BINDING OF HUMAN PLACENTAL RIBONUCLEASE INHIBITOR TO EXTRACELLULAR MATRIX MOLECULES

TAKAHISA SHINOMIYA¹, NOZOMI NISHIMURA¹, KOICHI TAMOTO² and TSUTOMU HONJOH³

¹Department of Experimental Surgery and Biotechnology, National Children's Hospital Medical Research Center, Taishido, Setagayaku, Tokyo 154, ²Department of Microbiology, Faculty of Pharmaceutical Sciences, Higashi-Nippon-Gakuen University, Ishikari-Tobetsu 061-02, and ³Morinaga Institute of Biological Science, Shimo-sueyoshi, Tsurumiku, Yokohama 230, Japan

ABSTRACT

The ribonuclease inhibitor from human placenta (HPRI) is a protein that inhibits pancreatic type ribonucleases (RNases) and is widely utilized in the field of molecular biology. We investigated the binding ability of biotinylated HPRI to the extracellular matrix (ECM) molecules by the use of an enzyme-conjugated streptavidin system. Biotin-HPRI bound to RNase A and to a number of ECM molecules such as collagens, laminin and fibronectin, whereas only weak binding activity was observed to some serum proteins such as albumin, transferrin, IgG, vitronectin and fibrinogen under the same experimental condition. The binding of biotin-HPRI to the ECM molecules was not inhibited by the addition of RNase A, suggesting that the binding mechanism for ECM molecules is different from that for RNase A.

Ribonuclease inhibitors have been found in many tissues of different animals (1, 7, 12). The inhibitor is a protein with a molecular mass of approximately 50 kDa that forms a very tight reversible complex with RNases of pancreatic type (2, 3). Angiogenin (13, 15), an RNase from HT-29 colon carcinoma cells (23), and RNase PL₁ and PL₃ from porcine liver (9) have also been found to bind to the inhibitor. Some RNase inhibitors have been purified from human placenta (14, 22) and porcine liver (8), and their primary structures have been determined. The primary structures of the ribonuclease inhibitor from human placenta and porcine liver are approximately 80% identical and are built from 15 homologous leucine-rich repeats. Interestingly, all cysteine residues, 32 in the human and 30 in the porcine inhibitor, were found to be in the reduced form. The inhibitor was irreversibly inactivated by sulfhydryl reagents (4).

We have been studying the metabolism of ¹²⁵I-labelled ribonuclease inhibitor from human placenta (HPRI) in cultured cells, and found it to bind directly to the dishes pretreated with fibronectin. This observation suggested that HPRI might bind to some types of extracellular matrix molecules. We labelled HPRI with a biotinylation reagent and test-

ed the binding activity to some extracellular matrix (ECM) molecules such as laminin (16), collagens type I to V (5, 17-19) and fibronectin (20) by the use of a microplate and streptavidin-enzyme assay. We report here the results, with a discussion of a possible physiological role of HPRI.

MATERIALS AND METHODS

Purification and Biotinylation of HPRI

The human placental RNase inhibitor was purified from human term placenta according to the method of Blackburn *et al.* (4) with slight modification by the use of RNase A-Sepharose and Mono Q ion exchange chromatography. The purity of HPRI was above 98% when analyzed by polyacrylamide gel electrophoresis containing sodium dodecylsulfate. Purified HPRI was dialyzed against phosphate-buffered saline (PBS) and subsequently biotinylated with *N*-hydroxysuccinimide-biotin ester incorporating a spacer arm in 0.05 M sodium borate buffer pH 8.5 by the use of a biotinylation reagent kit (PRN28, Amersham, U.K.) in accordance with the procedure recommended by the manufacturer. Biotinylated HPRI (biotin-HPRI) was separated from unreacted reagents with a Sephadex G-25

Fig. 1 The binding activity of biotin-HPRI. Each protein solution was diluted with PBS to various concentrations (horizontal axis) and coated for 2 h at room temperature in microtiter wells (0.1 ml/well). The washed plate was treated with 0.1 ml/well of 2.5 μ g/ml biotin-HPRI for 1 h. Binding of biotin-HPRI was measured using streptavidin-conjugated β -galactosidase. For details, see Materials and Methods. Proteins used for coating wells; RNase A ($\circ$ -- $\circ$), bovine collagen type II (∇ — ∇), human collagen type III ($\triangle$ — $\triangle$), mouse laminin ($\circ$ — $\circ$), human fibronectin ($\square$ — $\square$), human IgG ($\times$ --- $\times$) and bovine albumin ($\bullet$ — $\bullet$).

(Pharmacia LKB) column in PBS solution.

Purification of Human IgG and Fibronectin

Human IgG was purified from human serum by Protein-G affinity chromatography combined with G-200 gel filtration. Fibronectin was purified according to the method of Rouslahti *et al.* (21) by the use of gelatin-Sepharose.

Extracellular Matrix Proteins

Human collagen type I, III, IV and V were kind gifts of Dr T. Takahashi (Morinaga Institute of Biological Science, Yokohama, Japan), and bovine collagen type II was a product of Koken, Japan. These collagens were purified from the fractions solubilized with pepsin digestion. Mouse laminin and mouse collagen type IV, purified from EHS sarcoma, were products of Life Technologies, U.S.A.

Bovine vitronectin and human vitronectin were purchased from Koken and Takara Biomedicals, Japan, respectively.

Binding Assay

Wells of microtiter plates (Nunc Immunoplate Maxisorp F96, Nunc, Denmark) were coated with 0.1 ml of an appropriate protein freshly diluted with PBS for 2 h at room temperature. The wells were then washed with PBS and treated with 0.15 ml of buffer A (0.1 mM *d*-biotin/0.05% Tween 20/0.02% 2-mercaptoethanol/PBS/0.05% NaN_3) for 1 h to prevent non-specific binding. After washing with PBS, wells were allowed to react for 1 h with 0.1 ml/well of biotin-HPRI with or without a binding inhibitory substance (e.g. RNase A) in buffer A. Wells were then washed and incubated for a further hour with 0.1 ml/well of streptavidin-conjugated- β -galactosidase (Life Technologies, U.S.A.)

Fig. 2 Biotin-HPRI concentration and binding activity. Various proteins at 10 $\mu\text{g/ml}$ in PBS were coated for 2 h in a microtiter plate (0.1 ml/well). Binding to coated proteins was tested by the addition of serially diluted biotin-HPRI (horizontal axis). Proteins used for coating wells; RNase A ($\circ$ -- $\circ$), mouse laminin ($\circ$ — $\circ$), bovine collagen type II (∇ — ∇), human collagen type V ($\triangle$ — $\triangle$), human fibronectin ($\square$ — $\square$), and bovine albumin ($\bullet$ -- $\bullet$).

at 500-time dilution with buffer B (0.05% Tween 20/0.02% 2-mercaptoethanol/PBS/0.05% NaN_3). After washing the wells again, 0.1 ml of a 0.2 mg/ml solution of chlorophenol red- β -D-galactopyranoside (Boehringer Mannheim GmbH) in buffer B was added to each well and the reaction was stopped after 30 min by the addition of 0.05 ml 0.1 M Na_2CO_3 . The absorbance of red colored substrate solutions was measured by the microplate photometer with double filters at 550 nm–650 nm.

Other Materials

Bovine serum albumin, bovine pancreatic ribonuclease A, bovine fibrinogen, human transferrin and Tween 20 were purchased from Sigma Chemical, U.S.A.

RESULTS

Binding of Biotin-HPRI to the Microtiter Plate Wells Coated with Proteins

We tested the binding of biotin-HPRI to the various proteins coated the microtiter wells. Fig. 1 shows that serial dilution of proteins reduces the

binding of biotin-HPRI (constant concentration: 2.5 $\mu\text{g/ml}$). In the high concentration region ($\sim 40 \mu\text{g/ml}$) of coating proteins, the binding of biotin-HPRI to some proteins tended to increase, as shown for human IgG. The binding of biotin-HPRI to human IgG was reduced with the decrease in the concentration of coating proteins, and the binding became negligible at concentrations lower than 10 $\mu\text{g/ml}$. On the contrary, even in a lower concentration range, biotin-HPRI still exhibited high binding activities to RNase A, laminin and collagens. As shown in Fig. 2, similar binding characteristics were observed when the binding of serially diluted biotin-HPRI was measured using the microtiter wells coated with a constant concentration (10 $\mu\text{g/ml}$) of proteins. Proteins such as RNase A, laminin, collagen type II, collagen type III and collagen type V show high reactivities. Table 1 shows the binding activity of biotin-HPRI (2.5 $\mu\text{g/ml}$) to the wells coated with various types of proteins at the concentration of 10 $\mu\text{g/ml}$. The binding activities were represented as optical densities (mean $\pm$ SD, $n=4$). Based on the reactivity of biotin-HPRI, proteins used for coating wells could be divided into four groups. RNase A showed the

Table 1 *Binding of Biotin-HPRI to Various Proteins*

Reactivity with HPRI	Proteins	Absorbance $\pm$ SD
High	RNase A (bovine pancreas)	1.96 $\pm$ 0.17
Moderate	Collagen Type II (bovine)	0.344 $\pm$ 0.016
	Collagen Type III (human)	0.419 $\pm$ 0.041
	Collagen Type IV (mouse)	0.287 $\pm$ 0.045
	Collagen Type V (human)	0.403 $\pm$ 0.031
	Laminin (mouse)	0.492 $\pm$ 0.034
Low	Collagen Type I (human)	0.089 $\pm$ 0.004
	Collagen Type IV (human)	0.099 $\pm$ 0.010
	Fibronectin (human)	0.146 $\pm$ 0.041
Very low	Fibrinogen (bovine)	0.049 $\pm$ 0.008
	Vitronectin (human)	0.036 $\pm$ 0.002
	Vitronectin (bovine)	0.035 $\pm$ 0.005
	IgG (human)	0.066 $\pm$ 0.016
	Transferrin (human)	0.039 $\pm$ 0.011
	Albumin (bovine)	0.034 $\pm$ 0.007
Control	PBS	0.023 $\pm$ 0.003

Binding was measured in wells coated with protein solutions (10 μ g/ml, 0.1 ml/well) using streptavidin- β -galactosidase as described in Materials and Methods. The concentration of biotin-HPRI was kept constant at 2.5 μ g/ml and 0.1 ml/well.

highest binding reactivity. Human collagen I, IV and fibronectin constituted a group exhibiting a low reactivity. Bovine collagen II, human collagen III, V, collagen IV from EHS sarcoma and laminin were proteins having a moderate reactivity. As for plasma proteins such as bovine fibrinogen, vitronectins, human IgG, human transferrin and bovine albumin, their reactivities were very low and were nearly at the non-specific binding level.

Effect of RNase A on the Binding of Biotin-HPRI to ECM Molecules

RNase A and angiogenin were reported to bind to the same site on a HPRI molecule (24). We tried to determine whether the binding of HPRI to ECM molecules is of the same nature as that to RNase. An inhibition experiment with RNase A was performed by addition to the biotin-HPRI solution before the reaction in the wells coated with various proteins. Fig. 3 shows that clear inhibition occurs in the presence of RNase A in the binding of biotin-HPRI to the RNase A-coated wells. Nearly 80% inhibition in the binding of biotin-HPRI to RNase A was observed at a 1:1 molar ratio of RNase A to biotin-HPRI, and the inhibition pattern showed a stoichiometrical S-shaped curve. On the contrary, addition of RNase A did not result in any signifi-

cant inhibition on the binding of biotin-HPRI to other proteins, including collagens, laminin and fibronectin, which suggests that the RNase A binding site was not used directly for the binding to ECM molecules.

DISCUSSION

We examined the reaction of biotin-HPRI with various types of ECM molecules and serum proteins. There may be some arguments against HPRI binding to ECM molecules, such that the contaminating proteins were labelled and reacted with ECM molecules. However, this possibility will be very low, because the purity of HPRI was above 98%, and the binding of HPRI to ECM molecules was relatively high if it be resulted from contamination of other proteins. Furthermore, the binding of HPRI to laminin and collagens was confirmed by the radioimmunoassay using 125 I-labelled rabbit anti-HPRI antibody, instead of the biotin and streptavidin- β -galactosidase system (data not shown). Another question is whether the biotinylation reaction changes the properties of HPRI to cause the binding ability to ECM molecules. We obtained similar binding to ECM molecules using HPRI labelled with 125 I-Bolton-Hunter reagent. The specific activity of the 125 I-Bolton-Hunter reagent was

Fig. 3 Inhibitory effect of RNase A on biotin-HPRI binding. Each protein was coated at 10 $\mu\text{g}/\text{ml}$ and 0.1 ml/well for 2 h. Serial dilutions of RNase A were mixed with biotin-HPRI (2.5 $\mu\text{g}/\text{ml}$). After 1 h incubation at room temperature, 0.1 ml of the mixture was transferred to wells previously coated with proteins. Biotin-HPRI remained in wells was measured with streptavidin- β -galactosidase. Proteins used for coating; RNase A (○--○), mouse laminin (○—○), human collagen type III (△—△) and bovine collagen type II (▽—▽).

high enough (81.4 TBq/mmol), and almost all ^{125}I -labelled HPRI (0.8 TBq/mmol) contained only one lysine residue modified with the reagent. It seems unlikely that the modification of one lysine residue changed the reactivity of HPRI, though such a possibility can not be excluded conclusively.

RNase A had no effect on the binding of biotin-HPRI to ECM molecules (Fig. 3). Laminin was expected to inhibit binding of biotin-HPRI to laminin-coated wells, but no inhibition was observed even in the presence of a high concentration of laminin (100 $\mu\text{g}/\text{ml}$). A similar result was obtained with human collagen type III (data not shown). These results suggest that HPRI reacts with molecules fixed on the surface of wells.

Fujita *et al.* (6) have reported that intraperitoneal administration of a relatively low dose (100 ng/kg/day, for 20 days) of HPRI reduced metastases of Lewis Lung Carcinoma to the recipient (BALB/c) lung. In general, collagens, laminin and fibronectin are thought to play an important role when cancer cells attach to metastatic sites. A fibronectin peptide (GRGDS) and a laminin peptide (YIGSR), derived from the cell adhesion region of fibronectin

and laminin, respectively, are known to inhibit the metastasis of cancer cells (10, 11). This effect appears to be due to inhibition of interactions between cancer cells and basement membranes. HPRI injected into the peritoneal cavity may bind to ECM molecules, resulting in the suppression of metastasis. Further studies on the properties of HPRI and interactions between HPRI and ECM molecules may ultimately contribute to clarify roles of RNase inhibitors in physiological and pathological processes.

Received 14 December 1992; and accepted 18 February 1993

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