Pioglitazone reduces monocyte adhesion to vascular endothelium under flow by modulating RhoA GTPase and focal adhesion kinase

Yasutoshi Toriumi[a,b], Megumi Hiraoka[a], Mamoru Watanabe[b], Masayuki Yoshida[a,*]

[a] *Department of Vascular Medicine, Medical Biochemistry, Tokyo Medical and Dental University, 1-5-45, Yushima Bldg. D-256, Bunkyo-ku, Tokyo 113-8519, Japan*

[b] *Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan*

Received 1 July 2003; revised 25 August 2003; accepted 8 September 2003

First published online 3 October 2003

Edited by Beat Imhof

Abstract Thiazolidinediones (TZDs), potent peroxisome proliferator-activated receptor γ ligands, have been shown to improve endothelial function in vascular diseases. We investigated the effects of pioglitazone, a TZD, on monocyte–endothelial interaction under flow and found that pretreatment (20 μmol/l, 48 h) significantly reduced U937 adhesion to human umbilical vein endothelial cells. Integrin expression was not altered, however, the activation of RhoA GTPase was significantly reduced after treatment. Further, pioglitazone treatment significantly reduced phosphorylation of focal adhesion kinase (FAK) at 925Y, but not at 397Y, suggesting a specific role in FAK-dependent signaling. These results indicate a novel anti-inflammatory role for this compound.

Key words: Thiazolidinedione; Monocyte adhesion; Cytoskeletal organization; Atherosclerosis

1. Introduction

Thiazolidinediones (TZDs) are insulin-sensitizing compounds that have been used to treat patients with type 2 diabetes. TZD compounds have been identified as a ligand for peroxisome proliferator-activated receptor γ (PPARγ), which belongs to a nuclear hormone receptor superfamily, and also received attention for their potential anti-inflammatory effects. Further, PPARγ activators such as 15-deoxy-12,14-prostaglandin J2, and TZDs have been shown to inhibit the production of several inflammatory cytokines, including interleukin (IL) 1β, IL-6, and tumor necrosis factor α in monocytes [1], and inducible nitric oxide synthase, matrix metalloprotease-9, and scavenger receptor-A expression in macrophages [2], and have also been reported to have anti-inflammatory effects that are independent of PPARγ [3]. We investigated the inhibitory effects of pioglitazone, a TZD, on monocyte–endothelial interaction under flow conditions and examined the molecular consequences.

2. Materials and methods

2.1. Cell culture and reagents

A U937 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Life Technologies Oriental, Tokyo, Japan). Human umbilical vein endothelial cells (HUVEC) were isolated from normal-term umbilical cords and maintained as previously described [4,5]. Recombinant human IL-1β was obtained from Genzyme (Cambridge, MA, USA). BCECF-AM and FITC-conjugated phalloidin were obtained from Molecular Probes (Eugene, OR, USA). Pioglitazone hydrochloride was a gift from Takeda Chemical Industry (Osaka, Japan) and was stored as a 20 mmol/l stock solution in dimethyl sulfoxide. Mouse anti-CD11a monoclonal antibody (clone 38, Ancell, Bayport, MN, USA); mouse anti-CD11b monoclonal antibody (clone 44, YLEM, Avezzano, Italy), mouse anti-CD18 monoclonal antibody (clone MEM48, Southern Biotechnology Associates, Birmingham, AL, USA), and mouse anti-CD49d monoclonal antibody (clone A4-PUJ1, Upstate Biotechnology, Lake Placid, NY, USA) were used in the present study. Rho activation assay kit was obtained from Upstate Biotechnology. Anti-focal adhesion kinase (FAK) monoclonal antibody was obtained from BD Biosciences (Franklin Lakes, NJ, USA). Anti-phosphorylated (p) FAK (397Y) monoclonal antibody and anti-pFAK (925Y) monoclonal antibody were obtained from Biosource (Camarillo, CA, USA). Horseradish peroxidase-conjugated goat anti-mouse IgG and anti-rabbit IgG were obtained from Cal-tag (Burlingame, CA, USA).

2.2. Monocyte adhesion assay

Static adhesion assays were carried out as previously described in detail [6]. In brief, U937 cells were prelabeled with BCECF-AM for 20 min at 37°C, and then placed on HUVEC plated in a 96-well microtiter culture plate and incubated for 10 min at room temperature. The fluorescent intensity of the monolayer-associated U937 cells was quantitated using a fluorescent plate reader (Perseptive Biosystems). Adhesion assays under laminar flow were carried out as described previously [7]. In brief, HUVEC monolayers on coverslips were stimulated with 10 U/m of IL-1β for 4 h, positioned in the flow chamber mounted on an inverted microscope (l×70, Olympus, Tokyo, Japan). U937 cells (1×10^6/ml) were drawn through the chamber at a wall shear stress of 1.0 dyne/cm^2 for 10 min. The entire period of perfusion was recorded using a video tape recorder. The numbers of rolling and adherent U937 cells on HUVEC monolayer in 6–10 randomly selected 20× microscope fields were determined.

2.3. Filamentous actin content in U937 cells

U937 cells were washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS) and fixed with 1% paraformaldehyde in DPBS for 5 min, then permeabilized with 0.1% Triton X-100 in DPBS for 60 s. U937 cells were then incubated with FITC-conjugated phalloidin (1:100 dilution) in DPBS for 60 min. After washing twice with DPBS, U937 cells were lysed with 150 μl of 0.01% NaOH in 0.1% sodium dodecyl sulfate (SDS) and fluorescent intensity was quantitated using a fluorescent plate reader.

2.4. RhoA translocation in U937 cells

RhoA translocation in U937 cells was examined by Western blotting analysis as described previously [8,9]. In brief, U937 cells were incubated with pioglitazone and then lysed in 100 μl of ice-cold lysis buffer (0.1 mol/l Tris–HCl, 0.15 mol/l NaCl, and 5 mmol/l EDTA, pH 7.4) containing 0.1% Triton X-100, 10 μg/ml leupeptin, 60 U/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 100 μmol/l sodium

*Corresponding author. Fax: (81)-3-5800 3380.
E-mail address: masavasc@tmd.ac.jp (M. Yoshida).

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)01040-8

vanadate for 5 min, then centrifuged at 15000 rpm for 15 min. The supernatants were collected as the cytosol fractions of the cell lysate. Pellets were washed with phosphate-buffered saline and lysed for 5 min in 100 μl of ice-cold lysis buffer as described above, except that it contained 1% Triton X-100, after which they were centrifuged at 15000 rpm for 15 min. The supernatants were collected as the membrane fractions. To obtain a total cell lysate, U937 cells were lysed with 100 μl of ice-cold lysis buffer containing 1% Triton X-100 for 10 min. An equal amount of protein (10 μg) from each lysate was subjected to 12.5% SDS–polyacrylamide gel electrophoresis and Western blotting analysis was carried out using mouse anti-RhoA monoclonal antibody (1:500 dilution). Activation of RhoA was determined by using a glutathione *S*-transferase (GST) fusion protein of the Rho binding domain of the Rho effector Rhotekin (Rho activation assay kit) following the manufacturer's protocol.

2.5. FAK phosphorylation in U937 cells

Phosphorylation of FAK at both 925Y and 397Y in U937 cells was examined by Western blotting analysis using phosphorylation-specific monoclonal antibodies against FAK as described above.

2.6. Statistical analysis

Results are presented as mean ± S.D. Data were analyzed using analysis of variance. $P < 0.05$ was considered significant.

3. Results

3.1. Pioglitazone reduced IL-1β-induced U937 cell adhesion to HUVEC

We first examined the effect of pioglitazone on monocytic U937 adhesion to cytokine-activated HUVEC (IL-1β 10 U/ml, 4 h). U937 cells were pretreated with various concentrations (1, 5, 10 and 20 μmol/l) of pioglitazone for 48 h prior to an adhesion assay. As shown in Fig. 1A, a dose-dependent reduction of pioglitazone-treated U937 cell adhesion to HUVEC was observed at concentrations as low as 10 μmol/l of pioglitazone (control 21.8 ± 4.64% adhesion, 0.5 μmol/l 20.0 ± 2.89%, 10 μmol/l 8.45 ± 1.15% and 20 μmol/l 8.10 ± 1.82%). Since we did not detect significant cellular damage caused by these pioglitazone treatments using trypan blue staining (data not shown), we chose to treat the U937 cells with 20 μmol/l of pioglitazone in the following experiments. The inhibitory effects of pioglitazone on the adhesion of U937 cells to HUVEC were then examined under flow conditions at a laminar shear stress level of 1.0 dyne/cm^2. U937 cells were incubated in the presence of 20 μmol/l of pioglitazone for 48 h then perfused over HUVEC for 10 min. As shown in Fig. 1B, the adhesion, but not rolling, of U937 cells was significantly reduced after pioglitazone treatment (control 8.20 ± 3.03 cells/HPF (high power field); pioglitazone 2.40 ± 1.67 cells/HPF, $P < 0.006$).

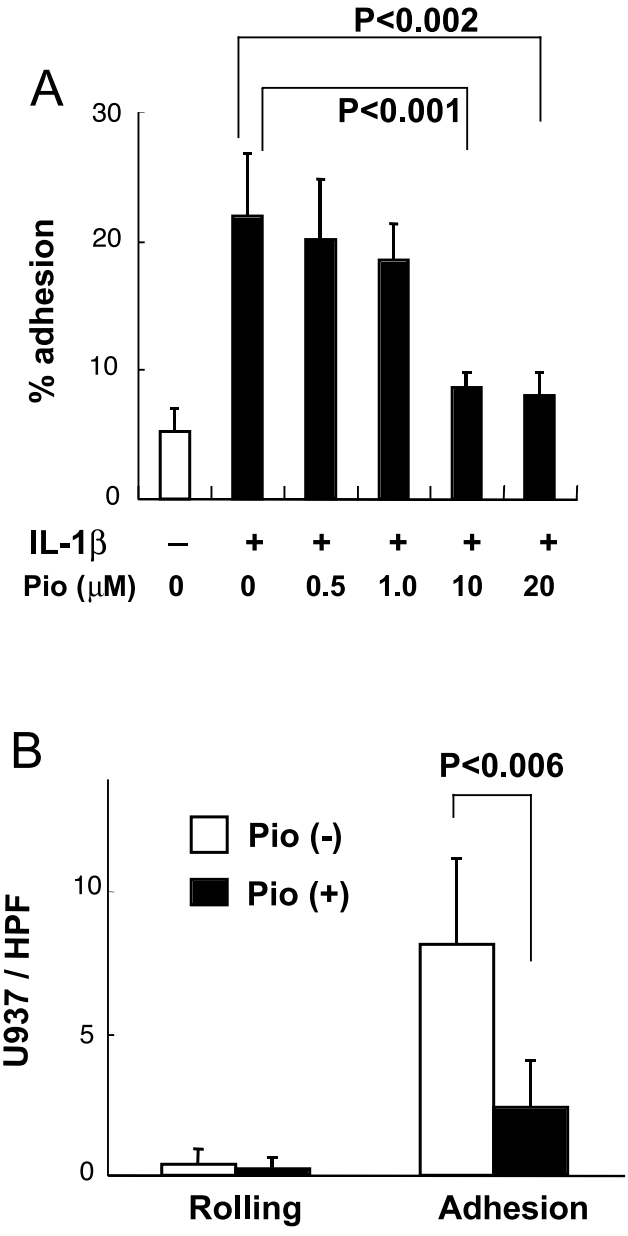

Fig. 1. Effect of pioglitazone on adhesion of U937 cells to HUVEC under static and physiological flow conditions. A: U937 cells (1×10^6/ml) were incubated with the indicated amounts of pioglitazone for 48 h and then placed on HUVEC activated with 10 U/ml of IL-1β for 4 h. An adhesion assay was carried out as described in Section 2. B: U937 cells were treated with (+) or without (−) 20 μmol/l of pioglitazone for 48 h and then perfused over activated HUVEC monolayers at a flow rate of 1.0 dyne/cm^2. Rolling and adherent U937 cells on HUVEC were counted in 10 different 20× microscope fields as described in Section 2. Data are representative of three separate observations.

3.2. Effect of pioglitazone on integrin expression in U937 cells

To elucidate the molecular mechanism of the pioglitazone-induced reduction of U937 cell adhesion, cell surface integrin expression after pioglitazone treatment was examined by flow cytometric analysis. The expression levels of CD11a, CD18, and CD49d on U937 cells were not significantly affected after treatment, as shown in Fig. 2A.

3.3. Pioglitazone reduced actin polymerization and RhoA activation in U937 cells

We then examined the effect of pioglitazone on the cytoskeletal network in U937 cells by detecting F-actin, which reflects actin polymerization. When U937 cells were incubated with 20 μmol/l of pioglitazone for 48 h, the F-actin content in U937 cells was significantly decreased (0.64-fold of control, $P < 0.05$) (Fig. 2B). The effect of pioglitazone on RhoA GTPase activity in U937 cells was also examined by determining RhoA translocation from the cytoplasm to membrane and Rho pull-down assay using a GST fusion protein of the Rho effector Rhotekin. Western blotting analysis revealed that the expression of RhoA protein in the membrane fraction and Rhotekin-bound activated RhoA were significantly decreased in U937 cells after pioglitazone treatment (Fig. 2C).

3.4. Pioglitazone inhibited FAK activation in U937 cells

We also investigated the involvement of FAK in U937 cells. Western blotting analysis was carried out using antibodies to detect phosphorylation of FAK at two distinct tyrosines. As shown in Fig. 3A, the expression of total FAK was not affected by pioglitazone treatment, however, FAK phosphorylation at tyrosine 925 was significantly inhibited. In contrast, phosphorylation of FAK at tyrosine 397 was not affected by pioglitazone treatment.

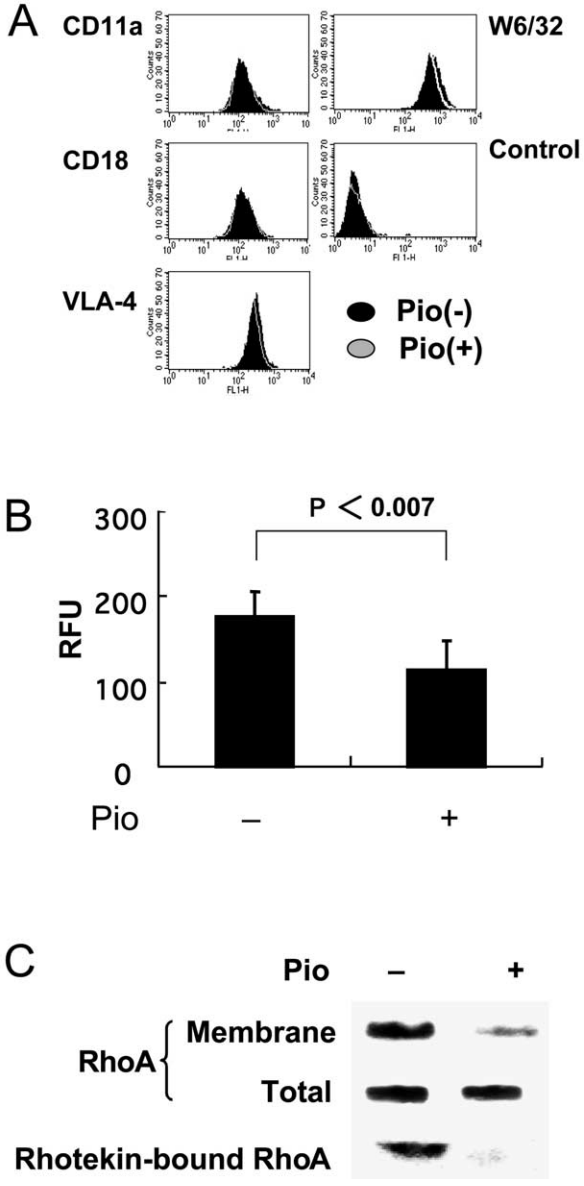

Fig. 2. Effect of pioglitazone on integrin expression, actin cytoskeleton, and RhoA GTPase in U937 cells. A: U937 cells (1×10^6/ml) were incubated with 20 μmol/l of pioglitazone (+) or medium alone (−) for 48 h. Integrin expression in U937 cells was analyzed by flow cytometry analysis using monoclonal antibodies to integrins (CD11a, CD18, VLA4) and compared to positive control HLA class I (w6/32). Five thousand cells were analyzed for each condition. Data are representative of four similar experiments. B: U937 cells (1×10^6/ml) were incubated with 20 μmol/l of pioglitazone or in medium alone (control) for 48 h. F-actin content in U937 cells was detected with phalloidin and quantitated using a fluorescent plate reader as described in Section 2, and then expressed as a percentage of that of the control. $^*P<0.05$ vs. control. Data are representative of three separate observations. C: U937 cells were incubated with medium alone (−) or 20 μmol/l of pioglitazone (+) for 48 h. Western blotting detected RhoA expression in the membrane and total lysate of U937 cells for each condition. RhoA activity was also determined using the Rhotekin assay. Blots are representative of three separate experiments.

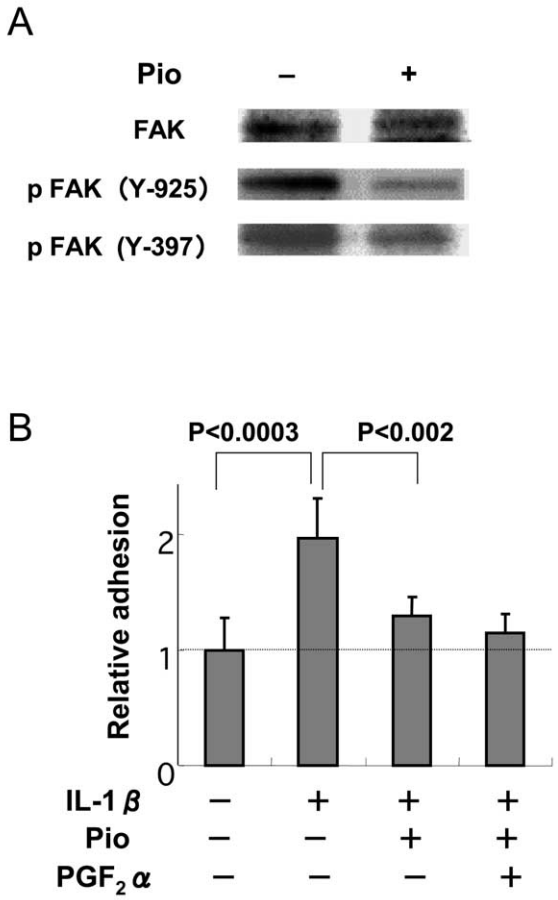

Fig. 3. A: Effects of pioglitazone on FAK activation in U937 cells. U937 cells (1×10^6/ml) were incubated with 20 μmol/l of pioglitazone (+) or in medium alone (−) for 48 h. The expressions of pFAK (397Y), pFAK (925Y), and FAK were detected in each condition by Western blotting analysis. Blots are representative of five separate experiments. B: Effect of PPAR antagonist on adhesion of U937 cells to HUVEC. U937 cells (1×10^6/ml) were incubated with 20 μmol/l of pioglitazone, PGF2α, for 30 min prior to incubation with 20 μmol/l of pioglitazone, or medium alone for 48 h, and then placed on HUVEC. An adhesion assay was carried out as described in Section 2.

3.5. PPAR antagonist failed to reverse the inhibitory effects of pioglitazone toward the adhesion of U937 cells to HUVEC

To examine whether the antagonizing PPARγ receptor has some effects on U937 adhesion, U937 cells were treated with prostaglandin F2α (PGF2α) for 30 min prior to pioglitazone treatment, in order to compete with the PPARγ binding of pioglitazone. When adhesion assays were carried out, pretreatment of U937 cells with PGF2α failed to reverse the inhibitory effects of pioglitazone toward the adhesion of U937 cells to the HUVEC monolayer (Fig. 3B).

4. Discussion

Recent studies have shown that TZDs have protective ef-

fects against atherosclerosis, as well as direct biological effects on monocytes, vascular endothelium, and vascular smooth muscle cells [10–12]. The present findings demonstrated that preincubation of monocytes with pioglitazone, a TZD, significantly reduced monocytic U937 adhesion to activated HUVEC under both static and flow conditions. Based on the results of flow cytometric analysis, pioglitazone did not have an effect on the expression levels of cell surface integrins in U937 cells. In parallel with this observation, recent reports have demonstrated that the surface expression levels of integrins do not exclusively modulate the adhesive interactions of leukocytes [13]. In the present experiments, we found a reduction of actin filament after pioglitazone treatment. Further, it has been reported that the actin cytoskeleton that is anchored to focal adhesion sites is critically important in monocyte adhesion to vascular endothelium [14], therefore, modulation of the cytoskeletal network leads to an increase or decrease of adhesive interactions. Pioglitazone significantly decreased the filamentous actin contents in U937 cells and the RhoA activity. This effect on RhoA inactivation and cytoskeletal modulation may show a novel intracellular mechanism by which pioglitazone inhibits monocyte adhesion. FAK is known to regulate cell adhesion and migration in various cell types, including non-adherent blood cells, by transferring signals to integrins at the cellular adhesion site [15]. Further, we showed that the phosphorylation of 925Y, which is reported to be associated with the Ras/mitogen-activated protein kinase pathway [16] was reduced by pioglitazone treatment. These data suggest that dephosphorylation at 925Y may be involved in the pioglitazone-mediated reduced adhesion of U937 cells. In contrast, the total amount of FAK and phosphorylation of FAK at tyrosine 397 was not affected by pioglitazone treatment. Interestingly, the PPARγ antagonist PGF2α failed to cancel the pioglitazone-mediated reduction of U937 cell adhesion, which implies that the observed phenomena occur independently of PPARγ agonistic stimulation. In agreement with our study, PPARγ-independent effects of PPARγ ligands were reported using TZDs [17]. Besides its activity as an agonist of PPARγ, pioglitazone may suppress the activation of RhoA GTPase. Careful examination in the future will be required to elucidate the responsible molecular mechanisms and other intracellular signaling important in cell adhesion. In summary, we demonstrated that pioglitazone, a novel antidiabetic agent, reduced IL-1β-induced monocyte adhesion to vascular endothelium, actin polymerization, and FAK activation. A reduction of RhoA GTPase activations may be involved in this process. Thus, in patients with diabetes, administration of pioglitazone is beneficial in reducing the serum level of glucose itself and may have clinical merits for modulating monocyte–endothelial interaction.

Acknowledgements: This study was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan, and from the Ministry of Health, Labour and Welfare of Japan. We gratefully acknowledge the expert assistance of Noriko Nitta in working with the cell cultures.

References

[1] Jiang, C., Ting, A.T. and Seed, B. (1998) Nature 391, 82–86.
[2] Ricote, M., Li, A.C., Willson, T.M., Kelly, C.J. and Glass, C.K. (1998) Nature 391, 79–82.
[3] Chawla, A., Barak, Y., Nagy, L., Liao, D., Tontonoz, P. and Evans, R.M. (2001) Nat. Med. 2001, 48–52.
[4] Yoshida, M. and Gimbrone Jr., M.A. (1997) Ann. NY Acad. Sci. 811, 493–497.
[5] Yoshida, M., Szente, B.E., Kiely, J.M., Rosenzweig, A. and Gimbrone Jr., M.A. (1998) J. Immunol. 161, 933–941.
[6] Kiely, J.M., Luscinskas, F.W. and Gimbrone Jr., M.A. (1999) Methods Mol. Biol. 96, 131–136.
[7] Yoshida, M., Sawada, T., Ishii, H., Gerszten, R.E., Rosenzweig, A., Gimbrone Jr., M., Yasukochi, Y. and Numano, F. (2001) Arterioscler. Thromb. Vasc. Biol. 21, 1165–1171.
[8] Li, S., Chen, B.P., Azuma, N., Hu, Y.L., Wu, S.Z., Sumpio, B.E., Shyy, J.Y. and Chien, S. (1999) J. Clin. Invest. 103, 1141–1150.
[9] Laufs, U. and Liao, J.K. (1998) J. Biol. Chem. 273, 24266–24271.
[10] Jiang, C., Ting, A.T. and Seed, B. (1998) Nature 391, 82–86.
[11] Murata, T., Hata, Y., Ishibashi, T., Kim, S., Hsueh, W.A., Law, R.E. and Hinton, D.R. (2001) Arch. Ophthalmol. 119, 709–717.
[12] Law, R.E., Goetze, S., Xi, X.P., Jackson, S., Kawano, Y., Demer, L., Fishbein, M.C., Meehan, W.P. and Hsueh, W.A. (2000) Circulation 101, 1311–1318.
[13] Kawakami, A., Tanaka, A., Nakajima, K., Shimokado, K. and Yoshida, M. (2002) Circ. Res. 91, 263–271.
[14] Takubo, T., Hino, M., Suzuki, K. and Tatsumi, N. (1999) Eur. J. Histochem. 43, 71–77.
[15] Zachary, I. and Rozengurt, E. (1992) Cell 71, 891–894.
[16] Schlaepfer, D.D., Hanks, S.K., Hunter, T. and van der Geer, P. (1994) Nature 372, 786–791.
[17] Palakurthi, S.S., Aktas, H., Grubissich, L.M., Mortensen, R.M. and Halperin, J.A. (2001) Cancer Res. 61, 6213–6218.