathy	5 (26.1)	3 (42.9)	
Chronic glomerulonephritis	3 (13.0)	0	
Unknown	14 (60.9)	4 (57.1)	
r-HuEpo exposure duration, months ± s.d. (range in months)	12.1 ± 7.8 (3–36)	22.3 ± 19.8 (6–60)	0.001*
r-HuEpo dose, U/kg/week ± s.d.	149 ± 82	171 ± 91	0.991
Hb before LOE, g/dl ± s.d.	10.8 ± 1.6	11.4 ± 0.7	0.458
Hemoglobin by the time of LOE, g/dl ± s.d.	5.6 ± 0.9	7.3 ± 0.7	<0.001*
Reticulocytes, cell/mm^3 ± s.d.	5978 ± 1217	13,128 ± 3,456	<0.001*
Serum ferritin, ng/ml ± s.d.	368.6 ± 83.1	370.3 ± 93.7	0.967
Transferring saturation, % ± s.d.	28.3 ± 6.6	28.8 ± 5.2	0.821
Serum folate, pg/ml ± s.d.	12.8 ± 4.5	12.5 ± 4.3	0.526
Serum B_{12}, pg/ml ± s.d.	258.2 ± 189.2	177.1 ± 84.4	0.123
CRP, mg/l ± s.d.	4.22 ± 2.98	3.62 ± 3.56	0.692
iPTH, pg/ml ± s.d.	241.4 ± 127.1	284.0 ± 151.6	0.518

Abbreviations: CKD, chronic kidney disease; CRP, C-reactive protein; Hb, hemoglobin; iPTH, intact parathyroid hormone; LOE, loss of efficacy; r-HuEpo, recombinant human erythropoietin.
*$P < 0.05$.

Table 2 | Total c.p.m. (%) by different sera dilution to detect anti-r-HuEpo-associated PRCA cases in patients using subcutaneous biosimilar r-HuEpo: antibody-positive and antibody-negative cases, negative control, and pure negative control

	Mean of percent c.p.m. ± s.d.					
	1:20 dilution	1:50 dilution	1:100 dilution	1:1000 dilution	1:10,000 dilution	1:20,000 dilution
Anti-r-HuEpo-positive cases (*N*=23)	18.2 ± 8.8	12.7 ± 9.7	10.5 ± 9.2	3.5 ± 5.0	1.0 ± 1.0	0.3 ± 0.8
Anti-r-HuEpo-negative cases (*N*=7)	0.2 ± 0.1	NA	NA	NA	NA	NA
Negative control (*N*=30)	0.2 ± 0.1	NA	NA	NA	NA	NA
Pure negative control (*N*=30)	0.2 ± 0.1	NA	NA	NA	NA	NA

Abbreviations: c.p.m., counts per minute; NA, not applicable; PRCA, pure red-cell aplasia; r-HuEpo, recombinant human erythropoietin.

titer assay, the mean values of anti-r-HuEpo was 18.2, 12.7, 10.5, 3.5, 1.0, and 0.3% of c.p.m. at 1:20, 1:50, 1:100, 1:1000, 1:10,000, and 1:20,000 dilutions, respectively (Table 2). Even at 1:10,000 dilution, anti-r-HuEpo was still detected, indicating that the antibody levels in the positive sera were extremely high. Through the neutralization assay, all 23 antibody-positive sera were able to inhibit the growth of UT7/Epo-dependent cell line by more than 25%.

The sera of the remaining seven patients were negative for anti-r-HuEpo. The mean percentage of total c.p.m. was $<0.9\%$ at 1:20 dilution, which was not different from the negative and pure negative controls.

Biosimilar r-HuEpo and PRCA

From bone marrow biopsy, all 23 anti-r-HuEpo-positive cases had PRCA. The bone marrow biopsy revealed normocellularity with normal myeloid and megakaryocytic lineages but absence of the erythroid precursor ($<5\%$ of erythroblasts in the bone marrow). Seven patients with antibody-negative sera did not show PRCA in the bone marrow biopsy. However, from the endoscopic examination, we were able to identify the etiology of LOE. The cause of sudden LOE in these antibody-negative patients was due to occult gastrointestinal bleeding.

Estimation of risk

Yearly prevalence of dialysis patients in 2008 receiving r-HuEpo was obtained from the Thai Renal Replacement Therapy Registry data. In 2008, there were a total of 26,511 chronic dialysis cases receiving r-HuEpo.[8] The prevalence of CKD stage IV (predialysis) was obtained from a recent epidemiologic survey conducted in Thailand.[9] From 63 million people, the prevalence of CKD stage IV was 0.2%. Out of this 0.2%, about 40% had Hb below 10 g/dl and needed r-HuEpo treatment. From the Thai ESA registry data, 78% of the patients had received biosimilar r-HuEpo.

Estimation of risk for anti-r-HuEpo-associated PRCA was calculated by $23/(26{,}511 + (63{,}000{,}000 \times 0.002 \times 0.4)) \times 0.78 = 23/59{,}990$.

Thus, an estimation of the actual cases using biosimilar r-HuEpo denominator with this complication was 1:2608. This indicated that 1 out of 2608 patients using biosimilar r-HuEpo would develop PRCA.

DISCUSSION

The results from our pilot study showed that there was an immunogenicity risk in using biosimilar r-HuEpo. Even at 1:10,000 dilution of the sera, we were able to detect anti-r-HuEpo in patients using biosimilar agents. Because of the nature of the pilot study and its aim to investigate whether biosimilar agents currently used in Thailand were capable of producing anti-r-HuEpo, the results could not provide sufficient information to determine exactly which specific biosimilar products are directly responsible for causing anti-r-HuEpo-associated PRCA. However, we can clearly state that repeated subcutaneous injections of biosimilar agents could result in the development of anti-r-HuEpo-associated PRCA. Further studies with a larger sample size and longer follow-up period are warranted to provide more information in the use of biosimilar r-HuEpo.

As a matter of fact, the results from this pilot study prompted the Nephrology Society of Thailand, the Thai Society of Hematology, the Association of Hospital Pharmacy (Thailand), and the Adverse Product Reaction Monitoring Center of the Food and Drug Administration of Thailand to set up a prospective, immunogenicity surveillance registry of erythropoiesis-stimulating agent (ESA) with subcutaneous exposure (http://www.clinicaltrial.gov; registration number NCT00799019). The primary objective of this registry is to estimate the incidence of anti-r-HuEpo-associated PRCA development in patients, and the secondary objective is to evaluate the efficacy of the currently available ESA products for the treatment of erythropoietin-deficiency anemia in Thailand. The study is a multicenter, prospective, cohort study of patients who are using ESA products according to normal practice, consistent with its medical indications. Subjects in the registry are adults receiving or about to receive, within 1 month, a marketed ESA product administered subcutaneously at the time of enrollment. Observations for the development of immunogenicity effects and PRCA will be followed in patients for a total of 3 years. The human leukocyte antigen-A, -B, -DR, and -DQ genotypes of anti-r-HuEpo-positive cases will be typed to investigate the association of human leukocyte antigen-associated and anti-r-HuEpo-associated PRCA. The reason for determining this relationship was based on results obtained from recent studies[5,10] that showed the genetic susceptibility of human leukocyte antigen DR B1*09 to anti-r-HuEpo-associated PRCA in innovative r-HuEpo cases. It will be another 2 more

years before we can analyze the data, but we are confident that the results of this registry study will enlighten us on how to deal with biosimilar r-HuEpo.

Our data suggested that biosimilar r-HuEpo should not be licensed through the conventional generic paradigm. A recent study[7] showed that the use of different cell lines used during the manufacturing processes can alter the biophysical characteristics of the protein. In Thailand, before 2010, biosimilar r-HuEpos were licensed using conventional generic paradigm that mainly focuses on bioequivalence study. The registration process did not require a clinical study data and full dossier was not required. Our data demonstrated that the lack of preclinical and clinical comparisons between each biosimilar r-HuEpo and its reference product can create potential gaps in the quality and safety of biosimilar r-HuEpo, and warrant guidelines on evaluation of biosimilar r-HuEpo. The generic approach is not suitable for the licensing biosimilar, as unlike chemical drugs, that comprise of small molecules and are easily reproducible with uncanny similarities to the reference products, biotherapeutic products consist of relatively large and complex structures that are difficult to characterize. It is essential that both preclinical and clinical comparisons between biosimilar agents and their reference products are required[11] to ensure the safety and efficacy of these biosimilar agents. A specific regulatory framework is now being developed in Thailand. The key principles for evaluating r-HuEpo biosimilar involve stepwise comparability exercises, starting with comparison of the quality characteristics of biosimilar with their reference biotherapeutic products, which were licensed based on a full dossier. The comparability exercises include physicochemical properties, biological activity, immunochemical properties, impurities, pharmacokinetic/pharmacodynamic studies, efficacy studies, and safety studies. As for most biotherapeutic products, data from prelicensing are usually too limited to identify all potential adverse effects; pharmacovigilance plans and risk management plan should be submitted and integrated as parts of evaluation.

As for countries that have already licensed biosimilar r-HuEpo products and are currently in use, such as Thailand, pharmacovigilance and antibody testing in a large cohort for a long period of time during the post-marketing phase is warranted, especially for products administered by subcutaneous injections. This is important because there is an inadequacy of post-marketing surveillance and a lack of reliable information in regards to the long-term effects of biosimilar agents. Although there are no reports of anti-r-HuEpo-associated PRCA from intravenous injection, the universal implementation of intravenous injection of r-HuEpo would be impractical for predialysis patients and is not realistic in countries with limited resources because higher doses are required to achieve the target Hb.[12]

In conclusion, we would like to caution against wholesale adoption of biosimilar r-HuEpo to decrease costs and increase access to therapy because the patients can develop anti-r-HuEpo antibodies after subcutaneous injection of biosimilar r-HuEpo. From this pilot study, the immunogenicity of biosimilar therapeutic agents is an important issue that deserves the attention of clinicians and drug regulators. It is highly recommended that a plan for strict licensing, continuous post-marketing monitoring, and pharmacovigilance should be implemented to detect, assess, and prevent these adverse effect in patients using biosimilar r-HuEpo agents.

PATIENTS AND METHODS

Patients

In 2008, 30 CKD patients were referred to our center. CKD patients who received only subcutaneous injections of biosimilar r-HuEpo and developed LOE were recruited in this study. Additional 30 CKD patients who received r-HuEpo but did not develop LOE served as negative controls. Another 30 CKD patients who have never received r-HuEpo served as pure negative controls. The study was approved by the Ethical Committee of Research, Faculty of Medicine, Chulalongkorn University Hospital, Bangkok, Thailand. Informed consent was obtained from each patient before entering the study.

Definitions

LOE was defined as a sudden drop in the Hb level more than 2 g/dl for 2 consecutive weeks resulting in anemic symptoms and the need for blood transfusions to alleviate anemic symptom every week in the absence of other common causes of r-HuEpo unresponsiveness, such as acute blood loss, acute hemolysis, infection, hyperparathyroidism, hypothyroidism, and deficiencies of iron, folic acid, and vitamin B12.

Radioimmunoprecipitation assay for anti-r-HuEpo

Sera from the recruited patients were assayed for anti-r-HuEpo. Radioimmunoprecipitation assay was used to quantitatively examine the presence of antibody to r-HuEpo in the sera.[13] The serum samples were incubated with ^{125}iodine-labeled Epo purchased from PerkinElmer Life Sciences (Boston, MA). The bound antibody was separated from the free-labeled Epo by using Protein G Sepharose 4 Fast Flow beads (GE Healthcare, Uppsala, Sweden). The beads were then thoroughly washed and counted on a gamma counter. The presence of anti-r-HuEpo antibody in the tested sera was determined by calculating the percent of total c.p.m. The serum analyzed in the screening assay was reassayed if the percent of total c.p.m. count was more than 0.3%. The titer assay was performed at dilutions of 1:20, 1:50, 1:100, 1:1000, 1:10,000, and 1:20,000. Positive serum for anti-r-HuEPO had a total c.p.m. count more than 0.9% at 1:20 dilution.

Neutralization assay

The sera identified as positive for anti-r-HuEpo were further assayed for its neutralization activity. Neutralization assay was used to determine whether the anti-r-HuEpo was capable of inhibiting the growth of UT-7/Epo-dependent cell line.[14] The UT-7/Epo cells were kindly obtained from Professor Nicole Casadevall (INSERM U 790). A cell bank was prepared, and the cells were stored in liquid nitrogen in 10% dimethyl sulfoxide and 90% fetal bovine serum. Thawed UT-7/Epo cells were grown in a basal medium containing Dulbecco's Modified Eagle's Medium, fetal bovine serum, 50 U/ml penicillin G, 50 μg/ml streptomycin sulfate, and L-glutamine. The ^{3}H-thymidine was obtained from Amersham Bioscience (Piscataway, NJ). The Epo levels in the sera were measured by the Human Erythropoietin Quantikine IVD ELISA Kit purchased from R&D Systems

(Minneapolis, MN). The test sera were diluted depending on the Epo and anti-r-HuEpo antibody levels, and were later added to the starved UT-7/Epo cells. r-HuEpo-α was also added at a final concentration of 10 mU/ml. The culture was incubated at 37 °C in 5% CO_2 for 48 h. The ^{3}H-thymidine was added, and after 6 h of incubation, the cells were harvested onto a microfilter to measure for radioactivity. The test sera were considered to be positive if more than 25% neutralization was observed.

Statistical analysis

The continuous values of variables were expressed as mean ± s.d. Comparisons of the variables were performed by the independent sample *t*-test or χ^2-test where appropriate. All statistical analyses were performed by using SPSS statistical package (version 11.5 for Windows, SPSS, Chicago, IL). A *P*-value of <0.05 was considered statistically significant.

DISCLOSURE

All the authors declared no competing interests.

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