Monoclonal antibodies that inhibit the enzyme activity of NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase

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Three hybridoma cell lines secreting antibodies against human placental NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase (15-OH-PGDH) were produced. Purified IgG_{2b} from these cell lines recognized a distinct band of M_r 28 000 on SDS/PAGE from the purified enzyme as well as a band of M_r 56 000 from the crude enzyme preparation. These three monoclonal antibodies inhibited 15-OH-PGDH activity to different degrees. Inhibition of the enzyme activity could be prevented by prior incubation of the enzyme with NAD⁺ but not with prostaglandin E₂ (PGE₂) or NADP⁺. Inhibition by monoclonal antibodies appears to be non-competitive with respect to NAD⁺ and PGE₂. An increased concentration of antibodies alters the apparent K_m for NAD⁺ but not for PGE₂, further supporting the notion that the antibodies bind to the coenzyme-binding site. The availability of these monoclonal antibodies should be valuable for probing the structure of the active site.

INTRODUCTION

NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase (15-OH-PGDH) catalyses NAD⁺-linked oxidation of the 15-hydroxy group of prostaglandins to a 15-oxo function [1]. It has been considered to be the key enzyme that controls the biological activity of prostaglandins, since 15-oxo-prostaglandins possess little activity [2]. The enzyme has been purified to homogeneity from human placenta [3–5], rabbit lung [6] and pig kidney [7] and found to be a dimer of subunit M_r of 25 000–29 000. Although the enzyme was found to be ubiquitous in mammalian tissues [8,9], the extent of structural analogy of the enzyme from different tissues and species remains unknown. The activity of the enzyme was shown to alter during development [10], pregnancy [11], hormone [12] and anti-inflammatory-steroid [13] administration. These studies suggest that an understanding of the regulation of this enzyme activity and of its intracellular abundance could shed new light on the function of the enzyme and of prostaglandins. Such studies would be greatly aided by the production of antibodies against the enzyme. Questions about the distribution, abundance and turnover of the enzyme under various conditions could be examined by measuring the quantities of the enzyme protein and with immunocytochemical techniques. The extent of structural analogy of the enzyme from different sources could also be probed by site-specific monoclonal antibodies. In this report, we describe our success in obtaining three monoclonal antibodies to the enzyme, each of which inhibits the enzyme to a different degree, suggesting that these monoclonal antibodies all interact with the enzyme near the active site, but in different ways.

MATERIALS AND METHODS

Materials

Freund's adjuvant, rabbit anti-mouse IgG, Protein A–Sephadex CL-4B, hypoxanthine, aminopterin, thymidine, pristane, dithiothreitol, NAD⁺, NADP⁺, bovine liver glutamate dehydrogenase and α -oxoglutarate were purchased from Sigma

Chemical Co. PGE₂ and 15-oxo-PGE₂ were supplied by Upjohn Co. 15(S)-[15-³H]PGE₂ was synthesized as described previously by Tai [9]. Other supplies for monoclonal antibody production were as described by Shen & Tai [14]. Human placenta was obtained from the University of Kentucky Hospital.

Enzyme assay

15-OH-PGDH was assayed by monitoring the reduction of NAD⁺ at 340 nm in the presence of PGE₂ [8] or by measuring the transfer of ³H from 15(S)-[15-³H]PGE₂ to glutamate by coupling 15-OH-PGDH with glutamate dehydrogenase as described previously [9]. The latter method was used primarily in this report. Briefly, the reaction mixture contained: NH₄Cl, 5 μ mol; α -oxoglutarate, 1 μ mol, 30 000 c.p.m.; glutamate dehydrogenase, 100 μ g; and enzyme preparation in a final volume of 1 ml of 0.05 M-Tris/HCl, pH 7.5. The reaction was continued for 10 min at 37 °C and terminated by the addition of 0.3 ml of 10% aqueous charcoal suspension. The radioactivity in the supernatant after centrifugation (2000 g, 5 min) was determined by liquid scintillation counting. Calculation of the amount of PGE₂ oxidized was based on the assumption that no kinetic isotope effect was involved in the oxidation of the 15(S)-hydroxy group of 15(S)-[15-³H]PGE₂ as a substrate.

Purification of 15-OH-PGDH from human placenta

15-OH-PGDH from human placenta was purified to homogeneity according to Mak *et al.* [3].

Production of monoclonal antibodies against human placental 15-OH-PGDH

Purified human placental 15-OH-PGDH was emulsified with Freund's adjuvant and injected into Balb/C mice as described previously [14]. Booster injections were continued at monthly intervals for 3 months. Mice were killed 3 days after the last injection. Fusion between mouse spleen cells and myeloma cells (NS1-AG4-1) was performed in 40% poly(ethylene glycol) according to the published procedure [14]. Cells were cultured

Abbreviations used: 15-OH-PGDH, 15-hydroxyprostaglandin dehydrogenase; PGE₂, prostaglandin E₂.

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in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and selected by the hypoxanthine/aminopterin/thymidine medium as described earlier [14]. Identification of positive clones was made by coating purified 15-OH-PGDH in a 96-microwell plate, and immobilized mouse IgG was revealed by ^{125}I -rabbit anti-mouse IgG. Following the identification of positive clones, cells were subcloned several times by the limiting dilution method. Mouse thymocytes or peritoneal cells were used as feeder cells (10^5 /well). Monoclonal cells were mass-cultured and injected intraperitoneally into Balb/C mice (10^6 cells/mouse) which had been primed 2 weeks earlier with pristane (0.5 ml/mouse) in order to produce antibody-rich ascites fluid.

Purification of IgG from ascites fluid

Monoclonal antibodies were purified from ascites fluid on a Protein A-Sepharose CL-4B column according to the procedure of Ey *et al.* [15]. Ascites fluid diluted 5-fold with 0.1 M-sodium phosphate buffer, pH 8.0, was applied to a Protein A-Sepharose column (1.5 cm $\times$ 3 cm) which had been equilibrated in 0.1 M-sodium phosphate buffer, pH 8.0. Antibodies were successively eluted with 0.1 M-sodium citrate buffers at pH 6.0 (elutes IgG_{2a} subclass), pH 4.5 (elutes IgG_{2a} subclass) and pH 3.0 (elutes IgG_{2b} subclass). After elution, IgGs were dialysed against phosphate-buffered saline and stored at -20°C .

SDS/PAGE and immunoblotting of 15-OH-PGDH

Proteins were fractionated by SDS/PAGE according to the method of Laemmli [16]. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose paper was performed according to the method of Towbin *et al.* [17]. Visualization of antigenic protein was performed according to Knecht & Dimond [18].

RESULTS AND DISCUSSION

Several stable monoclonal cell lines were obtained from a single mouse immunized with the purified human placental 15-

Fig. 1. Inhibition of human placental 15-OH-PGDH activity by monoclonal anti-15-OH-PGDH antibodies

Purified IgG_{2b} from PGDH-1, -2 and -3 monoclonal at the indicated concentrations were incubated with human placental 15-OH-PGDH (10 μ units) for 18 h at 4°C . The reaction mixtures were then assayed for the 15-OH-PGDH activity by the ^3H release method as described in the Materials and methods section. ○, Control IgG_{2b}; ■, IgG_{2b} from PGDH-3; ▲, IgG_{2b} from PGDH-2; ●, IgG_{2b} from PGDH-1.

Fig. 2. Inhibition of 15-OH-PGDH activity from different tissues by IgG_{2b} from the PGDH-1 monoclonal

Purified IgG_{2b} from the PGDH-1 monoclonal at the indicated concentrations was incubated with the cytosolic fraction of human placenta (○), rabbit lung (▲), pig kidney (■) or pig lung (●) for 18 h at 4°C . The reaction mixtures were then assayed for 15-OH-PGDH activity by the ^3H release method as described in the Materials and methods section. The amounts of enzyme activity from different tissues were adjusted to approximately the same activity (10 μ units) before incubation with the antibody.

OH-PGDH. Three of these monoclonal (PGDH-1, -2 and -3) were found to produce IgG_{2b}, which inhibited the enzyme activity of 15-OH-PGDH as shown in Fig. 1. PGDH-1 antibody showed the highest inhibitory activity, giving a maximum of 60% inhibition, whereas PGDH-2 and PGDH-3 antibodies gave 50% and 35% inhibition respectively. The PGDH-1 antibody did not only inhibit human placental 15-OH-PGDH activity, but it also inhibited 15-OH-PGDH activity from rabbit lung, pig kidney and pig lung as shown in Fig. 2. However, no complete inhibition can be seen with any of the three monoclonal antibodies, indicating partial blockade of the active site of the enzyme. Differential inhibition of the enzyme activity from different sources also suggests that the antibody cross-reacts with the enzyme near the active site to different degrees, reflecting structural differences in the binding domain of the enzyme from different sources.

In addition to inhibiting the enzyme activity, the IgG_{2b} produced by these monoclonal also recognized the 15-OH-PGDH from both the purified and the crude placental enzyme preparation, as shown in the SDS/PAGE immunoblotting studies (Fig. 3). A distinct band of M_r 28000 was observed in homogeneous, partially purified and crude cytosol preparations. As well as the M_r -28000 band, the antibodies also recognized a band of M_r 56000 in the crude cytosol fraction. This band could be due to part of the native enzyme that was not completely dissociated into subunits. A protein band of similar M_r has been reported on SDS/PAGE analysis by Jarabak & Watkins [4] and Tanaka *et al.* [5] in their isolated human placental 15-OH-PGDH.

Inhibition of 15-OH-PGDH activity by monoclonal antibodies can be prevented by prior addition of NAD⁺ but not of NADP⁺ or PGE₂ to the enzyme preparation, as shown in Fig. 4. Increasing concentrations of NAD⁺ increased the degree of protection of the enzyme from inhibition by monoclonal antibody. The fact that only NAD⁺ but not NADP⁺ or PGE₂ protected the enzyme

Fig. 3. Immunoblotting studies of homogeneous, partially purified and cytosolic fractions of human placental 15-OH-PGDH with PGDH-1 monoclonal antibody

Homogeneous (1 μ g, lane 1), partially purified (20 μ g, lane 2) and cytosolic (100 μ g, lane 3) preparations of human placental 15-OH-PGDH were electrophoresed, blotted and immunovisualized with PGDH-1 monoclonal antibody as described in the Materials and methods section.

from inhibition suggests that the antibody binds specifically to the coenzyme-binding domain but not to the substrate-binding site. This is also evident from the kinetic studies, in which inhibition of the enzyme activity by increasing concentrations of antibody was determined at various concentrations of NAD^+ (Fig. 5) or PGE_2 (Fig. 6). Increased concentrations of antibody altered the apparent K_m for NAD^+ but not that for PGE_2 , further supporting the notion that the antibody binds to the coenzyme-binding site and prevents NAD^+ from interacting effectively with

Fig. 5. Double-reciprocal plot of the inhibition of 15-OH-PGDH activity by PGDH-2 monoclonal antibody at different concentrations of NAD^+

Human placental 15-OH-PGDH (10 μ units) was incubated with increasing concentrations of IgG_{2b} from the PGDH-2 monoclonal (Δ , none; $\circ$, 5 μ g; $\blacktriangle$, 10 μ g; $\bullet$, 20 μ g) for 18 h at 4 $^{\circ}\text{C}$. The reaction mixtures were then assayed for 15-OH-PGDH activity at different concentrations of NAD^+ (0.05, 0.1, 0.2, 0.5 and 1.0 mM) and a constant concentration of PGE_2 (1 μM).

the enzyme. Since studies on the kinetic order of addition of coenzyme and PGE_2 to 15-OH-PGDH indicated that NAD^+ was the first to bind to the enzyme before oxidation of PGE_2 can occur [19], antibody apparently blocked the binding of NAD^+ to the active site and prevented the subsequent oxidation of PGE_2 .

In conclusion, these monoclonal antibodies should prove valuable for probing the structure of the NAD^+ -binding site of 15-OH-PGDH. Furthermore, the availability of these antibodies should be also useful for studying the distribution and abundance of the immunoreactive enzyme in different tissues and species by immunocytochemical techniques and for molecular cloning of the gene by immunoscreening of the cDNA expression library.

Fig. 4. Protection of antibody-induced inhibition of 15-OH-PGDH activity by NAD^+ , NADP^+ and PGE_2

Human placental 15-OH-PGDH (10 μ units) was incubated with the indicated concentrations of IgG_{2b} from the PGDH-2 monoclonal in the presence of NAD^+ (a), NADP^+ (b) or PGE_2 (c) at the following concentrations for 18 h at 4 $^{\circ}\text{C}$. (a) and (b): $\circ$, none; $\blacktriangle$, 10 μM ; $\bullet$, 30 μM ; $\blacksquare$, 60 μM ; (c): $\circ$, none; $\blacktriangle$, 0.5 μM ; $\bullet$, 2 μM ; $\blacksquare$, 5 μM . The reaction mixtures were then assayed for 15-OH-PGDH activity by the ^3H release method as described in the Materials and methods section.

Fig. 6. Double-reciprocal plot of the inhibition of 15-OH-PGDH activity by PGDH-2 monoclonal antibody at different concentrations of PGE_2

Human placental 15-OH-PGDH (10 μ units) was incubated with increasing concentrations of IgG_{2b} from the PGDH-2 monoclonal (Δ , none; $\circ$, 5 μ g; $\blacktriangle$, 10 μ g; $\bullet$, 20 μ g) for 18 h at 4 °C. The reaction mixture was then assayed for the 15-OH-PGDH activity at different concentrations of PGE_2 (0.2, 0.5, 1.0, 2.0 and 5.0 μ M) and a constant concentration of NAD^+ (1 mM).

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