Targeting of enzyme immobilized on erythrocyte membrane to collagen-coated surface

V.R. Muzykantov, D.V. Sakharov, M.D. Smirnov, S.P. Domogatsky and G.P. Samokhin

Institute of Experimental Cardiology, USSR Cardiology Research Center, Academy of Medical Sciences, 3rd Cherepkovskaya str., 15A, 7, Moscow 121552, USSR

Received 20 December 1984

It is suggested to use 'enzyme(s)-erythrocyte-antibody' complex for modulation of the microenvironment in definite compartments of blood circulation. A model system including peroxidase, human erythrocytes and anti-collagen antibodies was chosen to illustrate the principle. Peroxidase was conjugated to the erythrocyte surface via periodate-oxidized enzyme carbohydrate moiety; biotinylated antibodies were linked by avidin to the biotinylated erythrocytes. The properties of the immunocomplexes obtained have been investigated in an artificial system simulating an injured blood vessel wall. The advantages in using erythrocyte-mediated immunoenzyme complexes for enzyme (drug) targeting are discussed.

Drug targeting *Collagen* *Immobilized enzyme* *Antibody attachment* *Peroxidase* *Specific binding*

1. INTRODUCTION

The targeting of drugs included in synthetic (liposomes) or natural (erythrocytes) containers possessing high specific affinity for organ, tissue or cell target, can be an effective method in treatment of many diseases [1–5]. High local concentration of the drug in the focus of the pathological process is the main advantage of targeting. In recent years significant success in the improvement of targeted intracellular injection of drugs has been achieved [6–8]. However, in some diseases (e.g., mural thrombogenesis [9]), the desired locus of drug action may be exposed to the bloodstream. There are three main obstacles to the practical application of systems employing water-soluble drug delivery from targeted containers – liposomes or erythrocytes in these cases. First, drug release from the container needs to be associated with its fixation on the target surface. Second, a considerable portion of the drug released into the blood will be washed off by the bloodstream before it can diffuse into the target tissue. Third, a container can discharge only once and, therefore, cannot induce prolonged effects.

Another approach would be the continuous conversion of inactive drug precursor(s) circulating in the blood into an active form by the targeted enzyme(s). A number of enzymes have been successfully introduced into clinical practice to correct metabolic disorders. Co-immobilization of both enzymes and antibodies on a biodegradable carrier is expected to be advantageous over direct immunoenzyme conjugates in the following respects:

(i) Enzyme stability can be improved as a result of immobilization [10,11].

(ii) Several different enzymes can operate in concert performing sequential transformation of metabolite.

(iii) Cooperative interaction of antibodies can provide tight specific binding at much lower antibody to enzyme ratios. Cooperative interaction of monoclonal antibodies with different specificities can be realized.

We have shown previously that red blood cells conjugated with antibodies to human type I collagen selectively bind to denuded regions of a human artery ex vivo [5]. This work explores the possibility of concurrent antibody and enzyme immobilization on the erythrocyte surface and in-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

vestigates the properties of the complex obtained for enzyme targeting in vitro.

2. MATERIALS AND METHODS

The blood from healthy volunteers was collected into acid-citrate anticoagulant and kept at 4°C for not more than 3 days. Before use the erythrocytes were 5 times washed by centrifugation in phosphate-buffered saline (PBS) (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4).

Type I collagen was obtained from human placenta, and rabbit anticollagen antibodies were obtained according to [12]. Avidin from hen egg-white was isolated according to [13] and assayed spectrophotometrically as in [14]. Bovine serum albumin (BSA) (Sigma), horseradish peroxidase (HRP) (Fluka), and biotin *N*-hydroxysuccinimide ester (Sigma) were used in the study.

2.1. *HRP immobilization on erythrocyte surface*

The standard procedure was as follows: 25 μl freshly prepared aqueous $NaIO_4$ (40 mg/ml) was added to 100 μl aqueous HRP (10 mg/ml). After 25 min incubation (20°C) the oxidized HRP (ox.HRP) was gel filtered by centrifugation on a microcolumn with Sephadex G-25 into buffer (0.15 M NaCl, 1 mM CH_3COONa; pH 3.8). The washed erythrocytes were placed in buffer (100 mM NaCl, 50 mM Na_2CO_3; pH 9.3) and 100 μl of gel-filtered ox.HRP was added to 100 μl of erythrocyte suspension (hematocrit value 50%, v/v). After 2 h incubation at 20°C with constant vigorous agitation, 30 μl $NaBH_4$ (15 mg/ml in PBS) was added, incubated for 15 min under vortex-mixing, then erythrocytes were washed by centrifugation using 6 × 10 ml PBS. ^{125}I-labeled HRP was added to the initial HRP solution as a tracer to determine the amount of immobilized protein.

2.2. *Assay of immobilized HRP activity*

HRP substrate was prepared by dissolving 1 mg *o*-phenylenediamine in 0.1 ml methanol and mixing it with 5 μl 30% H_2O_2 in 10 ml PBS.

0.1 ml of HRP-carrying or intact erythrocyte suspension (hematocrit 0.5%) in PBS-BSA (PBS, containing 2 mg/ml BSA) was mixed with an equal volume of substrate solution in a well of a Microtest microtitration plate (Dynatex). After 10 min incubation the plates were centrifuged in a TH-4 rotor (Beckman) at 3000 rpm for 2 min. Then, 100 μl of supernatant from each well was quickly transferred to the corresponding well of the empty plate, 25 μl of 50% H_2SO_4 were added to each well and 10 min later the absorbance in the wells at 490 nm was determined with an MR-580 Autoreader spectrophotometer (Dynatex). The obtained data were compared with the calibration curve obtained in identical conditions using known amounts of HRP dissolved in PBS-BSA.

2.3. *Coupling of antibodies to erythrocyte surface and erythrocyte incubation in antigen-coated wells*

The procedure was carried out as described in [4]. Briefly, the antibodies or non-immune rabbit IgG and erythrocytes (intact or HRP-carrying) were biotinylated separately. Then the excess of biotin ester was removed and avidin and biotinylated protein were added to biotin-modified erythrocytes. To determine the amount of the coupled protein, ^{125}I-labeled IgG was added to the initial IgG solution as a tracer.

Multiwell plates (Falcon) were coated with type I collagen or BSA as in [4]. The non-bound excess of antigen was preincubated with PBS-BSA for 1 h. Then 300 μl of erythrocyte suspension (hematocrit 0.5%) were added to the wells and incubation and washing of non-bound erythrocytes were performed as in [4]. Each sample of erythrocytes was incubated in two identical wells.

2.4. *Quantitation of antigen-bound erythrocytes and associated peroxidase activity*

After washing, a standard volume of distilled water was added to a well, and hemoglobin absorbance at 405 nm in lysate was determined. The amount of erythrocytes in the well was estimated according to the calibration curve obtained with standard erythrocyte suspension lysates.

An identical well was washed out and supplemented with 500 μl substrate solution, incubated for 10 min with careful stirring, then 200 μl substrate was transferred to a well of a Microtest (Linbro) plate, 50 μl of 50% H_2SO_4 was added and 10 min later the absorbance at 490 nm was determined.

3. RESULTS AND DISCUSSION

Enzymes may be immobilized on the erythrocyte surface in various ways. HRP conjugation via its carbohydrate moiety oxidized by periodate was chosen because: (i) this precluded unwanted chemical modification of erythrocytes, (ii) this method is suitable for conjugation with immunoglobulins [16], (iii) it seemed to be adaptable for various carbohydrate-containing enzymes. For example, galactose oxidase was effectively linked to polymers in a similar manner [15]. Fig.1 demonstrates a linear dependence of HRP coupling to erythrocytes on initial ox.HRP concentration in the reaction mixture. It reaches 10^5 molecules of bound HRP per erythrocyte at the highest ox.HRP concentration used (5 mg/ml). The loss of bound HRP activity was attributed mainly to the oxidation step and did not exceed 25% of the initial activity (fig.2). In subsequent experiments it was possible to evaluate the number of immobilized HRP molecules according to its activity associated with erythrocytes.

The efficiency of coupling was strictly dependent on the degree of oxidation of HRP carbohydrate residues; non-oxidized HRP did not bind to erythrocyte membrane. It is seen from fig.3 that the coupling value reaches a plateau at a periodate/HRP molar ratio equal to 200, and further increase in periodate concentration had little effect. Evidently, at a molar ratio equal to 200 the majority of the reactive carbohydrate groups of the enzyme are oxidized. At this molar ratio further experiments were performed. It was necessary to remove thoroughly periodate after HRP oxidation, since periodate caused significant hemolysis. Fig.4 shows the time course of the coupling reaction. The reaction rate was constant over 6 h, and much lower than that of HRP coupling to immunoglobulins [16]. Further increase of incubation time was of no use because of hemolysis. In the technique described above both enzyme and

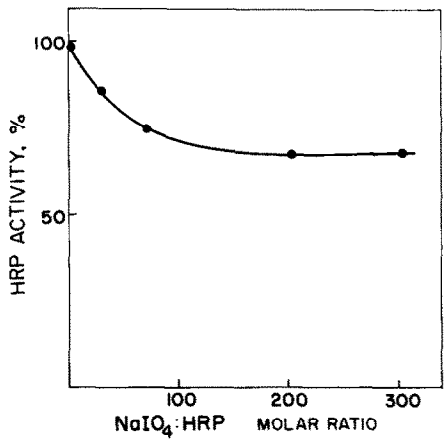

Fig.2. The effect of oxidation on HRP activity at different periodate/HRP molar ratios.

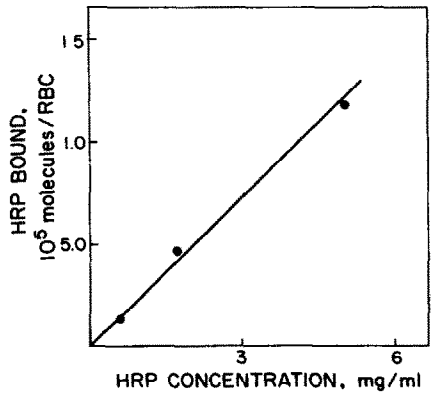

Fig.1. Dependence of HRP coupling to erythrocytes on ox.HRP concentration in reaction medium (after subtraction of the background of inherent erythrocyte peroxidase activity equal to the activity of 8×10^3 HRP molecules per erythrocyte). Incubation time 2 h; periodate/HRP molar ratio = 200.

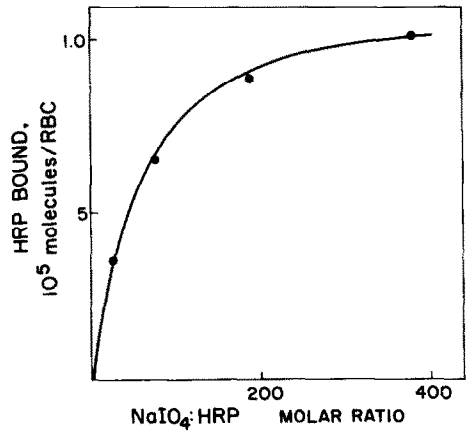

Fig.3. Dependence of HRP coupling to erythrocytes on the degree of HRP oxidation (periodate/HRP molar ratio). Incubation time 2 h, ox.HRP concentration in the reaction medium 3.5 mg/ml.

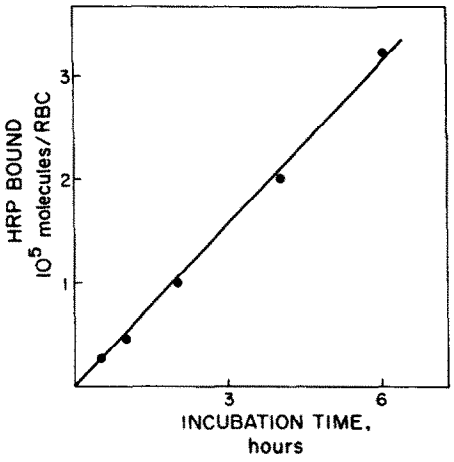

Fig.4. The kinetics of HRP coupling to erythrocytes. Conditions are described in section 2.

biotin ester reacted with amino groups. The amount of amino groups (or any other reactive groups) on the erythrocyte surface is limited, as well as the space accessible for protein conjugation. To optimize enzyme loading, it was necessary to determine the minimal quantity of antibodies sufficient to provide specific targeting. In previous experiments 4.5×10^5 antibody molecules per cell were successfully used. Fig.5 shows that equally effective attachment of immunoerythrocytes to antigen-coated surface could be achieved at 3×10^4 molecules per cell. Lowering of the ratio to 1×10^4 molecules per cell caused only a 2-fold decrease in attachment. It is worth mentioning, however, that this value will differ for various antibody preparations; so we have estimated an order of magnitude. Up to 10^6 enzyme molecules can be conjugated to the erythrocyte surface and antibody would not be a limitation. Using ^{125}I-labeled IgG, we compared the efficiency of immunoglobulin attachment via avidin-biotin interaction to intact and HRP-carrying erythrocytes. It was found that HRP immobilization did not effect the subsequent linkage of antibodies. In turn, the procedure of HRP-carrying erythrocyte biotinylation and subsequent antibody attachment did not influence the activity of immobilized HRP.

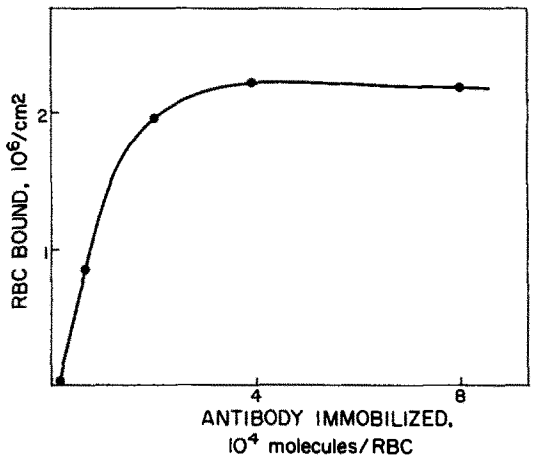

Fig.5. Dependence of antibody-erythrocyte complex binding to collagen-coated surface on the number of antibody molecules immobilized on erythrocyte.

Finally, we studied binding of peroxidase-erythrocyte antibody complexes to the collagen-coated surfaces. A system simulating denuded blood vessel wall has been described [3]. Peroxidase-conjugated immunoerythrocytes differ from

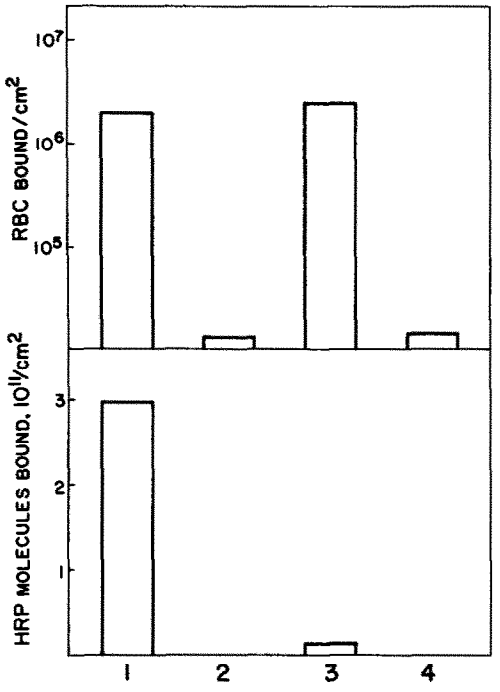

Fig.6. Binding of immunoerythrocytes to collagen-coated surface. (A) Number of erythrocytes bound per cm^2 surface. (B) HRP activity bound per cm^2 surface (HRP activity after incubation of samples 2 and 4 was negligible), expressed as the number of peroxidase molecules. (1) HRP-antibody-erythrocyte, (2) HRP-non-immune IgG-erythrocyte, (3) antibody-erythrocyte, (4) non-immune-IgG-erythrocyte.

enzyme-free counterparts neither in efficiency nor in specificity of binding to the corresponding collagen type (fig.6) (binding of erythrocytes to BSA-coated surfaces was less than 10^4 per cm^2). Substantial peroxidase activity was retained only in the collagen-coated wells incubated with peroxidase-erythrocytes carrying antibodies with respective specificity. An increased background level in the case of enzyme-free immunoerythrocytes was attributed to the inherent erythrocyte-associated peroxidase activity.

The results obtained illustrate the efficiency of enzyme-erythrocyte-antibody complexes in specific targeting and manifestation of enzymatic activity. With a choice of proper enzyme(s) this system may be practically used for prevention of mural thrombosis or for targeted thrombolysis. More complicated systems employing combination of both enzyme and drug targeting are now being designed.

REFERENCES

[1] Gregoriadis, G. (1977) Nature 265, 407–411.
[2] Torchilin, V.P. and Klibanov, A.L. (1981) Enzyme Microbiol. Technol. 3, 297–304.
[3] Chazov, E.I., Alexeev, A.V., Antonov, A.S., Koteliansky, V.E., Leytin, V.L., Lyubimova, E.V., Repin, V.S., Sviridov, D.D., Torchilin, V.P. and Smirnov, V.N. (1981) Proc. Natl. Acad. Sci. USA 78, 5603–5607.
[4] Samokhin, G.P., Smirnov, M.D., Muzykantov, V.R., Domogatsky, S.P. and Smirnov, V.N. (1983) FEBS Lett. 154, 257–261.
[5] Smirnov, M.D., Samokhin, G.P., Muzykantov, V.R., Idelson, G.L., Domogatsky, S.P. and Smirnov, V.B. (1983) Biochem. Biophys. Res. Commun. 116, 99–105.
[6] Papahajopoulos, D., Wilson, T. and Taber, R. (1980) In Vitro 16, 45–54.
[7] Docherty, P.A. and Aronson, N.N. (1982) Biochem. Biophys. Res. Commun. 109, 527–532.
[8] Godfrey, W., Doe, B. and Wofsy, L. (1983) Proc. Natl. Acad. Sci. USA 80, 2267–2271.
[9] Mustard, J.F., Pakham, M.A. and Kinglough-Rathbone, R.L. (1978) in: Thrombosis Animals and Clinical Models (Day, H.J. et al. eds) pp. 7–30, Plenum, New York.
[10] Chang, T.M.S. (1977) in: Biochemical Applications of Immobilized Enzymes and Proteins (Chang, T.M.S. ed.) pp. 93–104, Plenum, New York.
[11] Torchilin, V.P. (1983) in: Targeted Drugs (Goldberg, E. ed.) pp. 127–152, John Wiley, New York.
[12] Nowack, H., Gay, S., Wick, G., Becker, U. and Timpl, R. (1976) J. Immunol. Methods 12, 117–124.
[13] Green, N.M. (1970) Methods Enzymol. 18A, 414–417.
[14] Green, N.M. (1970) Methods Enzymol. 18A, 418–424.
[15] Zaborsky, O.R. and Oglette, J. (1974) Biochem. Biophys. Res. Commun. 61, 210–216.
[16] Wilson, M.B. and Nakane, P.K. (1978) in: Immunofluorescence and Related Staining Techniques (Knapp, W. et al. eds) pp. 215–244, Elsevier/North-Holland, Amsterdam, New York.