The Toxicity of a Chemically Synthesized Peptide Derived from Non-Integrin Platelet Collagen Receptors

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Abstract: A chemically synthesized peptide derived from platelet non-integrin collagen receptor has been shown to be an effective agent for inhibiting collagen-induced platelet aggregation and adhesion of washed radiolabeled platelets onto natural matrices and collagen coated microtiter plates. In order to be a therapeutic agent, we have used a cell culturing system and an animal model to test its cytotoxicities. In cell culture experiments, the peptide is not toxic to MEG-01, a megakaryoblastic cell line. Prior to performing experiments in rats, the existence of both platelet type I and type III collagen receptors and its functional roles in rat platelets had to be established. In this investigation, we report that rat platelets contain both receptors and the cHyB peptide inhibits both type I and type III collagen-induced rat platelet aggregation. In addition, analysis of the rat sera collected at various time intervals following an injection of cHyB into the rat-tail vein, did not show an increase in the activity of key enzymes which indicate tissue and/or organ damage. These results suggest that the cHyB peptide is safe and its development into a potential therapeutic agent for inhibiting thrombi formation is possible.

Keywords: collagen, platelet, platelet aggregation inhibitor, thrombosis

Introduction

Ligand-receptor interactions with the subsequent transduction of signals have long been recognized as a major mechanism for regulating cellular activities in eukaryotic cells. This mechanism is also operative when platelets interact with collagen exposed by damage to the endothelial surfaces of blood vessels. In blood vessels, type I and type III collagens are the major collagen components. The vessels also contain small amounts of types IV and V collagen and minor amounts of several other types of collagen. After platelets adhere to the exposed connective tissue, they aggregate, secreting biologically active substances, and effect hemostasis. Although many studies have centered on the role of the integrin and non-integrin collagen receptors in regulating platelet function, others have shown that platelets possess additional distinct reactive sites for type I collagen (Fitzsimmons et al. 1986) and type III collagen (Morton et al. 1987). Definitions of the platelet collagen receptors must be fully established before logical interventions can be identified and used to alter the course of abnormal hemostasis when platelets encounter denuded vascular surfaces. Development of an inhibitor(s) from platelet collagen receptors to inhibit the collagen-induced platelet activation and aggregation will prevent the risk of thrombosis.

We have characterized a non-integrin platelet receptor for type I collagen (Chiang et al. 1997) and defined its active peptide (Chiang and Kang, 1997; Chiang, 2000). In addition, we have characterized the platelet receptor for type III collagen and defined two active peptides in its sequences (Chiang et al. 2002). We have chemically synthesized a hybrid peptide (cHyB), which contains one of each active peptide of platelet types I and III collagen receptors with a linker of 12 amino acid residues. The cHyB can inhibit types I and III collagen-induced platelet aggregation and other functions in vitro (Du et al. 2007).

In the present investigation, we have studied the cytotoxicity of the cHyB in a human cell line and in rats, to determine its feasibility as a therapeutic agent.

Materials and Methods

Reagents

We have purchased Cytotoxicity Detection Kit (Roche Diagnostic Corp., Indianapolis, IL) to assay the activity of LDH). Assay Kits for ALT and AST were from Diagnostic Chemical Ltd (Oxford, CT).

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Reagents for the GS assay method were purchased from Sigma Chemical Inc. (St. Louis, MO). All other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO), Bio-Rad (Hercules, CA), Fisher Scientific (St Louis, MO), and Pierce (Rockford, IL). [^{3}H]-thymidine and MEG-01 cells were purchased from Perkin Elmer (Waltham, MA) and American Type Culture Collection (Manassas, VA), respectively.

Preparation of platelet-rich plasma (PRP)

Human blood (9 parts) from normal volunteers were collected in polypropylene tubes containing 1 part 3.8% Sodium Citrate following an overnight fast. PRP were prepared by centrifuging the citrated blood at room temperature for 10 min at 226 × g (Chiang et al. 1975). Whole blood and PRP were exposed to plastic surfaces or siliconized vessels only. Platelet counts of the PRP ranged from 200,000 to 300,000 per mm^3. The method to obtain PRP from rats was the same method as from humans for aggregation studies.

Platelet aggregation: Platelet aggregations were performed according to the method established by Born (1962).

Preparation of types I and III collagen

Type I and type III collagens were prepared from a human placenta of a normal delivery according the method developed by Seyer et al. (1976, 1980). Collagen concentrations were determined according the method described by Bergman and Loxley (1963).

Western blot analysis

Rat platelets were obtained from heparinized blood by centrifugation (225 × g) for 5 minutes and washed with 20 mM Tris-130 mM NaCl-1 mM EDTA, pH 7.3 (Tris-EDTA), sonicated, and protein concentration determined. Human and rat platelet lysates (100 μg) were separated on 7.5% SDS-PAGE and then transferred onto a nitrocellulose membrane. Following transfer, the membrane was incubated with 3% Difco skim milk in 20 mM Tris/500 mM NaCl/0.05% Tween 20 (TBST) for 1 hr at room temperature, then washed with TBST three times and probed with either anti-65-kDa antibodies (1:3000) or anti-47-kDa antibodies (1:5000) diluted in TBST/1% Difco skim milk at 4° overnight. The membrane was washed with TBST three times and probed with appropriate secondary antibodies (Cappel, Solon, OH) at 1:20000 dilution in TBST for 1 hr at room temperature, washed with TBS-T three times and visualized with chemiluminescent substrate (Pierce, Rockford, IL).

Culturing MEG-01 cells

MEG-01 cells were plated at a density of 1×10^5 cells/ml and cultured in RPMI 1640 medium with 2 mM L-glutamine, 10 mM HEPES, 1 mM Sodium pyruvate, 4.5 g/L glucose, adjusted to contain 1.5 g/L sodium bicarbonate, 10 mM HEPES, and 10% fetal bovine serum and grown at 37 °C and 5% CO_2. The sub cultivation ratio was 1:2 to 1:3 as recommended by the manufacturer.

The toxicity of the cHyB was examined in a cultured cell line to ensure the chemical synthesis of the cHyB did not contain cytotoxic component(s) (Chiang and Postlethwaite, 2007). First, the effect of cHyB on cultured MEG-01 cells was tested. We have used the cell line (5×10^5), cultured in the presence of various concentrations of cHyB and added [^{3}H]-thymidine for incorporation at different culturing times. Table1shows that the cHyB does affect the cell growth at 48 hr compared to 24 hr of different concentrations, which inhibit the adhesion of washed labeled platelets on rabbit aortic segments. These results are an acute effect. We will perform experiments with longer time exposures (1, 2, and 3 weeks), multiple additions, and higher doses to ensure the cHyB is safe to use.

Cytotoxicity studies in rats

The rats (two per group) were injected with either 0.25 ml of controls (0.5% DMSO in PBS or PBS alone) and 120 μg/kg cHyB as listed in the table. The initial venipuncture drawn immediately following injection counted as time zero. All withdrawn blood, time zero and each time point following injection (1, 3, 6, 24, and 36 hr), were placed in a microfuge tube containing 1/10 of heparin, centrifuged, plasma collected, and frozen until assay for activities of lactic acid dehydrogenase (LDH), glutamine synthase (GS), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) could be performed. The methods for the determining LDH, ALT, and AST were according to the manufacturer's protocol. The GS assay method was adapted from Kingdon et al. (1968).

Table 1. Cytotoxicity of cHyB on short-term cultured MEG-01 cells.

Treatments	24 hr culture	48 hr culture
Vehicle control	110950 ± 8269	185000 ± 8544
cHyB 1 μM	160500 ± 3500	174000 ±1527
cHyB 2 μM	155500 ± 9500	163500 ± 5500
cHyB 4 μM	158000 ± 3500	178000 ± 1000
cHyB 8 μM	170000 ± 3852	169666 ± 6807
cHyB 16 μM	162000 ± 4795	176000 ± 5031

MEG-01 cells were harvested, washed with PBS × 2, resuspended in growth media and aliquoted 5×10^5 cells/100ul/ well. The peptide, cHyB, was dissolved in DMSO, diluted with media (0.7% DMSO-final concentration), filter-sterilized and added to individual wells in appropriate concentrations. Various concentrations of hybrid peptide and vehicle control (0.7% DMSO) were added to appropriate wells for 24 and 48 hr, in triplicate. Following incubation, an aliquot of 1 μCi/10 μl/well of [^{3}H]-thymidine was added to each sample, cultured for an additional 24 hr, harvested using the Packard Harvester Filtermate 196 Cell Harvester and quantified with the Packard Matri × 96 Direct Beta Counter. Data were Mean ± S.D. of triplicate determinations. There is no significant effect by comparing the vehicle control and each individual concentration of cHyB (Student *t* test).

Results

Rat platelets possess 65-kDa and 47-kDa proteins

Rat platelets possess immunoreactive proteins to anti-65-kDa and anti-47-kDa antibodies. We have performed experiments showing that rat platelets possess the 65-kDa (panel A) and 47-kDa (panel B) receptors by Western blots. Figure 1 shows rat platelets possess anti-65-kDa active peptide antibody (panel A) and anti-47-kDa antibody (panel B) reactive bands. This result demonstrates that rat platelets contain immunoreactive proteins to human platelets. The cHyB inhibits collagen-induced rat platelet aggregation. The cHyB inhibits both type I and type III collagen-induced platelet aggregation similar to that of human platelets. Figure 2 shows the type I collagen-induced rat platelet aggregations are inhibiting dose-dependently by cHyB. Initially, we performed aggregation experiments with PRP from rats, which had not fasted overnight and observed that platelets required more collagen and longer delay time to induce platelet aggregation. We then performed experiments with the PRP from rats, which were fasted overnight and we did observe the inhibitory effect of cHyB on type III collagen-induced platelet aggregation (Fig. 3). The differences in lag-time of type I (PRP was prepared from rats' blood which had not fasted overnight) and type III collagen (PRP was prepared from rats who were fasted overnight).

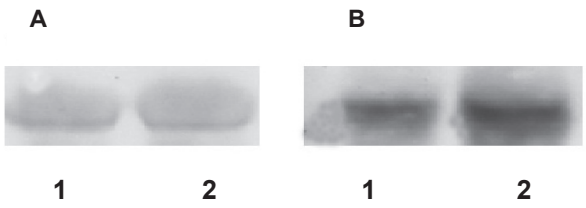

Figure 1. Platelets in rats possess immunoreactive bands with anti-65-kDa and 47-kDa antibodies. Human platelet membranes (100 μg, lane 1) and rat platelet membranes (100 μg, lane 2) Western blotted with anti-65-kDa (Panel A) and anti-47 kDa (panel B) antibodies. The dilution of first antibody was 1/1000 and the second antibody was 1/10000. Bands were visualized with enhanced chemiluminescence solutions.

Toxicity of the cHyB was Examined in Cultured the MEG-01 Cell Line

The cytotoxicity of the cHyB was examined using a cultured cell line to ensure that the chemical synthesis of the cHyB does not contain cytotoxic component(s). First, we tested the effect of cHyB on cultured MEG-01 cells. Cells were cultured (5×10^5) in the presence of various concentrations of cHyB (concentrations which inhibited the adhesion of washed labeled platelets on rabbit aortic segments) for various time points, then [^{3}H]-thymidine is added for incorporation by the cells. Table 1 shows that the cHyB does affect the cell growth at 24 hr nor 48 hr with differing concentrations of peptide. These results are an acute effect. Next, we tested the toxicity of cHyB in multiple doses and

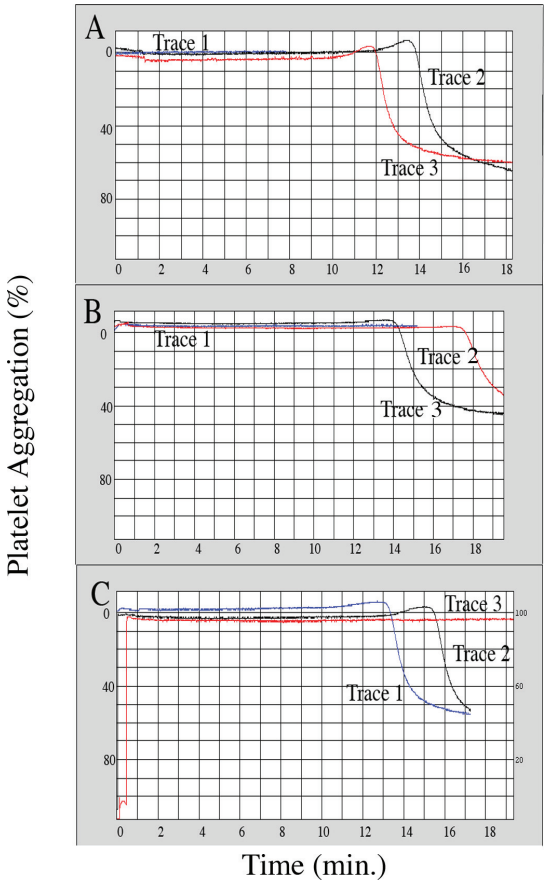

Figure 2. cHyB inhibits type I collagen-induced platelet aggregation. Panel A shows the platelet aggregation-induced by PBS (Trace 1) and various amounts of type I collagen (Traces 2 and 3 are 1 μg, and 2 μg, respectively). Panel B shows the effect of cHyB on type I collagen (1 μg)-induced platelet aggregation (Traces 1, 2, and 3 are 60 μg, 30 μg, and 15 μg, respectively). Panel C shows the inhibitory effect of cHyB (30 μg) on type I collagen-induced platelet aggregation can be reversed by adding higher amounts of type I collagen (Traces 1, 2, and 3 are 3 μg, 2 μg and 1 μg, respectively). Following pre-incubations of various amounts of cHyB with collagen, an aliquot of 0.45 ml PRP was added to the cuvette and platelet aggregation was immediately (at time 1, x-axis) monitored with a ChronoLog Lumi-Aggregometer.

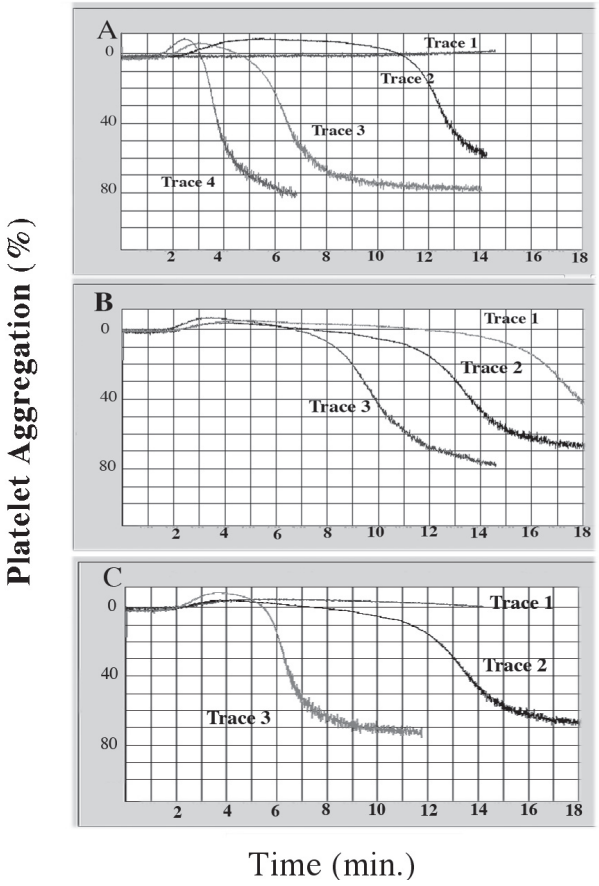

Figure 3. cHyB inhibits type III collagen-induced platelet aggregation. Panel A shows the platelet aggregation-induced by PBS (Trace 1) and various amounts of type III collagen (Traces 2, 3, and 4 are 0.25 μg, 0.5 μg, and 1 μg, respectively). Panel B shows the effect of cHyB on type III collagen (0.5 μg)-induced platelet aggregation (Traces 1, 2, and 3 are 60 μg, 30 μg, and 15 μg, respectively). Panel C shows the inhibitory effect of cHyB (30 μg) on type III collagen-induced platelet aggregation can be reversed by adding higher amounts of type I collagen (Traces 1, 2, and 3 are 0.25 μg, 0.5 μg and 1 μg, respectively). Following pre-incubations of various amounts with collagen, an aliquot of 0.45 ml PRP was added to a cuvette and platelet aggregation was immediately (at time 1, x-axis) monitored with a ChronoLog Lumi-Aggregometer.

long-term cultures and these results are in Table 2 and Table 3. These data demonstrated that there are no toxic effects by cHyB on MEG-01 cells. We intend to perform longer-term cultures (1, 2, and 3 weeks), additional multiple dosing, and extension the dose response curve to ensure that use of cHyB is safe.

Toxicity of the cHyB was Examined with Rats by I. V. Injection

We have also used the LDH Cytotoxicity Detection Kit (lactate dehydrogenase—LDH) to examine the plasma damage effect by cHyB in rats. We measured the release of LDH at different time intervals following injection of the tail veins. Results of the study (Table 4) show that the release/activity of LDH does not increase with the time, at the concentration of cHyB used. These results are also an acute effect.

In order to determine whether cHyB has a damaging effect on various tissues (heart, muscle, and lung), we assayed the activity of three enzymes i.e. glutamine synthtetase (GS), aspartate amino transferase (AST), and alanine amino transferase (ALT) of cHyB injected rat sera. Results show that these enzyme activities were undetectable in these samples (data not shown).

Table 2. The effect of multiple doses of various concentrations of cHyB on long-term MEG-01 cultured cells.

Treatments	Mean + S.D.
Med. Control	48541 + 1999
Med. + vehicle control (DMSO)	51634 + 4851
cHyB 1 μM	46797 + 1509
cHyB 2 μM	46779 + 3721
cHyB 4 μM	59068 + 3801
cHyB 8 μM	53304 + 4743

MEG-01 cells were harvested, washed with PBS, resuspended in growth media and aliquoted as in Table 1. The peptide was prepared (as in Table 1) and appropriately added to individual wells with final concentrations of 1, 2, 4, and 8 μM, respectively, in triplicate. Media and media with DMSO are controls. The cells were maintained at 37 °C with 5% CO_2. Following 24 and 48 hr of incubation, corresponding wells received a second and third dose of the peptide, respectively. After 72 hr of incubation, each well received 1 μCi/10 μl of [H^3]-thymidine and allowed to incubate an additional 24 hr. The cells were harvested and quantified as described in Table 1. There is no significant effect by comparing the vehicle control and each individual concentration of cHyB (Student *t* test).

Discussion

The use of an animal model is essential for the discovery of new drugs effective in the prophylaxis and treatment of arterial thrombosis. A modern approach used in attempts to unravel the physiological role of the various integrin and non-integrin receptors has been the use of specific "knock out" mice. Elimination of several integrin subunits (β1, β5, β6, and αv) has been performed and has lead to embryonic or prenatal lethal phenotype, however, ablation of ß3 or β1 does not affect development or viability. Conversely, mice lacking α2 or αIIb have still not been breed. This area has been reviewed (Chen and Sheppard, 2007). A knockout mouse of the non-integrin collagen receptor, GP IV (CD36) has been successful but its role in platelet function has not been defined (Goudriaan, 2002). Many other animal models have been used to test the effectiveness of an inhibitor on the thrombi formation (Philip et al. 1978; Hynes and Bader, 1997; Kuez et al. 1990; Gaber et al. 2004). Leger et al. (15) developed a guinea pig arterial thrombosis model to study a protease-activated receptor 1–4 heterodimer in platelet-mediated thrombosis. Hladovec (1971) has described an experimental model of arterial thrombosis in rat, which was induced in the carotid artery by electric current. Kurt et al. (1990) modified this model by using 30% $FeCl_3$ instead of an electric current to injure vessel walls thus preventing swift corrosion of the artery. Gaber et al. (2004) used the rat cremaster muscle to study the leukocyte-endothelial cell interactions in microvessels. This rat model appeared to be a good candidate for our purpose. The rat model was selected due to the manageable size of the rats and their blood vessels for our studies. Others, including

Table 3. The effect of various concentrations of cHyB on MEG-01 cell growth.

Treatments	Mean ± S.D.
Med. Control	45439 ± 1211
Med. + vehicle control (DMSO)	46182 ± 8045
cHyB 1 μM	51155 ± 6777
cHyB 2 μM	45613 ± 3288
cHyB 4 μM	43519 ± 6705
cHyB 8 μM	48658 ± 1904

MEG-01 cells were harvested, washed with PBS × 2, and aliquoted as described in Table 1. Also, the dissolution and initial distribution of the peptide, controls and culturing conditions were the same as described in Table 1. Following 48 hr of incubation, the cells received a second dose of peptide, incubated for an additional 24 hr before the addition of 1 μCi/10 μl of [H^3]-thymidine. Again, the cells were cultured for thymidine uptake, harvested, and quantified as described in Table 1. There is no significant effect by comparing the vehicle control and each individual concentration of cHyB (Student *t* test).

Table 4. The activity of LDH in cHyB injected rat sera.

Treatments	0 hr	1.5 hr	3 hr	6 hr	24 hr	36 hr
PBS control	0.076	0.131	0.045	0.072	0.023	0.022
Vehicle control	0.054	0.105	0.128	0.051	0.130	0.041
120 μg cHyB/kg	0.063	0.109	0.062	0.079	0.082	0.050

Rats (two per group) were injected with 0.25 ml of either: control (PBS) or vehicle control (PBS/0.7% DMSO), or cHyB as listed in the table, following venipuncture as zero time. At each time point, blood was withdrawn, placed in a microfuge tube with 1/10 heparin, centrifuged, the plasma collected separately, and frozen at –80 ºC until LDH activity could be measured. Determination of LDH activity for each time point was according to manufacturer's suggestions, using 100 μg/well, in triplicate. A positive control of 0.055U LDH yielded O.D 490 nm = 1.788).

Chen et al. (2000) have applied this model to study the effects of fish oil on arterial thrombogenesis, platelet aggregation, and superoxide dismutase activity. Hynes et al. (1997) have used targeted mutations in integrins and their ligands to study the receptor and ligand interaction. Although, we do not have animal model for the role of the 65-kDa and 47-kDa proteins, we have used rat thrombosis model to test the effective of cHyB on thrombi formation (Du et al. 2007). In that report, we have established that cHyB can inhibit types I and III collagen-induced platelet aggregation, adhesion of platelets onto types I and III collagen-coated microtiter wells, and rabbit aortic segments in a dose-dependent manner. To advance the peptide as a useful therapeutic agent, we tested its cytotoxicity in cells and rats. Results from the present study suggest that the cHyB is not toxic to cultured cells by using [^{3}H]-thymidine incorporation at different time intervals and multiple additions of the cHyB. The cHyB does not have a toxic effect on the MEG-01 cells suggesting it is safe for further studies in an animal model.

Results from our earlier studies have shown that the cHyB is a specific and functional inhibitor *in vitro* experiments (Du et al. 2007; Zhu et al. 2007). For the therapeutic usefulness, we have injected cHyB into tail vein of rats, taken blood samples at different time intervals, and measured the activity of LDH, AST, ALT, and GS in the duration of 36 hr. The activity of these enzymes did not increase in these samples suggesting that the cHyB did not damage organs (muscle, heart, and lung). We will perform experiments with multiple doses and longer time frames (1, 2, and 3 weeks) to study the effect of cHyB on tissue damage. Additional studies to fully characterize the cytotoxicity of selected organs (liver, heart, kidney, and brain) are needed to justify its usefulness.

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Abbreviations Used Were

PRP, platelet-rich plasma; cHyB, chemical synthesized peptide containing one of active peptide derived from both platelet type I and type III collagen receptor with 12 amino acid residues as a linker; LDH, lactic acid dehydrogenase, GS, glutamine synthase, ALT, alanine aminotransferase, and AST, asparate aminotransferase; Tris-EDTA, 20 mM Tris-130 mM NaCl-1 mM EDTA, pH 7.3; TBS, 20 mM Tris-500 mM NaCl; TBST, 20 mM Tris-500 mM NaCl-0.05% Tween 20.

Disclosure

The authors report no conflicts of interest.

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