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SUMMARY OF DOCTORAL THESIS

Name:　**Pinky Karim Syeda K. Fatema**

Title:　**Generation of monoclonal antibodies for prostaglandins of J_2 series and the application to their immunological assays**

（J_2シリーズのプロスタグランジン類に対する単クローン抗体の作製と免疫学的測定法への応用）

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is well known to be the potent natural ligand for the activation of peroxisome proliferator-activated receptor (PPAR) γ, a nuclear hormone receptor serving as a transcription factor. Since PPARγ is a master regulator of adipogenesis of adipocytes, 15d-PGJ_2 acts as a pro-adipogenic prostanoid. On the other hand, 15d-PGJ_2 has shown to have anti-inflammatory effects by suppressing the expression of cyclooxygenase (COX)-2 gene that is controlled by the NF-κB signaling pathway in a PPARγ-independent manner. These anti-inflammatory effects are known to involve covalent modifications of critical cysteine residues in the signaling cellular proteins. More recently, our laboratory has described that 15d-PGJ_2 inhibits the endogenous synthesis of anti-adipogenic prostanoids in preadipocytes through a PPARγ-independent mechanism. For the formation of PGJ_2 series, the arachidonate COX pathway requires the enzymatic reactions of COX and terminal PGD synthase (PGDS). The resulting product, PGD_2, can readily undergo the non-enzymatic dehydration to give J_2 series of PGs, including 15d-PGJ_2 and Δ^{12}-PGJ_2 through PGJ_2 as an unstable intermediate. Cultured mouse preadipogenic 3T3-L1 cells are a useful system for monitoring the changes in the life cycle of adipocytes. Recent studies have shown the selective gene expression of lipocalin-type PGDS (L-PGDS) in addition to COX isoforms in cultured 3T3-L1 adipocytes. Our colleagues have also reported endogenous synthesis of PGJ_2 derivatives during the maturation phase of adipocytes and suggested the contribution of those compounds to the up-regulation of adipogenesis. However, no attempts have been made until now to monitor the formation of 15d-PGJ_2 in the fresh culture medium of adipocytes due to the non-enzymatic degradation of PGD_2 under the different incubation conditions. The present study was undertaken to prepare monoclonal antibodies specifically recognizing 15d-PGJ_2 by immunizing mice with a protein conjugate of 15d-PGJ_2 as an immunogen. Then, using a specific monoclonal antibody for 15d-PGJ_2, I attempted to develop a solid-phase enzyme-linked immunosorbent assay (ELISA) specific for 15d-PGJ_2. Finally, the current immunological method was applied to the quantitative determination of 15d-PGJ_2 generated in the fresh maturation medium of cultured adipocytes for the evaluation of the specificity and accuracy of our methods.

15d-PGJ_2 is a biologically active molecule serving as a pro-adipogenic factor or an anti-inflammatory regulator. This compound is one of naturally occurring derivatives formed by the non-enzymatic dehydration of PGD_2. To determine the endogenous synthesis of 15d-PGJ_2, a convenient immunological approach is useful. At first, I established a cloned hybridoma cell line to secrete a monoclonal antibody specific for 15d-PGJ_2. For the development of a solid-phase ELISA, the immobilized antigen using a protein conjugate of 15d-PGJ_2 was allowed to react competitively with a monoclonal antibody in the presence of free 15d-PGJ_2. Under the optimized conditions, a sensitive calibration curve was generated able to determine the amount of 15d-PGJ_2 from 0.5 pg to 9.7 ng with 71 pg of 50% displacement in one assay. My monoclonal antibody did not recognize other related prostanoids except PGJ_2 with cross-reaction of 4%. The present ELISA was demonstrated to be reliable for the quantification of 15d-PGJ_2 in the maturation medium of cultured adipocytes by confirming the accuracy and specificity of its determination. The application of our assay revealed that

the non-enzymatic formation of 15d-PGJ_2 became more evident after several hours of the incubation with authentic PGD_2 at 37 °C. The results indicate the usefulness of our developed solid-phase ELISA with the monoclonal antibody for further studies on the endogenous synthesis of 15d-PGJ_2 and its roles in various cells and tissues.

A PGJ_2 series can be formed through the non-enzymatic dehydration of PGD_2 since it is especially unstable in aqueous solutions including serum albumin or in human plasma. Of these PGJ_2 derivatives, Δ^{12}-PGJ_2 as the ultimate metabolite of PGD_2 has been considered as an actual bioactive factor responsible for the growth inhibition of certain tumor cells. Our group has been studying the regulation of the arachidonate cascade leading to the biosynthesis of PGD_2 and the related PGJ_2 series at different stages of adipocytes. For these studies, preadipogenic mouse 3T3-L1 cells are useful because we can monitor the cultured adipocytes at different life stages of adipocytes under controlled culture conditions. Recently, we have shown that cultured 3T3-L1 adipocytes up-regulate the gene expression of L-PGDS using PGH_2 as a substrate during the maturation phase. By contrast, the accumulation of fats can be appreciably suppressed in the presence of COX inhibitors, suggesting the involvement of endogenous prostanoids in the stimulation of adipogenesis. Actually exogenous, each of 15d-PGJ_2 and Δ^{12}-PGJ_2 has been effective in rescuing the fat storage suppressed by each of the COX inhibitors. However, the generation of a specific monoclonal antibody for Δ^{12}-PGJ_2 and its utilization in the immunological approach for the quantification of the endogenous product in a cell culture system of adipocytes have not been described until now. This study was initially undertaken to establish a hybridoma cell clone secreting a monoclonal antibody specific for Δ^{12}-PGJ_2. Next, I tried to utilize the desired monoclonal antibody to develop a highly sensitive and convenient solid-phase ELISA for Δ^{12}-PGJ_2. Then, our developed immunological assay was applied to the cell culture system of adipocytes during the maturation phase associated with the progression of adipogenesis. I show here that our solid-phase ELISA for Δ^{12}-PGJ_2 is reliable and useful for studies on the endogenous formation of Δ^{12}-PGJ_2 derived from unstable PGD_2 biosynthesized by cultured adipocytes during the progression of adipocytes.

PGD_2 can be produced in adipocytes and dehydrated to PGs of J_2 series including Δ^{12}-PGJ_2 and 15d-PGJ_2, which serve as pro-adipogenic prostanoids through the activation of peroxisome proliferator-activated receptor γ. To accomplish the quantification of Δ^{12}-PGJ_2 in the cell culture system of adipocytes, the present study aims to develop a sensitive and specific immunological assay for Δ^{12}-PGJ_2. Here, I established a cloned hybridoma cell line secreting a monoclonal antibody specifically recognizing Δ^{12}-PGJ_2 and utilized for the development of its solid-phase ELISA. The immobilized antigen using a conjugate of Δ^{12}-PGJ_2 and γ-globulin was competitively allowed to react with the monoclonal antibody in the presence of free Δ^{12}-PGJ_2. The assay provided a sensitive calibration curve for Δ^{12}-PGJ_2 allowing us to determine a range from 0.16 pg to 0.99 ng with a value of 13 pg at 50% displacement in one assay. The monoclonal antibody showed almost no cross-reactivity with other related prostanoids since PGJ_2 and 15d-PGJ_2 were only recognized with much lower values of 0.5% and 0.2%, respectively. The accuracy for determining Δ^{12}-PGJ_2 in the culture medium of adipocytes was confirmed by measurement after the culture medium was fortified with known amounts of authentic Δ^{12}-PGJ_2 in a range from 10 to 200 pg/ml. The application of our ELISA revealed that the formation of Δ^{12}-PGJ_2 became more pronounced after several hours of incubation of PGD_2 at 37 °C in fresh maturation medium of cultured adipocytes. Furthermore, I provide evidence for the increased ability of cultured adipocytes to synthesize endogenous Δ^{12}-PGJ_2 during the progression of adipogenesis. These results indicate the reliability and usefulness of our solid-phase ELISA for stable Δ^{12}-PGJ_2 reflecting the biosynthesis of unstable PGD_2 in the culture system of adipocytes.

Taken together, the present immunological approaches using monoclonal antibodies for each species of PGJ_2 derivatives are convenient, reliable, and useful for monitoring the biosynthesis of PGD_2 in a variety of biological systems by the specific quantification of PGs of J_2 series including Δ^{12}-PGJ_2 and 15d-PGJ_2.