The level of pancreatic PLA_2 receptor is closely associated with the proliferative state of rat uterine stromal cells

Christian Tessier[a], Gian-Paolo Rossini[b], Jean-François Pageaux[a], Hélène Cohen[a], Michel Lagarde[c], Christian Laugier[a], Jean-Michel Fayard[a,*]

[a]*Laboratoire de Physiologie-Pharmacodynamie, INSERM U.352, INSA 406, 69621 Villeurbanne, France*
[b]*Dipartimento di Scienze Biomediche, Universita Degli Studi di Modena, 41100 Modena, Italy*
[c]*Laboratoire de Chimie Biologique, INSERM U.352, INSA 406, 69621 Villeurbanne, France*

Received 17 June 1996

Abstract Rat uterine stromal cells (U_{III}) express pancreatic type PLA_2 (PLA_2-I) receptor and internalize the enzyme bound to receptors. Here, we investigate the proliferating effect and alterations in binding of PLA_2-I. There is a dramatic decline in PLA_2-I binding in U_{III} cells as they progress from a non-confluent proliferating state (40,000 sites/cell) to a confluent state (1300 sites/cell). Intracellular concentration of PLA_2-I changed with the alteration in binding, suggesting that regulation in the PLA_2 binding capacity may have important implications in growth control mechanisms.

Key words: Pancreatic phospolipase A_2; PLA_2 receptor; Proliferation; Uterine stromal cell line; Rat

1. Introduction

Phospholipases A_2 (PLA_2; EC 3.1.1.4) are lipolytic enzymes that hydrolyze the sn-2 acyl ester bound in glycerophospholipids [1]. Many forms of PLA_2 enzymes have been described and classified into several groups [2–5]. Type I, II and III PLA_2 are low molecular weight (13–18 kDa), extracellular enzymes, including pancreatic and cobra venom PLA_2 (type I), rattle snake and inflammatory PLA_2 (type II) and bee venom (type III). Intracellular cytosolic PLA_2, on the other hand, belong to different groups, including the 85 kDa (type IV) and 40–60 kDa enzymes [5]. In recent years, evidence for the involvement of distinct types of PLA_2 in cell proliferation has been obtained. Particularly, type I PLA_2 has been shown to stimulate proliferation of 3T3 cells via a specific membrane receptor, and the contribution of Arita's group in this research field was determinant [6–8]. This proliferative effect can be clearly distinguished from its phospholipid hydrolyzing activity.

We have recently established a stromal cell line (U_{III}) derived from normal rat uterus which has retained some of the characteristics of uterine stromal cells, particularly progesterone and prolactine receptor expression, progesterone regulation of growth [9] and production of prostaglandins under hormonal regulation [10]. Immunocytochemistry studies revealed that U_{III} cells express the pancreatic type PLA_2 [10]. Proliferation of U_{III} cells was not affected by inhibitors of cyclooxygenase and lipoxygenases but was reduced and even arrested by different PLA_2 inhibitors [11]. Moreover, we have recently demonstrated that U_{III} cells express specific membrane receptors for PLA_2-I and that binding of PLA_2-I to its specific receptor is associated with internalization of the enzyme [12], which seems to be a general mechanism of membrane receptors for low molecular weight PLA_2 [13]. Since, in our experimental system, the internalized PLA_2 is not rapidly degraded, supporting the hypothesis that this enzyme is involved in the control of U_{III} cell functioning, we investigated the proliferative effect of PLA_2-I and the relationship between the number of PLA_2-I binding sites and the proliferative state of these non-transformed cells.

2. Material and methods

2.1. Chemicals

Tissue culture medium (M199), phosphate-buffered saline (PBS), L-glutamine, penicillin, streptomycin were obtained from TechGen International (Les Ulis, France), fetal calf serum (FCS) from GIBCO (Cergy-Pontoise, France). Trypsin 1×, trypan blue, PLA_2 from porcine and bovine pancreas and from bee, *Crotalus atrox* and *Naja mocambique mocambique* venom were purchased from Sigma Chimie (St. Quentin Fallavier, France). Iodo beads were purchased from Pierce Chemical Co. Bis-benzimide H 33258 was from Fluka. [6-^{3}H]thymidine (26 Ci/mmol) was obtained from Amersham (Les Ulis, France). Sodium (^{125}I) iodine (carrier-free, 100 mCi/ml) was from Du Pont/New England Nuclear (Les Ulis, France). All other chemicals were analytical grade.

2.2. Cell culture

For stock culture, rat uterine stromal cells (U_{III} cells) were grown in Falcon plastic flasks (75 cm^2) in an air/CO_2 (95 : 5%) humidified atmosphere at 37°C. M199 medium was supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Medium was changed every 48 h. Confluent cells were subcultured by incubation with 0.25% trypsin, centrifuged and seeded at a ratio of 1 : 2. Cell viability, determined by the trypan blue exclusion method, was consistently greater than 95%.

2.3. Growth experiments

Confluent cells from stock culture (50th–58th passages) were trypsinized inoculated in 24-well plates ($\approx 5\times10^4$ cells/well) in medium M199 with 1% FCS. Twenty-four hours after seeding, the medium with unattached cells was removed and fresh medium with or without the different PLA_2 was added. An index of cell number was achieved by assay of total DNA content per well after 48 h of culture, using a fluorimetric procedure [14]. The cells were washed twice with PBS. Then 2 ml of homogenization buffer (50 mM Na_2HPO_4, 2 M NaCl, 2 mM EDTA, pH 7.4) were added to each well. Well contents were briefly sonicated using a microprobe. Aliquots of the homogenate were mixed with buffer containing the fluorochrome H 33258 to a final concentration of 0.1 μg/ml. Fluorescence measurements were made 15 min after mixing, using a Jobin-Yvon spectrofluorimeter, with calf thymus DNA as a standard. For thymidine incorporation, 1 μCi of [^{3}H]thymidine was added to each well and plates were incubated for a further 24 h. The medium was removed, and the cells were washed twice with 1 ml of ice-cold PBS. Then 2 ml of ice-cold 10% trichloroacetic acid (TCA) were added to each well. Well contents were briefly sonicated using a microprobe, incubated for 30 min

*Corresponding author. Fax: (33) 72-43-85-24

Abbreviations: PLA_2, phospolipase A_2

PII S0014-5793(96)00680-1

at 0°C, removed and filtered through a nitrocellulose membrane filter (Millipore 0.45 μm, with 2.5 cm diameter). The filter was then washed 3 times with 5 ml of ice-cold 5% TCA, and the radioactivity associated with the filter was counted by liquid scintillation using 8 ml of Ultimagold (Packard).

2.4. Binding studies

For binding studies, U_{III} cells from stock cultures were seeded at different densities and grown for 72 h in medium M199 with 10% FCS in 60 mm Petri dishes. The cells were washed once with 2.5 ml of binding medium, consisting of M199 medium containing 0.1% (w/v) bovine serum albumin and 50 mM HEPES buffer, pH 7.2. Culture dishes received 1.5 ml of binding medium, and the cells were then incubated with 20 nM iodinated PLA_2 in the presence or absence of a 100-fold molar excess of non-radioactive PLA_2 as a competitor. Iodination of porcine pancreatic PLA_2 was carried out using the Iodo beads iodinating reagent, as described previously [12]. The specific radioactivity of iodinated PLA_2 was 100–200 dpm/fmol. The incubation with ligand was performed 2 h at 4°C or 90 min at 37°C. At the end of the incubation, the binding medium was removed, and cells were washed 5 times with 2.5 ml of PBS buffer at 4°C. The radioactivity associated with U_{III} cells was then determined after solubilization with 1 ml of 1 N NaOH and samples neutralization with 1 ml of 1 N HCl. The specific binding of PLA_2 to U_{III} cells was determined by subtraction of the radioactivity measured in cells incubated in the presence of competitor from that of paired samples incubated in its absence.

2.5. Statistical analysis

Non-parametric statistics (Kruskal-Wallis test) were used. A comparison was considered not significant when the calculated P value exceeded 5%.

3. Results and discussion

Our results show that porcine PLA_2-I exerts a significant proliferative effect on U_{III} cells grown in medium 199 supplemented with 1% FCS, a serum concentration that allows a very slow proliferating state [9]. Both DNA content per well (Fig. 1a) and [^{3}H]thymidine incorporation (Fig. 1b) were increased after 2 days of culture. These effects were dose-dependent and significant ($P < 0.05$) at the optimal concentration of 10 nM. Supplementation of the culture medium with concentrations of PLA_2-I ($\gtrsim$50 nM) reduced the cell viability (data not shown). Bee, *Crotalus* and *Naja* venom PLA_2s, which have no affinity for the pancreatic PLA_2 receptor present in U_{III} cell membranes [12], showed no growth promoting effect (Table 1). The bovine pancreatic PLA_2, although very similar to the porcine enzyme, was also inactive. This unexpected result could be related to the high specificity of U_{III} cell binding sites towards porcine pancreatic PLA_2. As a matter of fact, we have shown from displacement curves that the affinity of bovine PLA_2-I is at least 5-fold lower than that of the porcine enzyme [12]. A competitive effect was only observed at concentrations which reduced the viability of cells grown during 48 h in such supplemented medium. These results agree

Table 1
Effect of various secreted PLA_2 on U_{III} cell proliferation

PLA_2(10 nM)	DNA(ng/well)
None	643 ± 10
Porcine pancreas	903 ± 56*
Bovine pancreas	638 ± 91
Bee venom	735 ± 77
Crotalus venom	723 ± 40
Naja venom	715 ± 46

U_{III} cells were grown for 48 h in the presence of various secreted PLA_2 at a concentration of 10 nM as indicated in Section 2. Results are expressed as the mean ± S.D. of three independent determinations. *Significantly different ($P < 0.05$) from control values.

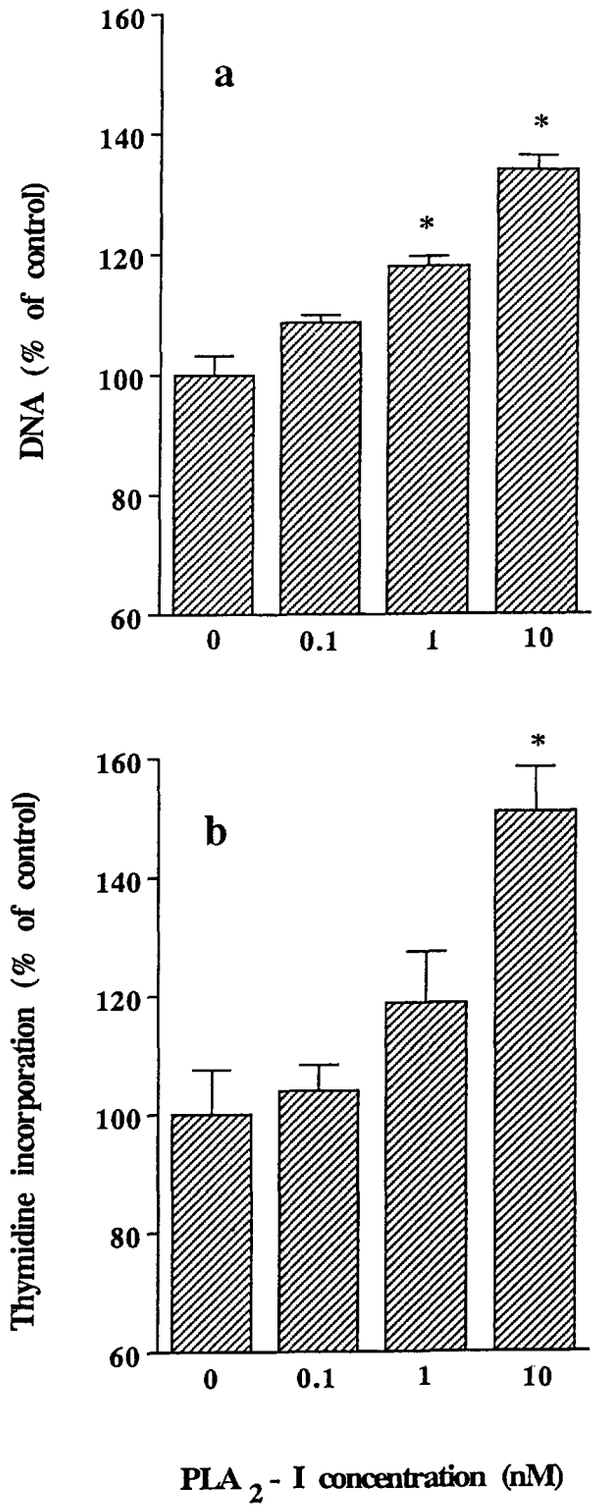

Fig. 1. Effect of pancreatic PLA_2 on U_{III} cell proliferation. Data are expressed as percent of control values (no PLA_2 added) and are the mean ± standard deviation of three independent experiments. U_{III} cells were grown on 24-well plates in M199 medium supplemented with 1% FCS, and the DNA content per well (a) or [^{3}H]thymidine incorporation (b) were determined as described in Section 2, 48 h after supplementation of the culture medium with 0.1, 1 or 10 nM PLA_2. *Significantly different ($P < 0.05$) from control values.

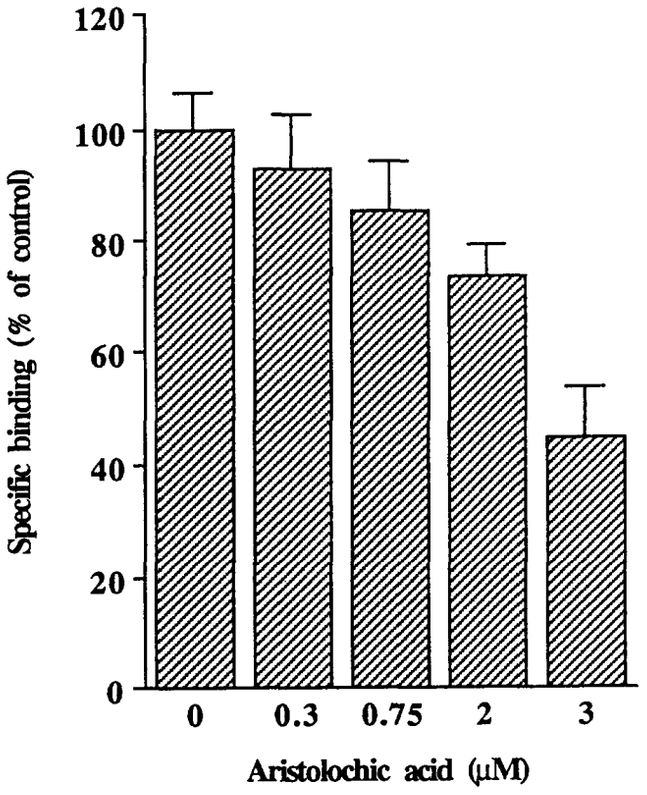

Fig. 2. Effect of aristolochic acid on PLA_2-I binding in U_{III} cells. Specific binding of iodinated porcine PLA_2-I was measured as described in Section 2 in the presence of indicated concentrations of aristolochic acid in the binding medium. Data are expressed as percent of control values (2217 ± 181 fmol/mg DNA) and are the mean ± standard deviation of three independent experiments.

with previously published data obtained on various transformed cellular models such as: Swiss 3T3 cells [6,15], rat aortic smooth muscle cells and rat synovial cells [16], rat chondrocytes [7] as well as on cancer cell lines such as A 549 lung adenocarcinoma cells [17] or human pancreatic cancer cells [18]. Moreover, they are consistent with the observation that PLA_2-I-induced proliferation is dependent on specific membrane receptor binding. We have shown previously that the PLA_2 inhibitor aristolochic acid, which binds directly to PLA_2 and alters the α-helical content of the protein [19], exerts a potent antiproliferative effect on U_{III} cells at micromolar concentrations [11]. We tested the hypothesis that this effect was related to change in binding activity of ^{125}I-labeled PLA_2-I. As shown in Fig. 2, addition of aristolochic acid to the binding medium reduced the binding activity of porcine PLA_2-I in a dose-dependent manner with an IC_{50} of about 3 μM, that agrees with its antiproliferative effect. It should be noted that dexamethasone, another PLA_2 inhibitor that reduced U_{III} cell proliferation at 100 μM [11], also reduces PLA_2-I binding activity by about 60% at this concentration [16].

In order to strengthen the relationship between PLA_2-I binding sites and U_{III} cell proliferation, we next investigated growth-dependent alterations in PLA_2-I binding. Results, summarized in Fig. 3, show that there is a drastic decrease in the number of specific PLA_2-I binding sites as U_{III} cells progress from an actively proliferating state to a contact-inhibited monolayer. The number of PLA_2-I receptors decreased during the growth of U_{III} cells from about 40,000 sites per cell in the earlier stages of proliferation to about 1300 sites per cell in confluent cultures. This decrease in the binding of PLA_2-I was not simply a consequence of increasing time of culture, since in these experiments U_{III} cells were grown during the same period after seeding at different densities. Since PLA_2-I is internalized after binding to its specific receptor [12], we studied the difference in internalization between confluent and non-confluent cells. We took advantage of the fact that cell washing with an acidic buffer removes PLA_2 bound to plasma membrane receptors [16] and we measured the PLA_2 levels in the extracellular (acid-soluble) and intracellular (acid-resistant) compartments of U_{III} cells. The data reported in Table 2 show that, after 90 min of incubation at 37°C, the levels of intracellular PLA_2 are about 4-fold higher than those

Table 2
Membrane-bound and intracellular levels of PLA_2-I in preconfluent and confluent U_{III} cells

	Confluent(n = 5)	Non-confluent(n = 3)
Membrane-bound	364 ± 55	1352 ± 130
Intracellular	1660 ± 228	6380 ± 141

Confluent and non-confluent cells were incubated for 90 min at 37°C with 20 nM iodinated porcine pancreatic PLA_2 in the presence or absence of a 100-fold molar excess of unlabelled competitor. At the end of incubation, cells were washed 5 times with 2.5 ml of PBS buffer and were further incubated with 2.5 ml of 50 mM glycine buffer, pH 3.0, containing 0.1 M NaCl, for 10 min at 4°C. The acidic buffer was then collected and used to determine the acidic-extractable, membrane-bound radioactivity. Cells were then solubilized with 1 N NaOH to measure the residual acid-resistant, intracellular radioactivity. Measurements were made in triplicate in n independent experiments. Data are expressed as fmol of iodinated PLA_2 bound per mg DNA and are the mean ± S.D.

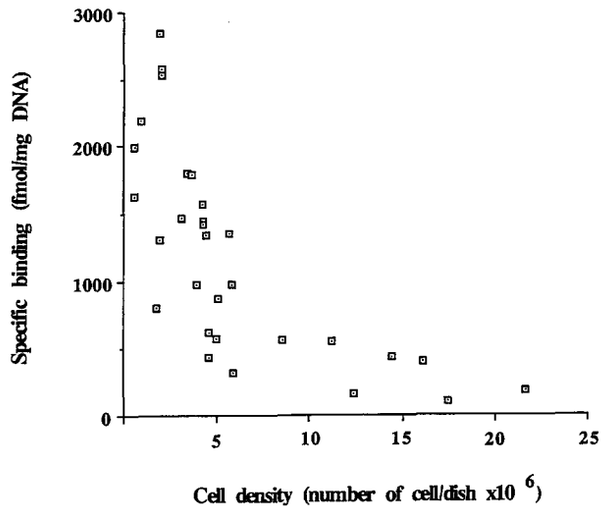

Fig. 3. Effect of cell density on specific binding of porcine pancreatic PLA_2 in U_{III} cells. Binding studies were performed as indicated in Section 2. Specific binding was calculated by subtraction of binding obtained in the presence of unlabelled competitor from that found in its absence.

detectable in the extracellular counterpart both in confluent (4.6 ± 0.7) and in non-confluent cells (4.8 ± 0.4). These results demonstrate that, at physiological temperature, PLA_2 is internalized at the same rate (about three molecules of PLA_2-I per receptor and per hour) in confluent and non-confluent U_{III} cells. Thus, decrease in the number of specific PLA_2-I membrane receptors results in a correlative reduction of intracellular concentration of PLA_2-I. This regulation in the number of PLA_2-I receptors might be a physiologically relevant mechanism implicated in the phenomenon of contact inhibition.

Many phenotypic changes occur in cultured cells as they progress from an actively dividing state to a contact inhibited monolayer. For example, metabolism of arachidonic acid in particular is highly modified and its release from phospholipids decreases when cells become confluent [20,21]. Changes in lipid metabolism occur in the process of normal or transformed cell proliferation [22,23] and variations in the cellular concentration of some particular phospholipids are even considered as cancer markers. The effects of eicosanoids on cell proliferation have also often been described in normal as well as in transformed cell lines [24–27]. All these results imply specific changes in PLA_2 activity rather than variations in the expression of PLA_2 itself. However, the secretory phospholipase A_2 genes have been recently described as a novel class of genes that influence tumor susceptibility [28]. Moreover, cancer cells may overexpress some receptors activated by specific growth factors such as EGF receptors, for example in the case of human pancreatic carcinoma cells [29]. Our results confirm the proliferative effect of PLA_2-I and indicate that the level of membrane PLA_2-I receptors varies according to the proliferative state of U_{III} cells. They suggest that, after binding to its receptor, internalized PLA_2-I might be involved, as a signaling molecule, in the control of U_{III} cell proliferation and we may speculate that the level of PLA_2-I receptor expression might be a new cell cancer marker.

Acknowledgements: We are grateful to Ms. Véronique Pommatau-Deschamps for her excellent secretarial assistance and Ms. Claudine Raffy for her excellent technical assistance. This work was supported by INSERM.

References

[1] Dennis, E.A. (1983) in: The Enzymes (Boyer, P.D., ed.), 3rd edn., Vol. 16, pp. 307–353, Academic Press, New York.
[2] Glaser, K.B., Mobilio, D., Chang, J.Y. and Senko, N. (1993) Trends Pharmacol. Sci. 14, 92–98.
[3] Kudo, I., Murakami, M., Hara, S. and Inoue, K. (1993) Biochim. Biophys. Acta 117, 217–231.
[4] Mayer, R.J. and Marshall, L.A. (1993) FASEB J. 7, 339–348.
[5] Mukherjee, A.B., Miele, L. and Pattabiraman, N. (1994) Biochem. Pharmacol. 48, 1–10.
[6] Arita, H., Hanasaki, K., Nakano, T., Oka, S., Teraoka, H. and Matsumoto, K. (1991) J. Biol. Chem. 266, 19139–19141.
[7] Kishino, J., Tohkin, M. and Arita, H. (1992) Biochem. Biophys. Res. Commun. 186, 1025–1031.
[8] Ohara, O., Ishizaki, J. and Arita, H. (1995) Prog. Lipid Res. 34, 117–138.
[9] Cohen, H., Pageaux, J.F., Melinand, C., Fayard, J.M. and Laugier, C. (1993) Eur. J. Cell Biol. 61, 116–125.
[10] Prigent, A., Fayard, J.M., Pageaux, J.F., Lagarde, M., Laugier, C. and Cohen, H. (1994) Prostaglandins 47, 451–466.
[11] Fayard, J.M., Tessier, C., Cohen, H., Lagarde, M., Pageaux, J.F. and Laugier, C. (1994) Eur. J. Pharmacol. 251, 281–289.
[12] Rossini, G.P., Fayard, J.M., Tessier, C. and Laugier, C. (1996) Biochem. J. 315, 1007–1014.
[13] Zvaritch, E., Lambeau, G. and Lazdunski, M. (1996) J. Bio. Chem. 271, 250–257.
[14] Labarca, C. and Paigen, K. (1980) Anal. Biochem. 102, 344–352.
[15] Kurizaki, T., Egami, H., Murata, K., Kiyohara, H., Okazaki, S., Yoshida, N. and Ogawa, M. (1992) Res. Commun. Chem. Pathol. Pharmacol. 78, 39–45.
[16] Hanasaki, K. and Arita, H. (1992) J. Biol. Chem. 267, 6414–6420.
[17] Tokumoto, H., Croxtall, J.D., Choudhury, Q. and Flower, R.J. (1993) Biochim. Biophys. Acta 1164, 236–242.
[18] Hanada, K., Kinoshita, E., Itoh, M., Hirata, M., Kajiyama, G. and Sugiyama, M. (1995) FEBS Lett. 373, 85–87.
[19] Vishwanath, B.S. and Gowda, T.V. (1987) Toxicon 25, 929–937.
[20] Whatley, R.E., Satoh, K., Zimmerman, G.A., McIntyre, T.M. and Prescott, S.M. (1994) J. Clin. Invest. 94, 1889–1900.
[21] Denizot, Y., Najid, A. and Rigaud, M. (1993) Cancer Lett. 68, 199–205.
[22] Piomelli, D. (1993) Curr. Opin. Cell Biol. 5, 274–280.
[23] Welsh, C.W. (1995) Free Rad. Biol. Med. 18, 757–773.
[24] Powis, G., Hill, S.R., Frew, T.J. and Sherill, K.W. (1995) Med. Res. Rev. 145 121–138.
[25] Ledwozyw, A. and Lutnicki, K. (1992) Acta Physiol. Hungarica 79, 381–387.
[26] Butcher, R.D., Wojcik, S.J., Lints, T., Wilson, T., Schoffield, P.C. and Ralph, R. (1993) Cancer Res. 53, 3405–3410.
[27] Bashir, N., Kuhen, K. and Taub, M. (1992) In vitro Cell. Dev. Biol. 28A, 663–668.
[28] MacPhee, M., Chepenik, K.P., Lidell, R.A., Nelson, K.K., Siracusa, L.D. and Buchberg, A.M. (1995) Cell 81, 957–966.
[29] Korc, M., Meltzer, P. and Trent, J. (1986) Proc. Natl. Sci. USA 83, 5141–5144.