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Generation of monoclonal antibody against 6-Keto $PGF_{1\alpha}$ and development of ELISA for its quantification in culture medium

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ABSTRACT

Prostacyclin or prostaglandin I_2 (PGI_2), a metabolite of arachidonic cyclooxygenase pathway, has been demonstrated as an effector of adipocyte differentiation. However, due to its instability in biological fluid, it is difficult to evaluate the role of PGI_2 in regulating adipocyte differentiation in different stages in culture. Therefore, this study aimed to establish a simple and rapid method for the production of monoclonal antibody against 6-Keto $PGF_1\alpha$, a stable PGI_2 metabolite, and its quantification to determine the role of PGI_2 in culture medium. Eight-week-old female BALB/c mice were immunized with the hapten of 6-Keto $PGF_1\alpha$ and BSA for several weeks until a higher antibody titer (absorbance value > 0.9 at 1000-times dilution) against 6-Keto $PGF_1\alpha$ was found. Then, fusion of antibody-producing spleen lymphocytes with SP-2 myeloma cells and thymocytes was performed and cultured in HAT-medium supplemented with hypoxanthine, aminopterin, and thymine. Specific antibody-producing cells (M2-A4-B8-D10) against 6-Keto $PGF_1\alpha$ were identified and separated. A standard ELISA calibration curve was developed with 100% reactivity for 6-Keto-PGF 1 α ranging from 0.26 pg to 6.44 ng corresponding to 90% and 10% of the maximum binding capacity for the immobilized antigen respectively. This method can easily be applied to monitor PGI_2 regulation in different stages of cultured adipocytes to reveal the regulatory roles of PGI_2 in maintaining homeostasis and adipocyte differentiation.

1. Introduction

Adipocytes and their progenitor cells produce different types of prostaglandins (PGs), through the arachidonic acid cyclooxygenase (COX) pathway, that have opposing effects on adipogenesis [1–4]. Furthermore, biosynthesis of individual PGs at various time points in the adipocyte life-cycle participates in the regulation of adipogenesis in an autocrine and/or paracrine manner. For example, endogenously synthesized prostaglandins such as Δ^{12}-PGJ_2 and 15-deoxy $\Delta^{12,14}$-PGJ_2 at the final stage of adipocyte life-cycle, activate peroxisome proliferator-activated receptor gamma (PPARγ) and thus promote adipogenesis and restore post-maturation fat stores in the cultured 3T3-L1 cells [5,6]. Conversely, PGE_2 and $PGF_2\alpha$ have been reported to inhibit adipocyte differentiation via E-type prostanoids receptor 4 (EP4) and prostaglandin F receptor (FP), respectively [7,8]. Previously, stimulation of terminal differentiation by PGI_2 analog, carbaprostacyclin, on pre-adipose Ob1771 mouse cells was described by different research groups [9,10]. However, pro-adipogenic action of PGI_2 still remains unclear for cultured 3T3-L1 cells as alteration of cell types and culture conditions might provide different results. In addition, endogenous PGI_2 is rapidly hydrolyzed to its inactive metabolite 6-Keto $PGF_1\alpha$ [11], which makes it more difficult to evaluate the effects of prostacyclin in cultured adipocytes.

Earlier, Syeda et al. described the procedures for monoclonal antibody production, and a solid-phase enzyme-linked immunosorbent assay (ELISA) for the quantification of Δ^{12}-PGJ_2 and 15-deoxy $\Delta^{12,14}$-PGJ_2 from the 3T3-L1 cell culture system [12,13]. Until now, very few approaches have been made to extensively check the regulation of 6-keto-$PGF_1\alpha$, an inactive hydrolytic stable metabolite of PGI_2 in the cultured 3T3-L1 cells. Therefore, the current study targeted to generate a hybridoma cell line producing monoclonal antibodies against 6-Keto $PGF_1\alpha$ and to develop an ELISA, which can be utilized for quantitative analysis of 6-Keto $PGF_1\alpha$ in cultured adipocytes.

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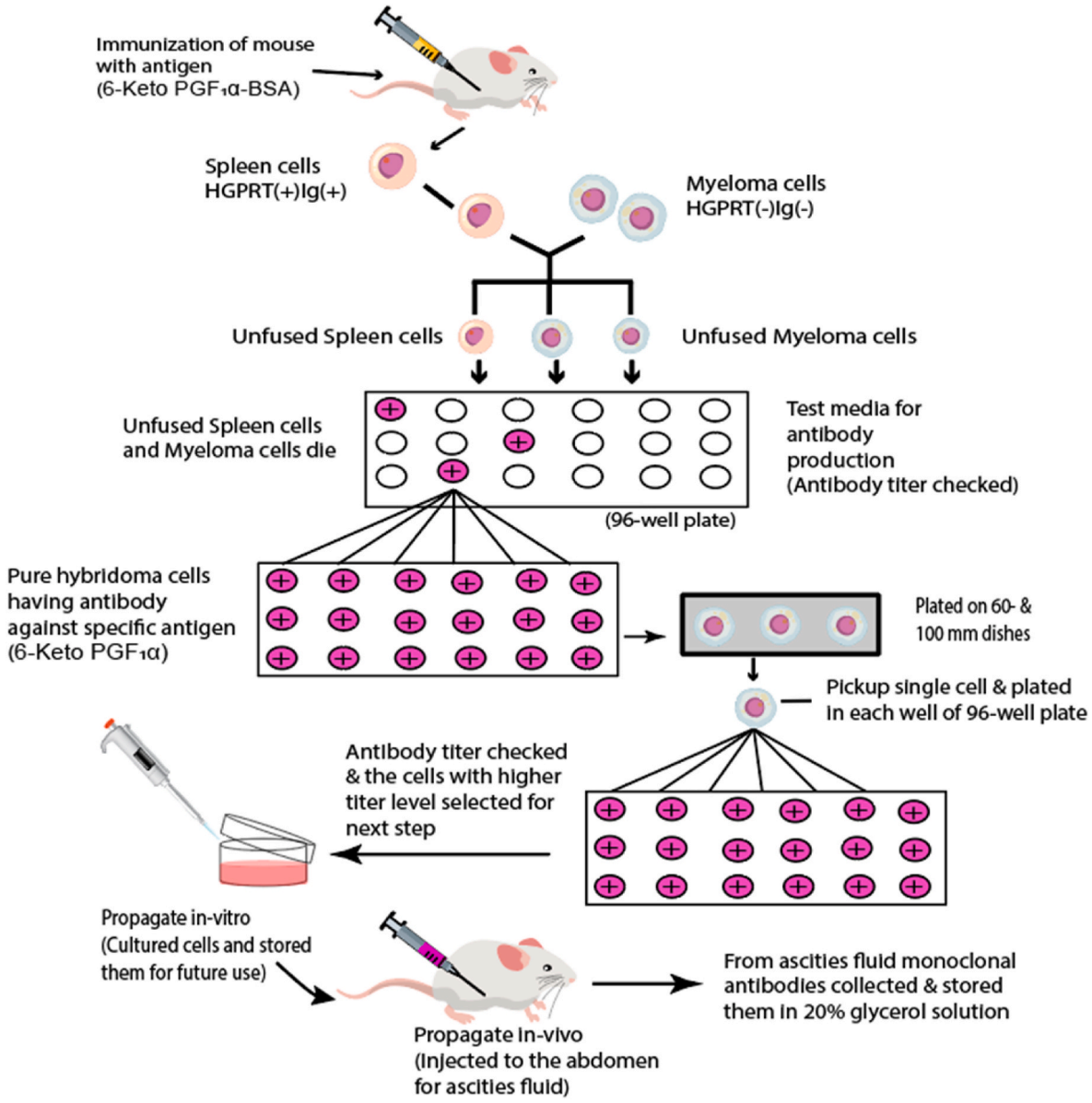

Fig. 1. Immunization, fusion of spleen cells from immunized mouse with myeloma cells for hybridoma preparation, in-vitro cloning of antibody producing cells and collection of monoclonal antibodies.

2. Materials and methods

Authentic 6-Keto-$PGF_1\alpha$ was purchased from Cayman Chemical (Ann Arbor, MI, USA). HEPES (25 mM)-supplemented Dulbecco's modified Eagle medium (DMEM-HEPES), penicillin G, streptomycin, dexamethasone, human insulin (recombinant), fatty acid-free bovine serum albumin (BSA) and γ-globulin, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl, N-hydroxysuccinimide, and ExtrAvidin-peroxidase conjugate were purchased from Sigma (St. Louis, MO, USA). Biotin-conjugated rabbit anti-mouse IgG antibody was supplied by Jackson Immuno Research Laboratories (West Grove, PA, USA). Fetal bovine serum (FBS) was purchased from MP Biomedicals (Solon, OH, USA). Aminopterin, L-Ascorbic acid, 3-isobutyl-1-methylxanthine (IBMX), Hypoxanthine, NCTC-109 medium, thymidine, pristane, o-phenylenediamine were purchased from Wako Pure Chemical Industries (Osaka, Japan); 96-well microplates for ELISA were the product of BD Falcon (Durham, NC, USA). BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan) and SP-2/O–Ag 14 myeloma cell line was supplied by the Japanese Cancer Research Resources Bank (JCRB) (Tokyo, Japan). All other chemicals, plastic and glassware used were of tissue culture grade.

2.1. ELISA method development for the quantification of 6-Keto $PGF_1\alpha$

A solution of 200 μg 6-Keto $PGF_1\alpha$ in ethanol was dried up and dissolved in 80 % dioxane. Then, 150 mg/ml N-hydroxy succinimide (NHSI) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide methiodide (EDC) in 80 % dioxane solution were added to it and subjected to shaking incubator for 3 h at 25 °C. Thereafter, equal volume of ethyl acetate and dH_2O were added to the above solution. The solution was centrifuged and evaporated in vacuum evaporator producing the dried activated NHSI ester of 6-Keto $PGF_1\alpha$. For the conjugate, 200 μl of 10 mg/ml bovine γ-globulin in 50 mM sodium phosphate buffer (pH 7.3) was added to it, mixed and kept overnight at 4 °C in a shaking incubator. The bovine γ-globulin and 6-Keto $PGF_1\alpha$ conjugate was then purified using a NAP-10 column. This conjugate was used as an immobilized antigen. 100 μl (equivalent to 10 ng protein) of this conjugate in 50 mM sodium phosphate buffer (pH 7.3) was used to coat each well of a 96-

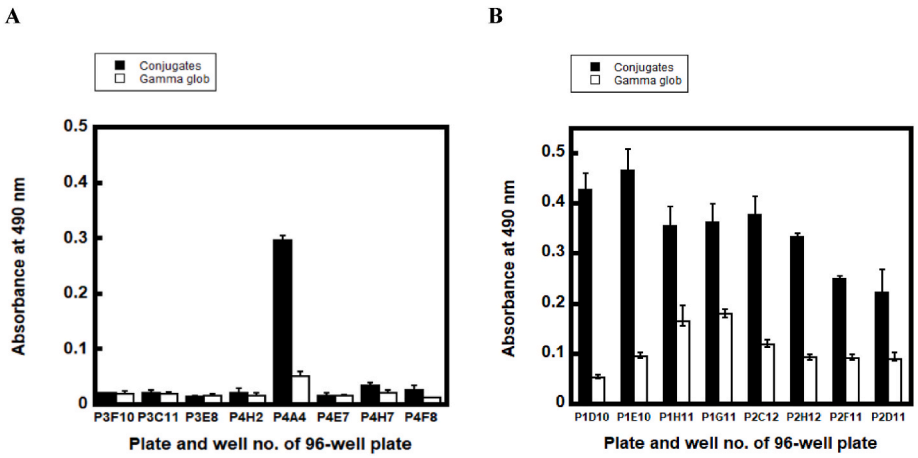

Fig. 2. Screening of cloned hybridoma cells. (**A**) Absorbances corresponding to antibody production by different cloned cells after initial fusion. To be noted that the clone P4A4 was selected for next culture. (**B**) Absorbances corresponding to antibody production by different cloned cells after second single cell pick up in culture. To be noted that P1D10 clone was finally selected for the *in-vivo* propagation for monoclonal antibody production as the titer ratio in comparison with γ-globulin was much prominent in P1D10. Data represent the mean ± S.E.M. of three experiments.

well ELISA microplate and incubated for 1hr at 30 °C [5,6,13–16]. ELISA microplate was blocked with 1 % gelatin in PBS(−) solution and washed with PBS(−) containing sodium azide (NaN_3). After washing, ELISA plate was incubated with 50 μl standard 6-Keto $PGF_1\alpha$ or 1000-times diluted mouse anti-serum against 6-Keto $PGF_1\alpha$ using PBS(−) with 0.5 % BSA solution for 1 h at 30 °C followed by overnight incubation at 4 °C. As a negative control, we used γ-globulin. Next day, after washing the immunocomplex in each well with PBS (−) containing NaN_3, 100 μl of 12×10^3 times diluted biotin-conjugated rabbit anti-mouse IgG was applied to each well and incubated for 2 h at 30 °C, followed by overnight incubation at 4 °C as described [13]. Following washing with PBS (−) containing thimersol, 10,000-fold diluted ExtrAvidin-peroxidase was applied and the plate was incubated at 30 °C for 30 min. Then, 50 μl of 5 N H_2SO_4 was applied to each well to stop the reaction, and the absorbance was measured spectrophotometrically at 492 nm wavelength.

2.2. Monoclonal antibody generation for 6-Keto $PGF_1\alpha$

Immunogen was chemically prepared from 6-Keto $PGF_1\alpha$ and BSA using the same protocol used to prepare 6-Keto $PGF_1\alpha$ and bovine γ-globulin conjugate [13,14,17]. Eight weeks old female BALB/c mice were immunized with the resulting hapten of 6-Keto $PGF_1\alpha$ and BSA in accordance with the guidelines approved by the Institutional Animal Care and Use Committee at of Shimane University (MZ4-21). First immunization was done with hapten using Freund's complete adjuvant, whereas Freund's incomplete adjuvant was continued for rest of the immunizations by 2-week intervals, until a higher antibody titer (absorbance value > 0.9 at 1000-times dilution) against 6-Keto $PGF_1\alpha$ was found. A boosting of 150 μL conjugate with the same volume of sterile PBS(−) at pH 7.2 was injected to the peritoneal cavity maintaining a 3-week resting period from last immunization. Fusion of antibody producing spleen lymphocytes with SP-2 myeloma cells was done using 50 % solution of PEG 4000, thymocytes of young mice were used as feeder cells [13]. Cell concentrations used for the fusion of

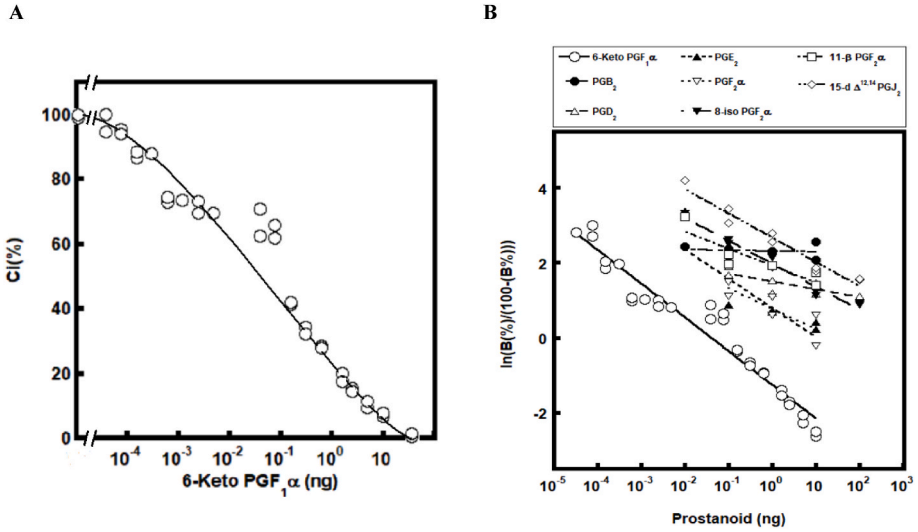

Fig. 3. ELISA calibration curves generated using the immobilized antigen and mouse heart anti-serum specific for 6-Keto $PGF_1\alpha$. (**A**) Sigmoid curve where the binding percentages of immobilized antigen were characterized by the corresponding amount of standard 6-Keto $PGF_1\alpha$. (**B**) Cross-reactivity of antiserum shown for 6-Keto $PGF_1\alpha$ with different prostanoids. Immobilized antigen with 4.5×10^4-times diluted mouse antiserum against 6-Keto $PGF_1\alpha$ was incubated with increasing amounts of standard prostanoids under the recognized condition. All data were plotted in duplicates. The data representing the graphs are available in Supplementary Table (S1).

Table 1
Cross-reactivity chart for mouse antiserum with different prostanoids.

Compounds	Cross-reaction (%)
6-Keto $PGF_1\alpha$	100
PGB_2	<0.01
PGD_2	<0.01
PGE_2	0.37
$PGF_{2\alpha}$	0.15
8-iso $PGF_{2\ \alpha}$	<0.01
11β-$PGF_{2\ \alpha}$	<0.01
15-deoxy $\Delta^{12,14}$ PGJ_2	<0.01

spleen cells, SP2/O–Ag14 myeloma cells and thymocytes were 3.06×10^7 cells/10 ml, 7.5×10^5 cells/5 ml and 1.9×10^8 cells/15 ml, respectively. These cells were then nourished with the selected HAT-medium supplemented with hypoxanthine, aminopterin, and thymine as described previously [18]. Cell suspension was dispensed into 96-well plate (n = 3) at 100 μl/well. Antibody titer for 6-keto PGF1α was measured from culture medium of growing hybridoma cells using the solid-phase ELISA. Independent cloned cells from hybridoma cells secreting the monoclonal antibodiy against 6-Keto $PGF_1\alpha$ recognizing specifically the immobilized 6-Keto $PGF_1\alpha$ antigen, were isolated by the limited dilution method. After second cloning, a hybridoma clone termed M2-A4-B8-D10 from clone P1D10 was obtained. Finally, these cloned hybridoma cells were injected into the mouse peritoneal cavity that was previously injected with a 500 μL of 2,6,10,14-Tetramethylpentadecane (pristine) injection [19]. After 10 days of cloned cells injection, collection, fractionation and precipitation of ascites fluid were done using 50 % saturation of ammonium sulfate when bulking of the mice peritoneum occurred (Fig. 1). The total protein was quantified by standard Lowry method using fatty acid-free BSA as a standard [20].

3. Results and discussion

Isolation of the cloned hybridoma cells producing antibody against 6-Keto $PGF_1\alpha$, from the mixture of spleen cells and SP2 myeloma cells was done by ELISA using 6-Keto $PGF_1\alpha$ - γ-globulin conjugate [14] and by limited dilution method [12]. At the beginning, out of 27 wells that became yellowish in four 96-well plates, only one well (P4A4) showed a relatively higher antibody titer ratio with γ-globulin (Fig. 2A). Promising hybridoma cells from this well were then transferred to a 24-well plate and finally, to 60-mm dishes for subsequent subcultures. After cloning twice using single cell pick up method, i.e. firstly, from clone P4A4 and then, from clone P1B8 (data not shown), a hybridoma cell line termed M2-A4-B8-D10 from clone P1D10, secreting the desired amount of antibody was eventually established (Fig. 2B). The resulting hybridoma cloned cells were then injected into the mice peritoneal cavity, and collection and fractionation of ascites fluid were done as described in the method section. The ascites was dissolved in PBS (−) with 20 % glycerol to obtain a protein concentration of 5.89 mg/ml.

A standard ELISA calibration curve (Fig. 3A) was developed and 6-keto PGF1α was quantified ranging from 0.26 pg to 6.44 ng corresponding to 90 % and 10 %, respectively of the maximum binding capacity for the immobilized antigen. A previous study showed a similar quantification ranging from 0.8 pg to 7.7 ng corresponding to 90 % and 10 %, respectively [15]. The linearized straight curve (Fig. 3B) was also obtained by fitting the logit value of corresponding binding percentage for individual standard 6-Keto PGF1α concentration, which might be used for the quantification of 6-Keto PGF1α in unknown samples. Using this regression line for 50 % maximal binding displacement, the calculated amount of standard 6-Keto PGF1α was observed 0.04 ng.

The cross-reactivity of the antibody used in the development of ELISA was checked by the immunoreactivities with different prostanoids and the results (Fig. 2B) indicate that our developed ELISA method showed 100 % reactivity for 6-Keto $PGF_1\alpha$ and exhibited cross-reaction values < 0.01 % with other prostanoids except PGE_2 and $PGF_2\alpha$, which had cross-reaction values 0.37 % and 0.15 %, respectively (Table 1). Thus, we demonstrate the precision of our antiserum for the quantification 6-Keto $PGF_1\alpha$ in unknown samples.

4. Conclusion

In addition to isolating a hybridoma cell line that secreted a monoclonal antibody against 6-Keto $PGF_1\alpha$, the present study developed an ELISA method which can quantify the 6-Keto $PGF_1\alpha$ directly from cell culture medium. Therefore, our ELISA method can be successfully applied for the quantification of 6-Keto $PGF_1\alpha$ and thus to monitor the status of PGI_2 secreted by cultured cells in the regulation of adipocyte differentiation.

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CRediT authorship contribution statement

Md. Mazharul Islam Chowdhury: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Nafisa Kabir:** Writing – original draft, Investigation. **Rezwana Ahmed:** Formal analysis, Writing – original draft. **Kazushige Yokota:** Supervision, Conceptualization. **Randy Mullins:** Writing – review & editing, Writing – original draft. **Hasan Mahmud Reza:** Writing – review & editing, Writing – original draft, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101748.

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