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Aspirin 於 p53 及其標的與調節基因之作用機制探討

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計畫主持人： 周明勇

計畫參與人員： 楊世煌

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Activation of p53 signalling in acetylsalicylic acid-induced apoptosis in OC2 human oral cancer cells

C.-C. Ho, X. W. Yang*, T.-L. Lee, P.-H. Liao, S.-H. Yang, C.-H. Tsai and M.-Y. Chou

Chung Shan Medical University Hospital, Taichung, Taiwan,
*Wuhan University, Wuhan, China

Abstract

Background Nonsteroidal antiinflammatory drugs (NSAIDs) such as acetylsalicylic acid (ASA, aspirin) are well known chemotherapeutic agents of cancers; however, the signaling molecules involved remain unclear. The aim of this study is to investigate the possible existence of a putative p53-dependent pathway underlying the ASA-induced apoptosis in OC2 cells, a human oral cancer cell line.

Materials and Methods The MTT (methyl tetrazolium) assay was employed to quantify differences in cell viability. DNA ladder formation on agarose electrophoresis was used as apoptosis assay. The expression levels of several master regulatory molecules controlling various signal pathways were monitored using the immunoblotting techniques. Flow cytometry was used to confirm the effect of ASA on cell cycle. Patterns of changes in expression were scanned and analyzed using the NIH image 1.56 software. All the data were analyzed by ANOVA.

Results ASA reduced cell viability and presence of internucleosomal DNA fragmentation. In the meanwhile, phosphorylation of p53 at serine 15, accumulation of p53 and increased the expression of its downstream target genes, p21 and Bax induced by ASA. The expression of COX-2 was suppressed. Disruption of p53-MDM2 (murine double minute-2) complex formation resulted in increasing the expression of MDM2 60 kd cleavage fragment. Inhibited the activation of p42/p44 MAPK (mitogen-activated protein kinase) by PD98059, a specific inhibitor of ERK (extracellular regulatory kinase), significantly decreased cell viability and enhanced the expression of p53 induced by ASA. The result of cell cycle analysis showed that ASA and PD98059 induced the cell cycle arrested at G0/G1 phase and resulted in apoptosis.

Conclusion NSAIDs inhibit cyclooxygenase is not the only or even the most important mechanism of inhibition. Our study presents evidences that activation of p53 signaling involved in apoptosis induced by ASA. Furthermore, the apoptotic effect was enhanced by blocking the activation of p42/p44 MAPK in response to treatment with ASA, thus indicating a negative role for p42/p44 MAPK.

Keywords acetylsalicylic acid, p53, COX-2, PD98059, cell cycle, apoptosis

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylic acid (ASA, aspirin) have been used as chemotherapeutic agents of cancers to induce apoptosis or reduce the incidence, to name a few, like colon [1], lung [2], stomach [3] and colorectum [4]. They are known to act by directly suppressing cyclooxygenase 1 and 2 (COX-1 and COX-2), the rate-limiting enzyme catalyzing the biosynthesis of prostaglandins, thereby blocking the production of proinflammatory prostaglandins. The current prevailing theory is that the mechanism for the suppressor effect of NSAIDs on carcinogenesis is attributed mainly to the inhibition of COX-2. However, ASA and selective COX-2 inhibitor NS-398 suppressed the proliferation and stimulated apoptosis in COX-2 negative colon cancer cell line (SW480) [5-6]. It implied a COX-2-independent mechanism of NSIADs. Increased in amount of COX-2 mRNA and protein are commonly found in pre-malignant and malignant conditions, including head and neck squamous cell carcinoma (HNSCC) [7-10]. Moreover, mutation of p53 is detected in both precancerous and cancerous lesions of the head and neck [11-12]. Subbaramaiah and coworkers (1999) indicated that levels of COX-2 protein and mRNA were markedly suppressed by wild type p53 but not by mutant p53 [13]; it rise the possibility that p53 status could be a determinant of COX-2 expression in HNSCC.

It well known that p53 is the most frequently mutated gene in human cancers and its functions have been described, including induction of G1 arrest or apoptosis following DNA damage or other cellular insults, moreover, in the maintenance of genomic stability and inhibition of angiogenesis. When the cell receives an appropriate stress signal, p53 will be phosphoreylated and the binding of MDM2 to p53 would be blocked or modified which results in increasing of p53 stability and accumulation. This in turn, leads to upregulation of p53-dependent genes required for cell growth arrest ($p21^{WAF1/CIP1}$) and activation of apoptosis (Bax), respectively [14-15].

Extensive studies of the mechanism of NSAIDs-induced apoptosis has been focused on physiopathological changes of cyclooxygenases and their regulatory pathways for decades. Compared to studies of the cyclooxygenase-centered mechanism, our understanding of p53-dependent apoptotic pathway induced by NSAIDs is far behind. We demonstrated that activation of p53 signaling may play an important role in cells undergoing aspirin-induced apoptosis.

Materials and Methods

Cell culture

Human oral cancer cell line (OC2) [16] was cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, streptomycin (10,000 U/ml) and penicillin (10,000 U/ml). The medium was buffered with sodium bicarbonate (2.0 gm/liter) as manufacturer recommended. Before exposed the cell to aspirin, cells were washed with PBS and cultured in serum-free medium. ASA (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO as 1 M stock solution and stored at -20℃. The volume of DMSO was equalized to 0.3 % in all culture dishes.

MTT (Methyl Tetrazolium) reduction assay

To examine the cytotoxic effect, cell viability was measured via MTT tetrazolium bromide mitochondrial activity assay described previously [17]. In short, a total of 5000 cells in 100 μl of medium were seeded per well in a 96-well plate for 24 hours before treatment with drugs. At the end of incubation, 20 μl of 5 mg/ml MTT (Sigma) solution in PBS was added to each well for 4 hours. After incubation, the medium was discarded and 100 μl DMSO was added to each well to dissolve the formazan crystals. The absorbance was determined at 540 nm.

DNA fragmentation analysis

The method of apoptotic DNA fragment analysis developed by Herrmann and colleagues (1994) was described in detail previously [18]. Briefly, cells were harvested, washed and pelleted by centrifugation. The cell pellets were then treated with lysis buffer (1% NP-40, 20 mM EDTA, in 50 mM Tris-HCl pH 7.5) for 10 seconds. After centrifugation, the supernatants were brought to 1% SDS and treated with RNase A (final concentration at 5 μg/μl, Sigma) at 56℃ for 2 hours followed by digestion with proteinase K (final concentration at 2.5 μg/μl, Sigma) at 37℃ for at least 2 hours. Apoptotic DNA fragments were separated by electrophoresis in 2% agarose gels.

Western blot

The expression level of p53, p53-pSer392 (Sigma), p53 -pSer15 (Calbiochem, San Diego, CA) and a small set of its regulatory proteins, including COX-2, MDM2, Bax, p21, active form of caspase-3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and MAPKs (Cell Signaling Technology, Beverly, MA) were analyzed by Western blot. The cells were washed twice with cold PBS prior to being extracted with 2.5 % Triton X-100. Samples were heated at 95℃ for 5 min in Laemmli buffer then chilled on ice. Subsequently after electrophoresis (50 μg /lane), the proteins were electro-blotted to ECL nitrocellular membrane (Amersham pharmacia Biotech, Buckinghamshire, England). The proteins of interest were detected with an ECL western blotting detection reagent (Amersham). Briefly, nonspecific binding on the nitrocellular membrane were blocked with 5 % non-fat dry milk in 20 mM Tris and 150 mM NaCl prior to incubating with primary antibodies against specific antigens.

After incubation with the conjugated second antibody, the blotted nitrocellular membrane were exposed to x-ray films and images of blotted patterns were analyzed with NIH image software (National Institutes of Health, U.S.A.). Blots were routinely re-probed with anti-actin to ensure equivalence of loading. If necessary, membranes were strip by incubation at 50℃ for 30 min in a solution of 62.5 mM Tris-HCl, pH6.7, 2% SDS and 100 mM 2-mercaptoethanol.

Flow cytometry

Cells were trypsinised, pelleted, fix and propiduim iodide (PI) stained as previous described [6]. After trypsinised and collected, cells were fixed with ice-cold 75% ethanol in PBS, washed twice with PBS and stored at -20℃. Before analysis, cells were incubated at 37℃ for 30 min with 5 μl RNase A (10 mg/ml, Sigma) and 1 ml PI (50 μg/ml, Sigma). The analysis of samples was performed by a flow cytometry (Coulter Epics XL, UK). Windows multiple document interface (WinMDI) software was used to calculate the cell cycle phase distridution from the resultant DNA histogram and expressed as a percentage of cells in G0/G1, S and G2/M phase. The apoptotic cells observed on a DNA histogram as a subdiploid peak [19].

Statistical analysis

All data were analyzed by ANOVA (analysis of variance) and expressed as mean ± standard deviation. A *P*-value of less than 0.05 is considered statistically significant.

Results

Aspirin inhibits cell proliferation and induces apoptosis in human OC2 cells

It was noted that by increasing concentrations of ASA (0, 0.5, 1, 2, and 4 mM) for 24 hours, drastic changes in morphology e.g. cell rounding, blebbing, and detachment leading to cell death took place (data not shown). In MTT assay, the effect of ASA on cell viability was dose dependent (Fig. 1A). To verify the coincided morphological changes and MTT results were caused by ASA-induced apoptosis, DNA agarose electrophoresis of genomic DNA extracted from ASA-treated cells were conducted. As shown in Figure 1B, DNA ladders were formed by typical internucleosomal fragments characteristic of apoptosis. This result agreed with previous study which shown a ladder pattern on agarose gel in human gastric cancer cells (AGS) treated with ASA (1, 10 mM) for 24 hours [5]. In contrast, no DNA fragmentation of HT-29 cells in response to ASA (3 mM, 72 hours) [6].

In addition, a distinct active form of caspase-3 expression induced early at 3 hours and 12 hours (Fig. 1C). Janicke and colleague (1998) reported that introduction of CASP-3 gene into MCF-7 breast carcinoma cell line resulted in DNA fragmentation [20]. It indicated that activated caspase-3 is required for DNA fragmentation of cells undergoing apoptosis. Markedly increased the expression of activated caspase-3 protein may help to explain ASA-induced formation of DNA ladder in this

experiment.

Upregulation of p53 signaling by ASA

To investigate the kinetic of p53 protein induced by ASA, we detected the expression of p53 protein in time- (3, 12, and 24 hours) and dose- (0, 0.5, 1, 2, 4 mM) dependent designs. Results of immunoblotting analysis of p53 protein showed that cells treated with ASA increased the accumulation of p53 protein (Fig 2A). In response to most stressors, the wild type p53 protein level increases rapidly within 1 to 12 hours after treatment with UV or ionizing radiation [21]. Moreover, the expression of phospho-p53 (p-p53), which phosphorylated at serine 15 ($pSer^{15}$), raised as expected (Fig. 2B), it in turn upregulates its downstream target genes; a key inhibitor for cell cycle progression, $p21^{WAF1/CIP1}$ and a pro-apoptotic member of the Bcl-2 family, Bax (Fig. 2B).

In an independent experiment, we decreased the concentration of ASA used to avoid the acidic effect of ASA. The concentration range (10^{-1}-10^{-4} mM) is consistent with the plasma salicylate concentrations in individuals taking therapeutic doses of aspirin or sodium salicylate [22]. Cells were treated with various concentrations (0, 0.01, 0.1 and 1 mM) for 24 hours. The expression of p53, p21 and Bax also increased (Fig. 2C).

The expression of COX-2 inhibited by ASA

In this experiment, the expression of COX-2 significantly decreased with increasing ASA concentrations (Fig. 2D). This result is agreed with previous study which suggested that salicylate exerts its anti-inflammatory action in part by suppressing COX-2 induction, thereby reducing the synthesis of prostaglandins [22].

ERK 1/2 plays a negative role in p53 regulation

ERKs are activated in response to growth factors and play a role in mitosis and differentiation. The effect of NSAIDs on activation of ERK1/2 is controversy. Treatment of HT-29 colorectal carcinoma cells with NS-398 caused activation of ERK1/2 [23]. However, sodium salicylate, metabolite of aspirin, blocked the phosphorylation of p42/p44 MAPK [24- 25]. Interestingly, the expression of phospho-ERK (pERK) induced after treated with ASA for 3 hours, then gradually down to the level of control at 12 hours and below the level of control at 24 hours in this study (Fig. 3A). To investigate the role of MAP kinase played in p53 signaling induced by ASA. A specific inhibitor of MEK (MAPK or ERK kinase), PD98059 (Calbiochem), was used in this study. The expression of p53 and p53 -$pSer^{15}$ upregulation by ASA was markedly enhanced when ERK1/2 activation was suppressed by 10 μM PD98059 for 3 hours (Fig. 3B) and 24 hours (Fig. , 3C), thus indicating a negative role for ERK1/2 in p53 signaling induced by ASA. The expression of other components of MAPKs, JNK and p38, can not be detected in this

study (data not shown). Overexpression of p53 implied that cells leaded to cell cycle arrest and induction of apoptosis. To confirm the effect of PD98059 on cell viability, cells were treated with 10 μM PD98059 for days 1, 2, 3 and 4. PD98059 alone decreased cell viability from ~0.89, ~0.69, ~0.61 to ~0.52. In the meanwhile, ASA (1 mM) coordination with PD98059 (10 μM) reduced cell viability from ~0.89, ~0.70, ~0.59 to ~0.43 compared with the level of control (data not shown).

ASA blocks p53-MDM2 complex formation

Murine double minute 2 (MDM2) acts as a master regulator of the p53 tumor suppressor protein. MDM2 and p53 are involved in a negative feedback loop. We found that ASA decreased the expression of p53-MDM2 complex and increased the expression of 60 kd MDM2 fragment early at 3 hours and 24 hours (Fig. 4). Previous studies indicated that MDM2 oncoprotein, encode a 90 kd protein, is cleaved by caspase-3 (CPP-32) during apoptosis and generating a 60 kd fragment [26-27]. In this aspect, increased the expression of 60 kD MDM2 fragment may contribute by overexpression of activated caspase-3 in this experiment, at least in part (Fig. 1C).

Effect of ASA on the cell cycle phase distribution

To identify the effect of ASA on p53 signaling, we studied it on cell cycle phase distribution using FACS analysis. The result showed that ASA increased the proportion of cells in G0/G1 phase, decreased the proportion of cells in G2/M phase and induced apoptosis in a dose-dependent manner with a typical subdiploid peak on the histogram (Fig. 5). Furthermore, cells treated with PD98059 (10 μ M) for 24 hours showed apoptosis and cell cycle arrest at G0/G1 phase (Fig. 5). It well known that p53 transduces the DNA damage signals to its downstream genes, p21 and bax, and induces the expression of those genes. This in turn, arrests the cell cycle at G1 phase and results in apoptosis.

Discussion

Phosphorylation at several different serine residues in p53 had been shown to occur after cells exposed to DNA damage agents. The expression of p53 phosphorylated p53 at serine 15 ($pSer^{15}$) and serine 392 ($pSer^{392}$) were detected. However, no signal can be detected at p53-$pSer^{392}$ in this experiment. Clearly, ASA induces p53 phosphorelated at serine 15 other than serine 392. Recent reports suggest that phosphorylation of serine 392 may be important for p53 oligomerization [28-29]. While, serine 15 and 37 become phosphorylated after DNA damage and that this phosphorylation reduces MDM2 biding to p53 [30-31].

The function and stability of the tumor suppressor p53 are tightly controlled by the negative regulator MDM2, which binds to p53, blocking DNA binding and targeting p53 for proteosome-mediated degradation. Pochampally and coworkers reported that

human tumor cell lines often expression high levels of a 60 kd MDM2 isoform in the absence of apoptosis [32]. This is help to explain why the OC2 cells used in this study expressed high levels of 60 kd MDM2 fragment. The 60 kd fragment is a product of caspase cleavage of full length MDM2 between residue 361 and 362 [27]. Whether the 60 kd MDM2 fragment functions in regulation of p53 remained unclear. Previous study indicated that cleavage of MDM2 by CPP32-like proteases may result in losing the ability to promote p53 degradation [26]. On the contrary, others suggested that p53 binding and inhibition functions of MDM2 are not affected by the cleavage [27, 32].

COX-2 is an isoform of cyclooxygenase that increased in response to growth factors, cytokines and other mitogenic stimuli. Xu and colleagues suggested that ASA blocked COX-2 mRNA and protein levels [22]. In our study, the expression of COX-2 protein decreased in a dose dependent manner after treatment with ASA. It seems plausible that salicylate exerts its anti-inflammatory action by suppressing COX-2 induction. However, a variety of research proposed that the effect of NSAIDs on cellular signal transduction pathways other than those involving prostaglandins, like effect on nuclear factor kappaB (NFκB) activity [33], regulation of mitogen-activated protein kinases (MAPKs) [24, 34] and regulation of p53 signaling [13, 35]. Here, we present evidences that ASA can induce apoptosis via p53-dependent pathway and inhibits p42/p44 MAPK activity enhanced the accumulation of p53.

Conclusion

NSAIDs inhibit cyclooxygenase may not the only or even the most important mechanism of inhibition. Our study presents evidences that activation of p53 signaling involved in apoptosis induced by ASA. Furthermore, the apoptotic effect was enhanced by blocking the activation of p42/p44 MAPK in response to treatment with ASA, thus indicating a negative role for p42/p44 MAPK.

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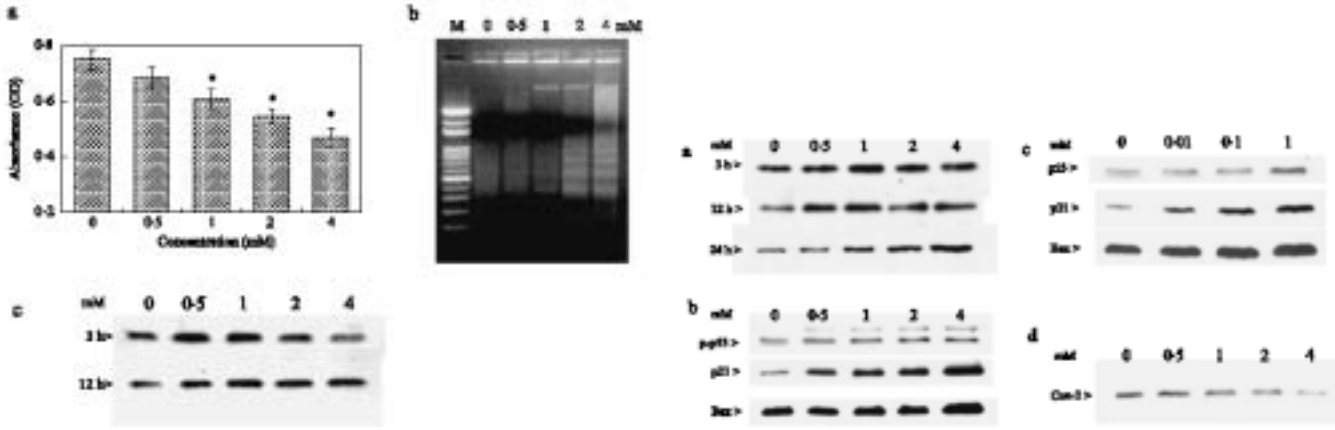

Fig. 1. Effect of aspirin on cell proliferation and apoptosis. OC2 cells were treatment with various concentrations (0, 0.5, 1, 2, 4 mM) of aspirin for 24 hours in serum-free medium.
(A) Cell viability was measured by MTT assay as described in Materials and methods. The values are expressed as means ± s.d. from four independent experiments ($p<0.05$). (B) The formation of oligonucleosomal fragments was determined by 2% agarose gel electrophoresis. Lane M: 100 bp DNA ladder as the size marker. (C) OC2 cells were exposed to aspirin for 3 and 12 hours, the expressions of activated caspase-3 protein were determined by Western blot. Experiments were performed three times and representative blot is shown.
Fig 2. Effect of aspirin on activation of p53 signaling and expression of COX-2. OC2 cells were treated with aspirin for 3, 12 (A) and 24 hours in serum-free medium. (A) The kinetic of p53 in response to aspirin. The expression of p53 induced early at 3 hours. (B) The expression of phospho-p53 (p-p53) was determined by an antibody reacted with p53 which phosphorylated at serine 15. (C) We decreased the concentrations of aspirin used to avoid the acidic effect. The expression of p53 and its downstream genes, p21 and Bax, induced by aspirin. (D) The expression of COX-2 was suppressed by aspirin. Experiments

were performed three times and representative blot is shown.

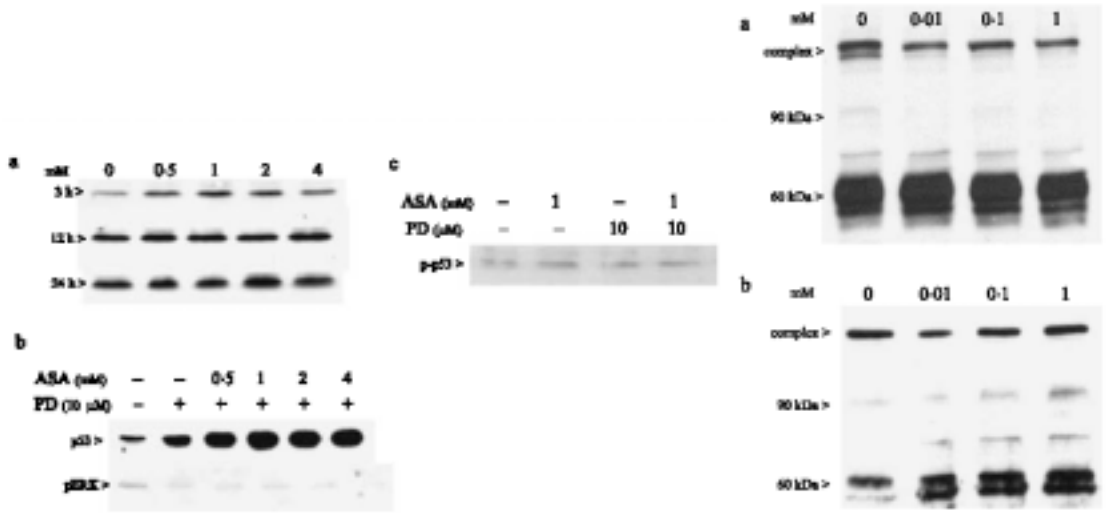

Fig. 3. The activation of p42/p44 MAPK in p53 signaling induced by aspirin. OC2 cells were treated with ASA in serum-free medium. (A) Aspirin induced the activation of ERK 1/2 early at 3 hours, then gradually down below to the levels of control at 24 hours. (B and C) PD98059, a specific inhibitor of ERK kinase, was used in this study to investigate the role of p42/p44 MAPK played in p53 signaling induced by aspirin. Cells were exposed to aspirin and/or PD98059 for 3 (B) and 24 (C) hours. Experiments were performed three times and representative blot is shown.

Fig. 4. Disruption of p53-MDM2 complex in response to aspirin. OC2 cells were treatment with aspirin for 3 (A) and 24 (B) hours in serum-free medium. The antibody used in this experiment can react with p53-MDM2 complex, 90 kd intact MDM2 and 60 kd fragment. Experiments were performed three times and representative blot is shown.

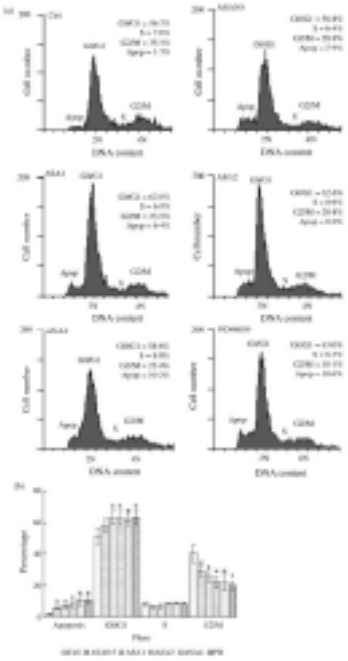

Fig. 5. The cell cycle phase distribution of OC2 cells treated with aspirin. Cells were treated with aspirin for 24 hours in serum-free medium and the DNA content was determined by FACS, as described in Materials and methods. (A) DNA histogram. (B) The cell cycle phase distribution of OC2 cells treated with aspirin and PD98059. The cells distributed in each cycle were determined by flow cytometry. The apoptotic cells observed on a DNA histogram as a subdiploid peak. The data are expressed as means ± s.d. from three independent experiments ($p<0.05$).