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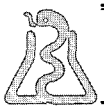

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Antigenicity of HM10760 in Guinea Pigs

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ABSTRACT. HM10760 is a recombinant human erythropoietin that has been developed as a drug for anemia. In this study, antigenic potential of HM10760 was examined by active systemic anaphylaxis in guinea pigs and passive cutaneous anaphylaxis in a guinea pig-guinea pig system. HM10760 was subcutaneously administered at 0, 2, and 20 μg/kg and also as a suspension with adjuvant (20 μg/kg + FCA). Ovalbumin as a suspension with adjuvant was administered to induce positive control responses. In the active systemic anaphylaxis test, no symptoms except rubbing or licking nose and urination that were considered as physiological phenomena were observed at 0 μg/kg. Four of 5 animals at 2 μg/kg and all the 5 animals at 20 μg/kg showed cyanosis and lying on side. All animals in the adjuvant mixture group showed relatively mild symptoms such as rubbing or licking nose, urination, and evacuation. In the passive cutaneous anaphylaxis test, 0/5, 3/5, and 5/5 serum samples from the animals immunized with 0, 2, and 20 μg/kg, respectively, showed positive reactions against HM10760. All 5 sera collected from the animals immunized with an adjuvant mixture contained HM10760-specific antibodies. These results suggest HM10760 have antigenicity in guinea pigs.

Keywords: Erythropoietin, Antigenicity, Active systemic anaphylaxis, Passive cutaneous anaphylaxis.

INTRODUCTION

Human erythropoietin (EPO) is an acidic glycoprotein cytokine with a molecular mass of 34 kD. It is produced primarily by cells of the peritubular capillary endothelium of the kidney, is responsible for the production and homeostasis of red blood cell. EPO production is stimulated by reduced oxygen content in the renal arterial circulation, bleeding, and anemia caused by various disease (Goldberg *et al.*, 1994). In the healthy population, serum EPO levels were maintained at a 54 ± 31 mIU/ml, but as may be expected in patients with chronic renal failure, serum EPO levels remain low despite the severe anemia (Wallner *et al.*, 1977; Saito *et al.*, 1984). After Eschbach *et al.* (1987) had demonstrated the effectiveness of recombinant human erythropoietin (rhEPO) in treating the anemia of end-stage renal disease at late 1980's, rhEPO had been the main therapeutic agents in various anemic disease. The glycosylation of erythropoietin is essential for *in vivo* activity maintenance and the contents of sialic acids (N-acetyl neuramic acid) is prominently important for activity. Goldwasser *et al.* (1974) showed that desialylated erythropoietin still maintained the proliferation activity *in vitro*, but had been completely lost the erythropoietic activity *in vivo*. Morell *et al.* (1968) proposed hepatic removal mechanism of asialoglycoproteins by galactose receptor in the liver to explain the role of sialic acids *in vivo*. Laura *et al.* (2001) reported that the sialic acids content of EPO is reversely proportional to the affinity to the receptor, suggesting that the sialic acid content is crucial to avoid receptor clearance as well as hepatic removal mechanism. Using this clearance mechanism, several biotech or pharmaceutical companies have been developing long-acting rhEPOs such as hyperglycosylated erythropoietin or pegylated version (Osterborg, 2004). In addition, there are other groups using dimeric EPO (Sytkowski *et al.*, 1999) or chimeric protein fused with other proteins such as the Fc fragment of immunoglobulins (Way *et al.*, 2005) to improve the physicochemical properties

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of EPO *in vivo*.

These modifications to enhance the stability and efficacy of rhEPO may cause immunogenicity problems when used in treating patients. Especially, the most severe adverse effect of rhEPO administration to patients with chronic renal failure was antibody-mediated pure red cell aplasia (PRCA), a profound anemia characterized by the absence of reticulocytes in the bone marrow. The antibodies against rhEPO produced in patients neutralize not only the exogenous rhEPO but also endogenous EPO and blocked the generation of red blood cells (Gershon *et al.*, 2002). Immunological mechanism for developing antibody-mediated PRCA is not known, but a variety of factors may increase the immunogenicity of rhEPOs (Macdougall, 2005). Protein related factors that have the potential to impact on immunogenicity include sequence variations in proteins, the degree and nature of glycosylation, handling and storage, formulation. Patient-related factors include administration routes, immune status, skin reactions, and treatment history. In this study we performed active systemic anaphylaxis (ASA) shock and passive cutaneous anaphylaxis (PCA) tests to evaluate the antigenicity of HM10760, our newly developed rhEPO fused with an Fc fragment.

MATERIALS AND METHODS

HM10760

rhEPO linked to the Fc portion of a human immunoglobulin, via amine residue specific non-peptidyl GRAS linker *in vitro*. The Fc portion was produced in a prokaryotic host without altering the natural sequence. The molar ratio between the rhEPO and the Fc portion is 1 : 1, and the total molecular mass was approximately 83.4 kDa.

Reagents

HM10760 was dissolved in 50 mM potassium phosphate buffer (pH 6.0) containing 200 mM NaCl and 10% maltose. Reagents including ovalbumin (OVA), Evans blue, and Freund's complete adjuvant (FCA) were purchased from Sigma (St. Louis, MO, USA).

Animals and housing conditions

Four-week old specific pathogen free Hartley guinea pigs were obtained from Charles River Laboratories (188 LaSalle, St.-Constant, Quebec, J5A 1Y2, Canada) and used following one week of acclimation. Randomization was carried out using the Path/Tox System (Version 4.2.2., Xybion Medical Systems Corporation, USA). The animal room was maintained at a temperature of 23 ± 3°C, relative humidity of 55 ± 10%, air ventilation of 10~20 times/hour and the light intensity of 150~300 Lux with 12 hour light/dark cycle was used. Pelleted food for guinea pigs (Harlan Teklad; Madison, WI, USA) and UV-irradiated and filtrated tap water were given ad libitum. All procedures were approved by Institutional Animal Care and Use Committee (IACUC) in Korea Institute of Toxicology.

ASA

ASA test was performed as previously described (Kang *et al.*, 1998). The animals were sensitized subcutaneously with HM10760 described in Table 1. as was using a 26-gauge syringe. The animals in Group IV and V were sensitized with HM10760 and OVA, respectively, after emulsifying with FCA. Fourteen days after the last sensitization, animals were challenged intravenously with the challenging antigens (Table 1) and the anaphylactic shock symptoms were monitored for the following 30 minutes.

PCA

PCA was performed as previously described (Mota and Wong, 1969). Blood samples were collected from the orbital plexus of the sensitized animals described in ASA 13 days after the last sensitization. Each serum sample was diluted 10 to 5120-fold in saline and injected intradermally (50 μl/site) into the clipped back of 2 recipient animals (Table 2). Four hours later, the challenging antigens were mixed with the equal volume of 1% Evans blue solution and injected intravenously. Thirty minutes after the antigen challenge, the clipped back skins of recipient animals were removed to evaluate the positive blue spots. When the mean of long and short diameters of blue spot was larger than 5 mm, the spot was evaluated as a positive response. The last dilution fold showing a positive reaction was considered as antibody titer. Average titer of serum

Table 1. Experimental design of sensitization

Group	Antigen	Animal ID	Dose (μg/kg)	Volume (ml/kg)	Frequency
I	Vehicle alone	1~5	0	1	4 times/2 weeks
II	HM10760 (Low dose)	6~10	2	1	4 times/2 weeks
III	HM10760 (High dose)	11~15	20	1	4 times/2 weeks
IV	HM10760 (High dose) + FCA	16~20	20	1	3 times/4 weeks
V	OVA + FCA	21~25	2500	1	3 times/4 weeks

Table 2. Experimental design of PCA

Groups	No. of animals	Challenge sera[a] (50 μl, i.d.)	Challenge antigens (μg/kg, i.v.)	Volume (ml/kg)
A	10	I	HM10760(20)	1
B	10	II	HM10760(20)	1
C	10	III	HM10760(20)	1
D	10	IV	HM10760(20)	1
E	10	V	OVA(2500)	1

[a]Each serum from an animal sensitized with antigen was tested for titer in duplicate.

samples from each group was calculated with following method.

Average titer = $\sum$(PCA titer × Ratio)

Ratio=proportion of positive response observed in specific dilution fold

Statistical analysis

Body weights collected during the study were analyzed with multiple comparison test. Multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Bartlett's Test. Since Bartlett's Test indicated no significant deviations from variance homogeneity, the ANOVA multiple comparison test (Dunnett Test) was conducted to determine which pairs of group comparison were significantly different. The level of significance was taken as $P < 0.05$ or 0.01. Statistical analyses were performed by comparing the different dose groups with the vehicle control group using Path/Tox System (ver4.2.2, Xybion Medical Systems Corporation, USA) and Statistical Analysis Systems (SAS/STAT Version 8.1, Cary, NC, USA).

RESULTS

Sensitization

Daily performed observations revealed neither treatment-related clinical signs nor deaths during the sensitization period. In body weight changes as shown in Table 3, the adjuvant mixture (Group IV) and positive control (Group V) group showed statistically significant decreases of body weight gain compared with the vehicle control group (Group I) on Day 22. In addition, the decrease in body weight gain was observed in the low dose (Group II), high dose (Group III), and the positive control group compared with the vehicle control group on Day 25.

ASA

In the vehicle control group (Group I), 3/5 animals showed moderate symptoms such as rubbing or licking nose and/or urination as shown in Table 4. In the low dose group (Group II), 4/5 animals showed severe symptoms such as cyanosis and/or lying on side. All 5 animals in the high dose group (Group III) also showed cyanosis and/or lying on side. In the FCA mixture group (Group IV), 4/5 animals showed moderate symptoms such as rubbing or licking nose, urination, and evacuation, which are similar with observed in vehicle control group. All 5 animals in the positive control group (Group V) showed severe symptoms (more than two symptoms among dyspnea, cyanosis, staggering gait, jumping, gasping and writhing, convulsion, lying on side, and Cheyne-Stokes respiration were shown in each animal), and 1/5 animal died. Necropsy findings were examined following the 30 minutes of anaphylaxis shock monitoring as summarized in Table 5. In the vehicle control group, petechia and ecchymosis in the lung were found in 1/5 animal, and hydropericardium in the heart and

Table 3. Body weight changes during sensitization

Groups	I	II	III	IV	V
DAY 1	363 ± 24.4	357 ± 17.2	356 ± 11.7	354 ± 12.4	360 ± 17.0
DAY 8	439 ± 25.9	428 ± 21.2	428 ± 15.7	429 ± 8.9	427 ± 19.0
DAY 14	495 ± 33.3	482 ± 26.1	486 ± 17.9	476 ± 14.3	451 ± 19.7
DAY 22	583 ± 31.3	558 ± 31.7	544 ± 23.3	539 ± 26.3*	532 ± 16.0*
DAY 25	608 ± 28.0	560 ± 27.6*	549 ± 29.0^{+}	571 ± 30.0	554 ± 12.3*
DAY 29	N/A	N/A	N/A	596 ± 30.6	563 ± 17.5
DAY 36	N/A	N/A	N/A	630 ± 32.8	590 ± 22.1
DAY 43	N/A	N/A	N/A	683 ± 47.1	627 ± 31.0

Significantly different from control group (* $p < 0.05$, $^{+}$ $p < 0.01$).1
NA : Not applicable.

Table 4. Active systemic anaphylaxis in male guinea pigs

Group	Sensitizing antigen	Challenging antigen	Symptom	Ratio[a)]
I	HM10760 (0 μg/kg)	HM10760 (20 μg/kg)	Rubbing or licking nose	1/5
			Urination	3/5
II	HM10760 (2 μg/kg)	HM10760 (20 μg/kg)	Rubbing or licking nose	3/5
			Sneezing	2/5
			Urination	1/5
			Evacuation	1/5
			Dyspnea	1/5
			Cyanosis	3/5
			Lying on side	3/5
III	HM10760 (20 μg/kg)	HM10760 (20 μg/kg)	Rubbing or licking nose	1/5
			Coughing	1/5
			Cyanosis	5/5
			Lying on side	3/5
IV	HM10760 (20 μg/kg) + FCA	HM10760 (20 μg/kg)	Rubbing or licking nose	1/5
			Urination	4/5
			Evacuation	1/5
V	OVA (2.5 mg/kg) + FCA	OVA (2.5 mg/kg)	Rubbing or licking nose	2/5
			Sneezing	2/5
			Coughing	3/5
			Hyperpnea	3/5
			Evacuation	1/5
			Dyspnea	4/5
			Cyanosis	5/5
			Staggering gait	2/5
			Jumping	1/5
			Gasping and writhing	2/5
			Convulsion	1/5
			Lying on side	3/5
			Cheyne-Stokes respiration	1/5
			Death	1/5

[a)]Number of animals with the symptom/Total number of animals observed.

Table 5. Necropsy findings of active systemic anaphylaxis test animals

Group	Sensitization		Challenging		Lesion	Ratio[a)]
	Antigen	Route	Antigen	Route		
I	Vehicle control (0 μg/kg)	s.c.	HM10760 (20 μg/kg)	i.v.	Petechia in the lung	1/5
					Ecchymosis in the lung	1/5
					Hydropericardium in the heart	2/5
					Hemorrhage in the diaphragm	2/5
II	HM10760 (2 μg/kg)	s.c.	HM10760 (20 μg/kg)	i.v.	Petechia in the lung	1/5
					Hemorrhage in the diaphragm	1/5
III	HM10760 (20 μg/kg)	s.c.	HM10760 (20 μg/kg)	i.v.	Ecchymosis in the lung	1/5
					Petechia in the lung	3/5
					Hydropericardium in the heart	1/5
					Hemorrhage in the diaphragm	3/5
IV	HM10760 (20 μg/kg) + FCA	s.c.	HM10760 (20 μg/kg)	i.v.	Petechia in the lung	1/5
					Hemorrhage in the diaphragm	1/5
V	OVA (2.5 mg/kg) + FCA	s.c.	OVA (2.5 mg/kg)	i.v.	Hemorrhage in the trachea	1/5
					Petechia in the lung	4/5
					Ecchymosis in the lung	1/5
					Hydropericardium in the heart	2/5
					Hemorrhage in the diaphragm	2/5

[a)]Number of animals with the lesion/Total number of animals observed.

Table 6. Four-hour homologous passive cutaneous anaphylaxis test in male guinea pigs with sera from sensitized male guinea pigs

Group	Sensitizing antigen	Challenging[a] antigen	PCA titer[b]	Ratio	Average[d] titer	Positive ratio[e] in serum sample
I	HM10760 (0 μg/kg)	HM10760 (20 μg/kg)	0[c]	10/10	0	0/5
II	HM10760 (2 μg/kg)	HM10760 (20 μg/kg)	0	6/10	9	3/5
			10	1/10		
			20	2/10		
			40	1/10		
III	HM10760 (20 μg/kg)	HM10760 (20 μg/kg)	0	1/10	30	5/5
			10	2/10		
			20	4/10		
			40	1/10		
			80	2/10		
IV	HM10760 (20 μg/kg) + FCA	HM10760 (20 μg/kg)	640	2/10	2816	5/5
			1280	1/10		
			2560	4/10		
			5120	3/10		
V	OVA (2.5 mg/kg) + FCA	OVA (2.5 mg/kg)	5120	10/10	5120	5/5

[a]Challenging antigen was intravenously injected 4 hours after sensitization of guinea pigs with sera.
[b]PCA titer represents the maximum dilution factor of original serum which gives positive reaction.
[c]Specific antibodies were not detected in 10-fold dilution of original sera.
[d]Average titer = ∑(PCA titer × Ratio).
[e]The ratio of guinea pigs that showed positive response among the animals of each group.

hemorrhage in the diaphragm were shown in 2/5 animals. In the low dose group, petechia in the lung and hemorrhage in the diaphragm were found in 1 animal. In the high dose group, petechia and ecchymosis in the lung were found in 3/5 and 1/5 animals, respectively, and hydropericardium in the heart and hemorrhage in the diaphragm were found in 1/5 and 3/5 animals, respectively. In the adjuvant mixture group, petechia in the lung and hemorrhage in the diaphragm were observed in 1 animal. In the positive control group, hemorrhage in the trachea was found in 1 animal, and petechia and ecchymosis in the lung were found in 4/5 and 1/5 animals, respectively.

PCA

The PCA test was performed in duplicate (2 guinea pigs/serum) to analyze the existence of anti-HM10760 specific antibodies. The results are summarized in Table 6. No positive reactions were monitored in the vehicle control group (Group I). In the low dose group (Group II), 3/5 serum samples showed positive responses with an average titer of 9. In the high dose group (Group III), 5/5 serum samples showed positive responses with an average titer of 30. In the adjuvant mixture group (Group IV), 5/5 serum samples showed positive responses with an average titer of 2816. In the positive control group (Group V), all samples showed a titer of 5120.

DISCUSSION

In this study, antigenicity of HM10760 were elucidated in two facets, ASA and PCA. ASA is the antibody-mediated systemic shock response observed when the antigen was systemically reexposed to the animals while PCA is a way that may demonstrate the existence of antibody against the antigen in the animals. Anaphylatic shock responses are mediated by histamine released from mast cells on which immunoglobulins are cross-linked by antigens, and guinea pigs are mainly used for this purpose (Karol and Graham, 1997). PCA is the antigen-antibody reaction induced by the i.v. injection of antigen mixed with dye into the animals in which the local basophils and mast cells were passively sensitized. It measures the dye accumulation due to the increased infiltration through peripheral blood vessels by the released chemical mediators from the cells (Mota and Wong, 1969).

There was no treatment-related clinical signs or deaths found during the sensitization. Regarding to the anaphylatic shock responses, the vehicle control group showed rubbing or licking nose and urination considered as physiological symptoms in 3/5 animals, whereas the low and high dose groups showed severe symptoms such as cyanosis and lying on side in most animals. However, all the animals in the adjuvant mixture group showed relatively moderate symptoms such as

rubbing or licking nose, urination, and evacuation, suggesting that HM10760 induces no or mild anaphylaxis shock responses when immunized by an emulsion with adjuvant. Considering that all serum samples from animals receiving the adjuvant mixture showed strong positive responses in the PCA, no or mild anaphylatic shock responses observed in these animals imply that there may be differences in immunoglobulin types produced mainly by adjuvant mixture and responsible for anaphylaxis. The positive control group as anticipated showed severe anaphylaxis shock responses such as cyanosis and lying on side in all 5 animals and death in 1 animal. Meantime, although the necropsy performed after the shock response observation showed many anaphylaxis shock-related lesions in all groups, only the petechia in the lung observed more often in the high dose and positive control group than in the vehicle control group is considered to be related with HM10760 sensitization.

PRCA, the most undesirable side effect observed in patients receiving rhEPO, was known to be mediated by immunogenicity of this drug. Antibodies induced by rhEPO administration also have cross-reactivity to endogenous EPO and result in the depletion of EPO. This depletion in turn arrests the generation of new red blood cells in the bone marrow to cause aplasia in patients. Although the administration of HM10760 to guinea pigs in this study showed positive responses in PCA reaction, it is probably because HM10760 is a recombinant human protein and considered as a foreign protein to guinea pigs. In the most cases that showed the pure red cell aplasia, rhEPO was administered subcutaneously as same as in present study. Compared to the other administration routes, subcutaneous administration is known to produce relatively high immunogenicity.

HM10760, a new drug candidate, is shown to have moderate antigenicity in guinea pigs. However, it is still unclear whether or not the antibodies neutralize HM10760 and/or endogenous EPO. And it is also not clear if HM10760 is immunogenic in human because the amino acid sequences of rhEPO and Fc fragment are from their respective human proteins even with different glycosylation and fused status. This antigenicity of HM10760 observed in this study is probably because it was considered as a foreign antigen in guinea pigs and because of the administration route of subcutaneous injection for sensitization.

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