Asialo-rhuEPO

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Asialo-rhuEPO can be prepared by enzymatic removal of sialic acid residues from rhuEPO^{M} (asialo-rhuEPO^{E}) or by expressing human *EPO* gene in glycoengineered transgenic plants (asialo-rhuEPO^{P}). Both types of asialo-rhuEPO, like rhuEPO^{M}, displayed excellent neuroprotective effects by regulating multiple cellular pathways in cerebral I/R animal models.

multimodal neuroprotectant erythropoietin hematopoietic activity

1. Asialo-rhuEPO Structures and Properties

EPO lacking terminal sialic acid residues is known as asialo-rhuEPO, which contains neutral galactose residues as new terminal sugars instead of acidic sialic acid residues. Removal of sialic acid residues does not impact protein folding [1], but has shown to dramatically alter protein charge, binding capacity to the homodimeric $(EPOR)_2$, in vitro and in vivo hematopoietic activities [2][3], and circulatory half-life [4][5]. In contrast to sialylated EPO with acidic pI, asialo-rhuEPO is a basic protein with a pI of 8.5 and a short circulatory half-life of ~2.5 min [5]. It has been reported to bind to the EPOR four times faster than its sialylated form in vitro [6]. Most importantly, asialo-rhuEPO was demonstrated to lack in vivo hematopoietic activity (non-erythropoietic) even at very high doses [5], and could cross the BBB to display excellent neuroprotective effects [5][7][8][9][10][11].

2. Methods of Asialo-rhuEPO Production

Currently, there are two methods used to produce asialo-rhuEPO. It is commonly prepared by enzymatic removal of sialic acid residues (desialylation) from rhuEPO^{M} [1][5]. Researchers designate this enzymatically prepared one as asialo-rhuEPO^{E}. Plants have been successfully glycoengineered to produce asialo-rhuEPO [12][13][14][15], which is designated as asialo-rhuEPO^{P}.

2.1. Enzymatic Method

Asialo-rhuEPO in small quantities for basic research was produced by the enzymatic method. In this method, asialo-rhuEPO^{E} was prepared by desialylation of rhuEPO^{M} with commercially available enzymes called neuraminidases (also known as sialidases) [1][5][6]. Neuraminidases catalyze the hydrolysis of α2,3-, α2-6-, α2-8-, and α2-9-linked Neu5Ac (a type of sialic acid typically present on mammalian glycoproteins) from glycoproteins. Although this is a simple and straightforward method to obtain asialo-rhuEPO without the loss of biological activity, it is not an economically viable method for large-scale production because of the high cost (~4000 USD/mg

protein) of rhuEPO^{M} [16]. In addition, neuraminidases for the above purpose are unavailable in bulk quantities for large-scale desialylation of rhuEPO^{M}. A mammalian cell-based expression system is also not available to directly express asialo-rhuEPO. Hence, the neuroprotective properties of asialo-rhuEPO^{E} could not be translated into clinical practice. Alternative methods to produce asialo-rhuEPO inexpensively were therefore sought to realize its full therapeutic potential.

2.2. Plant-Based Expression Method

Plants have been used as an inexpensive expression system to produce asialo-rhuEPO because they lack sialylation capacity (as they lack the entire enzymatic machinery necessary for the synthesis and transfer of sialic acid residues to glycoproteins) but have the ability to synthesize similar complex biantennary *N*-glycans like mammalian cells [17][18][19]. Moreover, transgenic plants expressing wild-type or chimeric human *GalT* can sufficiently add galactose residues on the *N*-glycans of produced glycoproteins [20][21]. The other advantages of using a plant-based expression system are low production cost, lack of human pathogen contamination, and ease of scaling up in production [18][22]. Researchers produced asialo-rhuEPO in tobacco plants by stably co-expressing human *EPO* and β1,4-galactosyltransferase (*GalT*) genes [13][14][15], while Parson and co-workers produced it in moss [12].

3. Unique Properties of Asialo-rhuEPO^{P}

3.1. Asialo-rhuEPO^{P} Carries Plant-Specific Biantennary *N*-Glycans

Asialo-rhuEPO^{P} accumulates as 28–30 kD bands representing different glycoforms in transgenic tobacco plants [14]. It is 162 amino acids long because of the proteolytic removal of the extreme *C*-terminal region Thr^{163}-Arg^{166} [23]. This lost region is not important for the biological activity of EPO [24]. Asialo-rhuEPO^{P} is a basic protein with a theoretical pI of 8.75. All three *N*-glycosylation sites in asialo-rhuEPO^{P} are occupied with *N*-glycan chains each bearing terminal mammalian-type β1,4-galactose residues [14][15]. The proportion of β1,4-galactose residues on asialo-rhuEPO^{P} was high (72%) when chimeric *GalT* was co-expressed with *EPO* [15], whereas it was low (8%) in the case of co-expressing wild-type *GalT* [14]. The *N*-glycan chains in asialo-rhuEPO^{P} are slightly different from that in asialo-rhuEPO^{E}. Asialo-rhuEPO^{P} carries biantennary *N*-glycans containing plant-specific β1,2-xylose and α1,3-fucose residues, while those *N*-glycans in asialo-rhuEPO^{E} lack β1,2-xylose but contain a core α1,6-fucose instead of α1,3-fucose. The presence of biantennary *N*-glycans with plant-specific β1,2-xylose and α1,3-fucose residues on asialo-rhuEPO^{P} showed no impact on in vitro $(EPOR)_2$ binding, since asialo-rhuEPO^{P} displayed similar affinity for the $(EPOR)_2$ as rhuEPO^{M} [13]. Furthermore, it provided a similar level of protection to the brain as rhuEPO^{M} after I/R injury [10], suggesting that the biantennary *N*-glycans bearing plant-specific sugars have no impact on its in vivo neuroprotective activity.

3.2. Asialo-rhuEPO^{P} Is Non-Erythropoietic and Non-Immunogenic

RhuEPO^{M} doses used for neuroprotection are typically higher (~40 µg/kg bw in a rodent stroke model and 4–36 µg/kg bw in clinical trials) than that used to improve hemoglobin (Hb) levels in anemia patients (~0.8 µg/kg bw) [25]. Therefore, rhuEPO^{M} or any of its derivatives at high doses can increase RBC levels and pose a thrombosis risk if they possess hematopoietic activity. Researchers confirmed a lack of erythropoietic activity in asialo-rhuEPO^{P} by repeated IV injection (twice a week for 5 weeks) in female BALB/c mice at a dose of 44 µg/kg bw (neuroprotective dose), which is 55 times higher than the dose of rhuEPO^{M} typically used to stimulate RBC production. At this high dose, asialo-rhuEPO^{P} showed no increase in Hb concentrations, while rhuEPO^{M} significantly increased Hb concentrations as early as a week after two injections [10]. Consistent with this observation, at the end of the 5-week period, the RBC count in mice receiving asialo-rhuEPO^{P} was 7.8 × 10^{6}/mm^{3}, similar to the saline group (7.5 × 10^{6}/mm^{3}), while that in mice administered rhuEPO^{M} was 11 × 10^{6}/mm^{3}, corresponding to a ~47% increase [10]. These results confirmed that asialo-rhuEPO^{P} is also non-erythropoietic, like asialo-rhuEPO^{E}.

Concerning the plant-specific sugars on asialo-rhuEPO^{P}, there is an ongoing debate whether plant-specific sugars on therapeutic glycoproteins are immunogenic [26][27][28][29]. The immunogenicity of plant-specific sugars on asialo-rhuEPO^{P} was investigated by immunizing BALB/c mice with 44 µg/kg bw (neuroprotective dose) and 88 µg/kg bw protein, along with the same doses of rhuEPO^{M} as a negative control and horse radish peroxidase as a positive control. Reseasrchers detected no antibodies against plant-specific sugars in the sera of mice immunized with asialo-rhuEPO^{P}, indicating that it is non-immunogenic even at high doses [10]. These results suggested that asialo-rhuEPO^{P} is safe for use in clinical practice.

4. Neuroprotective Effects of Asialo-rhuEPO

Asialo-rhuEPO is a nonerythropoietic EPO derivative that has been proven to have neuroprotective functions in various studies (**Table 1**) [5][9][10][11]. Erbayraktar et al. [5] demonstrated that asialo-rhuEPO^{E} is non-erythropoietic and neuroprotective in animal models of cerebral ischemia, spinal cord compression, and sciatic nerve crush. It has also been shown to attenuate neuronal cell death in the hippocampal CA1 region after transient forebrain ischemia [9]. In addition, asialo-rhuEPO^{E} was reported to improve motor behavior and reduce motoneuron loss in the cervical spinal cord of wobbler mice, an animal model of amyotrophic lateral sclerosis, without affecting hematocrit values [11]. Despite its non-erythropoietic nature and excellent neuroprotective effects, no clinical trials have been conducted for ischemic stroke treatment or for other organ injury treatment, likely due to its limited availability and high cost. To circumvent this problem, researchers produced asialo-rhuEPO^{P} in tobacco plants. Researchers describe the neuroprotective properties of asialo-rhuEPO^{P} and discuss its potential as a multimodal drug for ischemic stroke treatment.

Table 1. In vitro and in vivo neuroprotective effects of asialo-rhuEPO.

Author/Year	Cell Line/Animal	Treatment/Model	Outcome
Erbayraktar et al. [5]/2003	PC-12 cells	Nerve growth factor (NGF) absence-triggered cell death	34% protection

Author/Year	Cell Line/Animal	Treatment/Model	Outcome
Erbayraktar et al. [5]/2003	P-19 cells	Hypoxia for 15 h	43% protection
Mennini et al. [11]/2006	Motoneuron culture	Kainate-induced cell death	Increased survival rate by 57%
Ishii et al. [30]/2012	PC-12 cells	Nerve growth factor (NGF) absence-triggered cell death	No observed protection
Kittur et al. [14]/2013	N2A cells	Staurosporine-induced cell death	44% protection
Erbayraktar et al. [5]/2003	Sprague Dawley male rats	MCAO model	Reduced infarct volume by ~50%
Erbayraktar et al. [5]/2003	Sprague Dawley male rats	Spinal cord compression	Restricted injury with better neuron survival and motor score
Erbayraktar et al. [5]/2003	Sprague Dawley male rats	Sciatic nerve crush model	Reduced functional loss and improved motor testing score
Wang et al. [7]/2004	Wistar rat pups (7 days old)	Hypoxia–ischemia model	Reduced infarct volume by 52%
Grasso et al. [31]/2006	Sprague Dawley rats	Spinal cord compression	Significantly recovered affected motor function
Mennini et al. [11]/2006	Homozygous wobbler mice	Amyotrophic lateral sclerosis model carrying a mutation of Vps54 gene	Improved motor behavior and reduced inflammation
Price et al. [8]/2010	Sprague Dawley male rats	MCAO model	Significantly reduced infarct volume with reduced cell death
Yamashita et al. [9]/2010	Mongolian male gerbils	Occlusion of the common carotid arteries	Improved learning and memory function with better neuron survival
Ishii et al. [30]/2012	Wistar male rats	MCAO model	Significantly reduced cerebral I/R injury
He et al. [10]/2022	BALB/c male mice	MCAO model	Significant decreased neurological deficits, infarction volume, and edema volume with better neuron survival

Researchers evaluated the in vitro neuroprotective effect of asialo-rhuEPO^{P} by studying its ability to protect neuronal-like cells (N2A) against staurosporine (STS)-induced apoptosis. The results showed that simultaneous treatment of N2A cells with 1 μM STS and 20 IU/mL asialo-rhuEPO^{P} or rhuEPO^{M} (a positive control) resulted in lower cytotoxicity (47% and 66%, respectively) while treatment with 1 μM STS alone caused 84% cytotoxicity [14]. These results suggest that asialo-rhuEPO^{P} is not only neuroprotective but also more effective (~2-fold) than rhuEPO^{M} in vitro.

The in vivo neuroprotective effect of asialo-rhuEPO^{P} was evaluated using a mouse model of I/R injury and compared with rhuEPO^{M}. Researchers used a dose of 44 µg/kg bw because asialo-rhuEPO^{E} has been found to be neuroprotective at this dose in a mouse model of I/R injury [5]. Following IV administration of asialo-rhuEPO^{P} (rhuEPO^{M} as well) immediately at the restoration of blood flow after 1 h occlusion, researchers observed a significant decrease in neurological deficits (from 3.1 in I/R–saline group to 1.8–1.9 in asialo-rhuEPO^{P} and rhuEPO^{M} groups), cerebral infarction volume (from 33.0% in I/R–saline group to 15.0% in asialo-rhuEPO^{P} and rhuEPO^{M} groups), and edema volume (from ~30% in I/R–saline group to 14% in asialo-rhuEPO^{P} and rhuEPO^{M} groups) [10]. Consistent with these observations, HE and Nissl staining of brain sections showed lesser cellular damage and higher neuron density in the asialo-rhuEPO^{P}-treated group than in the I/R–saline group. Immunostaining for NeuN, a marker commonly used to assess the functional state of neurons, also revealed a higher number of NeuN-positive cells in the asialo-rhuEPO^{P}-treated group than that in the I/R–saline group [10]. The results demonstrated that asialo-rhuEPO^{P} is neuroprotective and equipotent to rhuEPO^{M} in reducing brain damage induced by I/R injury.

4.2. Neuroprotective Mechanism of Asialo-rhuEPO^{P}

Asialo-rhuEPO^{E} has been shown to exert excellent neuroprotection against cerebral I/R injury [5][7][8][9][11][30], but its neuroprotective mechanism has not been dissected yet. Regarding asialo-rhuEPO^{P}, the studies suggested that it protects the brain from I/R injury by restoring mitochondria fusion–fission-related proteins, preventing I/R injury-induced mitophagy and autophagy markers, and inhibiting apoptosis [10]. Mitochondria are both a source and target of I/R injury and cell death, and their dysfunction occurring via fission and fusion imbalance is considered one of the hallmarks of I/R-induced neuronal cell death [32][33]. Fission regulates the amounts of mitochondria and removes damaged mitochondria, whereas fusion maintains normal mitochondrial activity by complementing damaged mitochondrial contents with the components of healthy mitochondria [33][34]. I/R injury and other cerebral insults promote fission, leading to disturbed mitochondrial dynamics and compromised mitochondrial functions, thereby promoting the release of pro-apoptotic factors, such as cyt *c* [32][33][35]. The western blotting and immunofluorescence studies showed that the induced fission-related proteins dynamin-related protein 1 (p-Drp1) and Drp1 receptor fission 1 protein (Fis1) in the brain of the I/R-saline group were significantly restored in both asialo-rhuEPO^{P}- and rhuEPO^{M}-treated groups [10]. Similarly, the fusion-related proteins mitofusin 1 and 2 (Mfn1 and Mfn2) and optic atrophy protein 1 (OPA1), whose levels were reduced in the I/R-saline group, were significantly restored in asialo-rhuEPO^{P} and rhuEPO^{M} groups [10]. These results suggested that, like rhuEPO^{M}, asialo-rhuEPO^{P} can maintain mitochondrial fission–fusion balance under I/R conditions.

The results also showed that asialo-rhuEPO^{P} treatment and rhuEPO^{M} as well can regulate mitophagy-related markers. Mitochondrial fission is followed by mitophagy to remove damaged organelles [36][37]. In mammalian cells, the PINK1 (PTEN-induced putative kinase protein 1) and parkin (an E3 ubiquitin ligase PARK2) cooperatively sense cellular stress and mediate the removal of damaged mitochondria [36][37]. The Western blotting results showed that asialo-rhuEPO^{P} (also rhuEPO^{M}) treatment was able to restore both PINK1 and PARK2 levels that were elevated by I/R injury [10], consistent with lower mitochondrial fission anticipated from the restoration of fission-related proteins in EPO-treated groups. In addition to mitophagy, the Western blotting and

immunofluorescence results of general autophagy markers (LC3B, p62, and Beclin1) revealed that their increased levels in the I/R–saline group were reinstated back in asialo-rhuEPO^{P} and rhuEPO^{M} groups to the levels similar to the sham group [10]. Furthermore, investigation of apoptotic markers and TUNEL staining revealed that asialo-rhuEPO^{P}-treatment (also rhuEPO^{M}) significantly attenuated I/R-induced Bax/Bcl2 ratio, cyt *c* release, and caspase 3 caspase cleavage, and reduced TUNEL-positive cells by 50% compared to the I/R–saline control, indicating that both asialo-rhuEPO^{P} and rhuEPO^{M} have similar suppressive effects on I/R injury-induced apoptosis [10]. Together, these results suggested that asialo-rhuEPO^{P}- and rhuEPO^{M}-treatment can restore I/R injury-affected mitochondrial fission–fusion-related proteins and mitophagy- and autophagy-related markers, leading to anti-apoptotic effects and cell survival.

Regarding signaling pathways responsible for displaying the neuroprotective effect of asialo-rhuEPO^{P}, the Western blotting results showed a significant increase in phosphorylation of STAT5, PI3K, AKT, and ERK1/2 in asialo-rhuEPO^{P}- and rhuEPO^{M}-treated groups compared with the I/R–saline group [10]. These results suggest that, like rhuEPO^{M}, the neuroprotective effects of asialo-rhuEPO^{P} are mediated through the activation of STAT5, PI3K/AKT, and MAPK/ERK1/2 signaling pathways as reported previously by others for rhuEPO^{M} [38][39][40][41][42]. These findings also indicate that asialo-rhuEPO^{P}, like rhuEPO^{M}, exhibits pleiotropic effects through these pathways. However, at this time, it remains unknown what the relative contribution of each of these asialo-rhuEPO^{P}-induced signaling pathways is to the regulation of mitochondrial fission–fusion, mitophagy, autophagy, and apoptosis to display neuroprotective effects. In addition, the receptor responsible for transducing asialo-rhuEPO^{P} tissue-protective signal remains to be identified.

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