Peplomycin induces G_1-phase specific apoptosis in liver carcinoma cell line Bel-7402 involving G_2-phase arrest[1]

Hong-ying CHEN[2], Cong-yi ZHENG[2], Guo-lin ZOU[2], Da-xing XIE[3], Jian-ping GONG[3]

[2]College of Life Sciences, Wuhan University, Wuhan 430072; [3]Cancer Research Institute, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430072, China

KEY WORDS peplomycin; Bel-7402 cells; apoptosis; cyclins; cell cycle; flow cytometry

ABSTRACT

AIM: To investigate the mechanism of peplomycin (PEP)-induced apoptosis in liver carcinoma cell line (Bel-7402). **METHODS:** Growth inhibition by PEP was analyzed using 3- 4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptotic cells were detected using Hoechest 33258 staining, and confirmed by flow cytometric analysis and DNA fragmentation analysis. The expression of cyclin A and B1 were determined by flow cytometry and Western blot. Annexin V assay was measured by flow cytometric analysis. **RESULTS:** PEP induced apoptosis and then inhibited cell proliferation in liver carcinoma cell line Bel-7402. Cells treated with PEP 50 μmol/L for 15 h were arrested in G2-phase with dramatical expression of cyclin A and a little change in cyclin B1. Almost all the apoptosis occurred in cells undergoing the G_1-phase after treatment for 24 h. **CONCLUSION:** Peplomycin induced G_1-phase specific apoptosis in Bel-7402 involving G_2-phase arrest.

INTRODUCTION

Apoptosis is an important genetically encoded cell death program that differs from necrosis in eukaryotic cells. The morphological changes associated with necrosis are cell swelling, rupture of membranes, and lysis of organized structure. In contrast, during the process of apoptosis, condensation and fragmentation of the cytoplasm and nucleus occur in the target cells while normal organelle structure is maintained. Mechani-stically, necrosis results from loss of lysosomal enzymes at a late stage. During apoptosis, internucleosomal DNA digestion is caused by the activation of an endogenous endonuclease, which is proposed to play a key role in apoptosis[1].

It has been repeatedly demonstrated that apoptosis is the mode of tumor cell death occurring as the result of radio- or chemotherapy[2,3]. Since activation and execution of apoptosis are regulated by complex molecular machineries, there are numerous points of interaction between the regulatory pathway of the cell cycle and apoptosis[4]. Most antitumor drugs that induce apoptosis target the cell cycle-specific events, so analysis of apoptosis with respect to the cell cycle position is of particular interest. This information is very helpful in designing treatment protocols and understanding the mechanism of action of antitumor drugs. Classical studies on the cell cycle phase specificity of antitumor drugs had been done by exposing cultured cells, synchronized

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[2] Correspondence to Prof Guo-lin ZOU.
Phn 86-27-8764-5674. Fax 86-27-8766-9560.
E-mail zouguolin@whu.edu.cn
[3] Co-Correspondence to Prof Jian-ping GONG.
Phn 86-27-8766-3301. Fax 86-27-8766-3302.
E-mail jpgong@tjh.tjmu.edu.cn
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at particular phases of the cell cycle, to the drug and studying their subsequent viability or reproductive capacity[5]. While these studies provided valuable evidence of the cell cycle phase sensitivity of cells to different drugs, they did not reveal the mode of cell death (eg, apoptosis or necrosis), or the actual phase of the cell cycle in which the cells were dying. During the past decade various of methods for analysis of apoptosis have been developed[6]. The methods utilizing flow cytometry are the most widely used in detection of apoptosis.

Peplomycin (PEP: 3-[(S)-1′-phenylethylamino] propylaminobleomycin), a new biosynthetic bleomycin (BLM)[7], has been used clinically in Japan since 1981. It shows less pulmonary toxicity and a more potent antitumor activity than bleomycin[8]. Both PEP and BLM belong to a class of important clinical anticancer drugs that interact with DNA to exert their biological activities. These reactions are mediated by metal ions with an intriguing mode of action[9]. It has been reported recently that PEP induced apoptosis in oral squamous carcinoma cells[10]. It is not known, however, whether PEP induces apoptosis in liver carcinoma cell line, and the mechanism by which it occurs. Our present work aimed at identifying the mode of cell death and pinpointing the phase of the cell cycle in which cells enter the apoptotic pathway.

MATERIALS AND METHODS

Materials Liver carcinoma cell line (Bel-7402) was obtained from China Center for Typical Culture Collection. Peplomycin was purchased from Taihe Medical Factory (Tianjing, China). Stock solution (10 mmol/L) were prepared in distilled water and added to the culture media to obtain the appropriate concentrations. Me_2SO, MTT, Hoechst 33258, PI, RNAse A, Triton X-100, PBS, digitonin, and proteinase K were obtained from Sigma Chemical Co (St Louis, USA). FITC-Annexin V, mouse anti-human cyclin B_1 and cyclin A, anti-mouse HRP polymer secondary antibody, and BCIP/NBT substrate kit were provided by PharMingen (San Diego, CA, USA). PVDF membrane was obtained from Bio-Rad (CA, USA).

Cell culture and treatment Cells were cultured in RPMI-1640 supplemented with 10 % fetal calf serum, 1 % (w/v) glutamine, 100 kU/L benzylpenicillin and 100 mg/L streptomycin. All media and serum were obtained from Gibco (Grand Island, NY, USA). For MTT assay the exponential cells were treated with PEP 1, 5, 10, 50, 100, 150, and 200 μmol/L for 24 h in RPMI-1640. For the DNA electrophoresis assay, the cells were treated with PEP 50, 100, and 200 μmol/L at 37 ºC for 42 h. For DNA content and cell cycle analysis, cells were treated with PEP 50 μmol/L for 9, 15, and 24 h. And for analysis of cyclin B1 and cyclin A, cells were treated with PEP 50 μmol/L for 9 and 15 h.

Cell growth inhibition assay The cytotoxicity of the pepleomycin was determined by a colorimetric MTT assay. Cells in the logarithmic growth phase were seeded in a 96-well plate and treated with different concentrations of the drug at 37 ºC for 48 h. Then 10 μL MTT (5 g/L) was added into each well of the plate and cells were incubated at 37 ºC for an additional 4 h. To dissolve formazan, Me_2SO 100 μL was added to all wells and mixed thoroughly at 25 ºC for 10 min. The absorbance values of each well at 540 nm were read using an ELISA reader (Molecular Device, Menlo Park, USA). Control wells contained medium alone. Cell survival rate was calculated as the percentage of MTT inhibition as follows: % survival=(mean experimental absorbance/mean control absorbance)×100

Cell morphology The method was according to Zheng *et al*[11]. Cells were collected, washed with PBS, fixed by methanol-acetic acid (3:1, v:v) at 25 ºC for 10 min. Following centrifugation, the samples were seeded separately onto a cover slides, stained with Hoechest 33258 dissolved in Hanks' buffer in the dark for 40 min. Then the cover slides were washed with distilled water three times and dried in the dark at 25 ºC. By placing a drop of mounting medium on the cover slide, the samples were observed on Olympus BH-2 fluorescence microscope (Japan).

DNA content and cell cycle analysis Untreated and treated cells were collected, after cultured in the presence or absence of PEP for the indicated time, rinsed with PBS and suspended in 75 % ethanol at -20 ºC overnight. Fixed cells were centrifuged at 1200×*g* and washed with PBS twice. For detecting DNA content, cells were contained in the dark with PI 50 mg/L and 0.1 % RNAse A in 400 μL PBS at 25 ºC for 30 min. Stained cells were analyzed on FACSort (Becton Dickinson). The percentage of apoptotic cells was determined using the CellQuest software program.

DNA electrophoresis Total cellular DNA was isolated from untreated and treated cells. Cells were lysed in a solution containing Tris-HCL (pH 7.8) 10 mmol/L, EDTA 1 mmol/L, NaCl 10 mmol/L, 1 % (w/v) SDS and

proteinase K 1g/L at 60 ºC for 2 h. The lysate was extracted twice with phenol-choloroform (1:1, v/v), and followed by precipitation with ethanol at -20 ºC overnight. The precipitation was washed once in 70 % ethanol and resuspended in 100 μL TE (EDTA 1 mmol/L, Tris-HCl 10 mmol/L, pH 8.0) and 10 μL (1 g/L)RNAse at 37 ºC for 30 min. DNA was electrophoresed for 3 h at 60 V in 2.0 % agarose gels containing Ethdum Bromide (EB). PCR marker was used as DNA size marker. The gels were photographed under UV light.

Flow cytometry for cyclin A and cyclin B1 Cells were washed with PBS and suspended in 75 % ethanol at -20 ºC overnight[12]. Fixed cells were centrifuged at 1200×*g*, washed with PBS and treated with 0.25 % Triton X-100 in PBS for 5 min. After the addition of PBS and centrifugation, the samples were incubated with mouse mAb to human cyclin B_1 and cyclin A at 4 ºC overnight. The antibody was diluted in PBS containing 1 % BSA and applied at a ratio of 0.25 μg mAb/5×10^5 cells. After washed with 1 % BSA, the cells were incubated in the dark with FITC-conjugated goat anti-mouse IgG for 30 min. After being washed with 1 % BSA again, the cells were stained with PI 50 mg/L and 0.1 % RNAse A in 400 μL PBS for 30 min. Cyclin detection was performed with a FACSort flow cytometry.

Western blotting for cyclin A and cyclin B1 The cells were washed twice with PBS. The washed cells were lysed in 2×SDS sample buffer and boiled for 5 min, then the lysates were centrifuged at 1800×*g* at 4 ºC for 15 min. The protein concentration was determined by Bradford's method[13] using bovine serum albumin as a standard. The supernatants were used for a Western blot analysis. Equivalent protein extract (50 μL) from each sample was subjected to electrophoresis using 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then the proteins were transferred to PVDF membranes at a constant current of 320 mA for 80 min. The membranes were blocked with 5 % non-fat milk in TBST (Tris-HCl 10 mmol/L, pH 8.0, NaCl 150 mmol/L, Tween-20 0.15 %) at 25 ºC for 1 h. They were subsequently incubated at 4 ºC overnight with the primary monoclonal antibodies anti-cyclinB1, anti-cyclin A. All primary antibodies (in TBST plus 5 % non-fat milk) were used at 1 mg/L. After the membranes were washed three times with TBST, they were incubated at 25 ºC for 1 h with anti-mouse HRP polymer secondary antibody at a 1:2000 dilution in TBST (plus 5 % non-fat- milk). After being washed the membrane with TBST, the protein bands on the membranes were detected with the BCIP/NBT system.

Annexin V assay According to universal method with modifications[14], the cells were collected and washed with PBS, followed by being resuspended in binding buffer (HEPES-NaOH 10 mmol/L, pH 7.4, NaCl 140 mmol/L, $CaCl_2$ 2.5 mmol/L). The samples were incubated with 5 μL Annexin V in dark for 15 min, washed with binding buffer and resuspended in 1% formaldehyde in the binding buffer at 4 ºC for 30 min. After washed with binding buffer again, the cells were stained with 500 μL PI in PIPES containing digitonin for 15 min then measured on FACSort flow cytometry.

Statistical analysis All of the assays were set up in triplicate. The results were expressed as mean±SD. Statistical analysis was determined by *t*-test.

RESULTS

MTT assay We examined the cytotoxicity effect of PEP on the Bel-7402 by treating with different concentrations of PEP for 48 h. The experiments clearly showed that PEP induced cytotoxicity in the Bel-7402 cell line in a concentration-dependent manner (Fig 1).

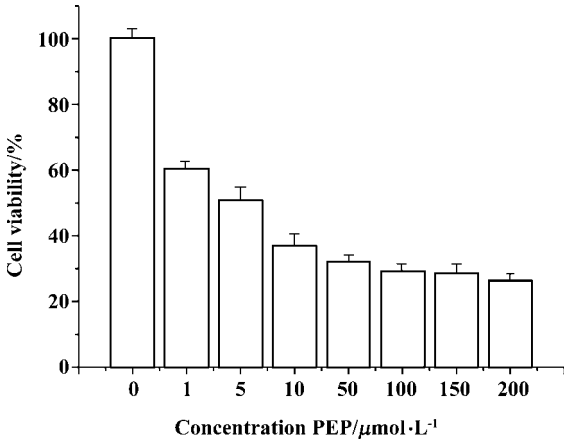

Fig 1. Cytotoxic effects of various concentration PEP on Bel-7402 cells for 48 h. The activity was compared to the control well of the same cell lines and the results were presented as mean±SD for triplicate.

Hoechest 33258 staining We used Hoechest 33258 to investigate the changes in the nucleus of cells, and lots of apoptotic bodies containing nuclear fragments were found in PEP-treated cells but none in untreated cells. At the same time, cytoplasmic shrinkage was observed in cells cultured with PEP (Fig 2).

DNA electrophoresis When the supernatants

were extracted from treated cells and examined by 2.0 % agarose-gel electrophoresis, "DNA laddering" was detected, indicating the presence of double-strand breaks (Fig 3). No fragment was detected in cells incubated in the absence of PEP. Increased DNA laddering was observed with higher doses of PEP. This result showed that DNA fragmentation was dose-dependent.

DNA content and cell cycle analysis Fig 4 illustrated changes in DNA content distribution in Bel-7402 treated with PEP 50 μmol/L for 9, 15, and 24 h. As the treatment time increases, the percentage of cells in sub-G_1 increased accordingly. Sub-G_1 and G_2/M portions were higher in the PEP-treated group than in the untreated group, 14.6 % *vs* 1.9 % for sub-G_1 and 48.2 % *vs* 18.4 % for G_2/M, whereas the G_1-phase and the S-phase were lower in the PEP-treated group than in the untreated group. These results illustrated that PEP arrested the cells at G_2/M phase *in vitro*.

Cyclin A and cyclin B1 analysis Cyclin A is expressed in late S and G_2 phase and degraded during mitosis just prior to metaphase. Another "mitosis" cyclin B1 is expressed and degraded later[15]. Based on the difference in time of expression of cyclin A *vs* cyclin B1 it was possible to discriminate between G_2 and mitotic cells by multiparameter (cellular DNA content versus cyclin expression) flow cytometry. Therefore, we used both flow cytometry and Western blot to examine cyclin B1 and cyclin A levels in cells treated with PEP 50 μmol/L for 9 and 15 h. We got the same results from those two experiments (Fig 5). The level of cyclin A in the cells treated with PEP was much higher than that of the control cells, while the level of cyclin B1 in the cells treated with PEP had a little change compared with the control cells. As to the amount of the cyclin A and B1 in treated cells at 15 h, the level of cyclin A was much higher than that of cyclin B1. These results indicated that treatment of Bel-7402 cells with PEP led to G_2 arrest which was associated with a dramatic increase in the levels of cyclin A and a little change expression of cyclin B1.

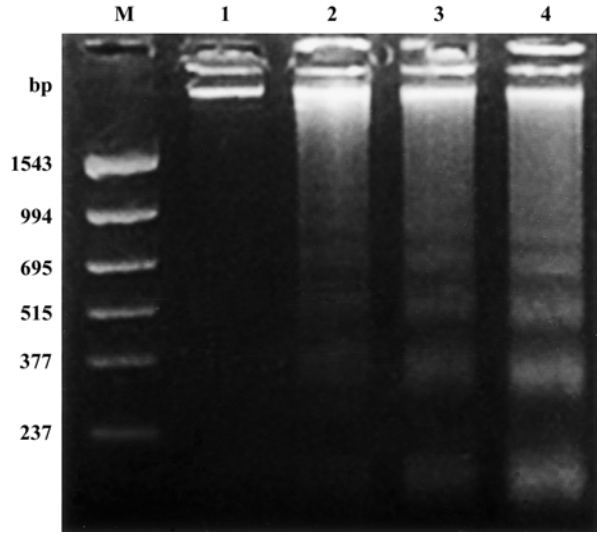

Fig 3. Agarose gel electrophoresis of DNA extracted from Bel-7402 untreated and treated with different concentrations PEP for 42 h. Lane M, PCR marker; Lane 1: untreated; Lane 2: PEP 50 μmol/L treated; Lane 3: 100 μmol/L treated; Lane 4: 200 μmol/L treated.

Annexin V assay All untreated cells were Annexin V negative, which meant no apoptosis happened. In contrast, when Bel-7402 was treated with PEP,

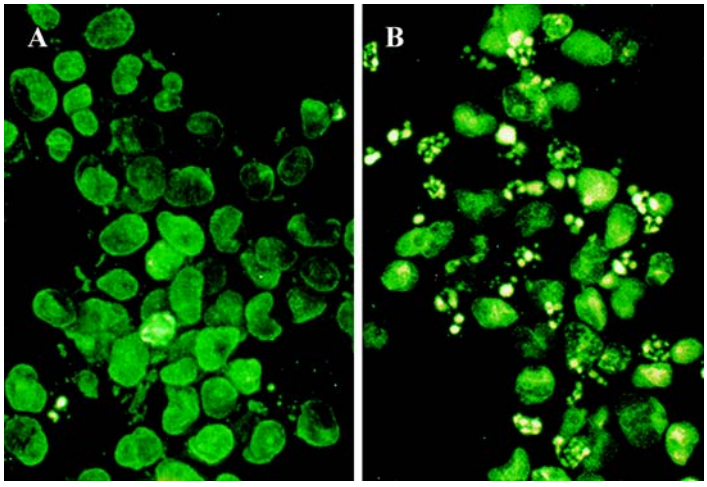

Fig 2. Morphological of Bel-7402 cells, untreated (A) and treated (B) with PEP 50 μmol/L for 24 h. The cells were cytocentrifuged, stained with Hoechst 33258. The photographs were taken under Olympus BH-2 fluorescence microscope (× 40).

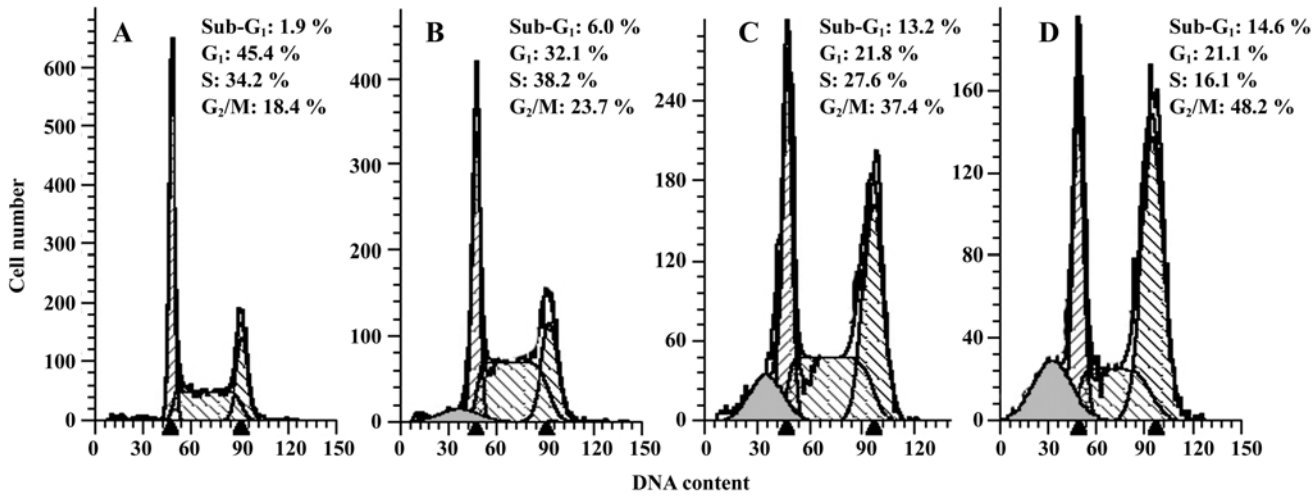

Fig 4. Time-dependent alteration of cell cycle distribution induced by PEP 50 μmol/L. (A) untreated, (B) treated for 9 h, (C) treated for 15 h (D) treated for 24 h. Results are representative of three independent experiments.

apoptotic cells became Annexin V positive and almost all the apoptosis occured in cells undergoing the G_1-phase (Fig 6). It meant that PEP could induce G_1-phase specific apoptosis.

DISCUSSION

In the present paper, we used MTT assay to examine the cytotoxicity effect of PEP on the Bel-7402 cells. Okamura *et al* [10] reported that at concentrations of 5-50 μmol/L, PEP triggered the apoptosis of both oral squamous carcinoma SCCKN and SCCTF cells. The cytotoxicity of Bel-7402 cells was shown at higher PEP concentrations than that of SCCKN cells. We used various methods to detect whether PEP induced apoptosis in Bel-7402 cell lines. Nucleus condensation, apoptotic bodies appearance, and "DNA ladder" are universal characteristic in the cells undergoing apoptosis. Furthermore, apoptotic cells showed a decreased DNA content below the G_0/G_1 level. DNA by cell fixation results in the appearance of cells with a fractional DNA content, which was defined "sub-G_1" peak. From the evidence in our experiments, we concluded that PEP could induce Bel-7402 apoptosis and it was time- and concentration-dependent.

To clarify the mechanism of PEP-induced apoptosis, we conducted further experiments. Accumulated evidence has shown that cell cycle arrest might result in apoptosis due to the existence of cell cycle checkpoints and feedback control[16]. In recent papers, several evidences have suggested that apoptosis may occur via a signaling pathway independent of mitotic arrest[17]. We wanted to detect if PEP–induced apoptosis required cell cycle arrest. After treating cells with PEP 50 μmol/L for various time periods, we used the common cell-cycle analysis that based on cellular DNA content measurement and found that the cell line exhibited G_2/M arrest after exposure to PEP. To discriminate cells that had the same DNA content but reside in different phases of the cycle, we used the bivariate, an analysis of cyclin-associated immunofluorescence versus DNA content of individual cell to correlate expression of these proteins with cell-cycle position. Cyclins, regulatory subunits of their respective cyclin-depentdent kinases (cdks), were the key components of the cell-cycle progression machinery. Growth imbalance can be reflected by the apparent changes in the expression of cyclins, such as cyclin E, B1, A and their synthesis and degradation being strictly scheduled[18]. Cells in G_2 phase had a very high level of cyclin A and a little change expression of cyclin B1[15]. The result of immunocytometry was coincided cell cycle analysis which revealed PEP arrested Bel-7402 cells on G_2 phase. And the results from the Western blot also confirmed this conclusion.

Annexin V-FITC is a sensitive probe for identifying apoptotic cells. This assay has been found to detect apoptotic cells significantly earlier than the DNA-based assay. Annexin V-FITC stains cells in various phases of apoptosis ranging from the early phase, in which no morphological changes of the nucleus are detected on the EM level, to the late phase with py-

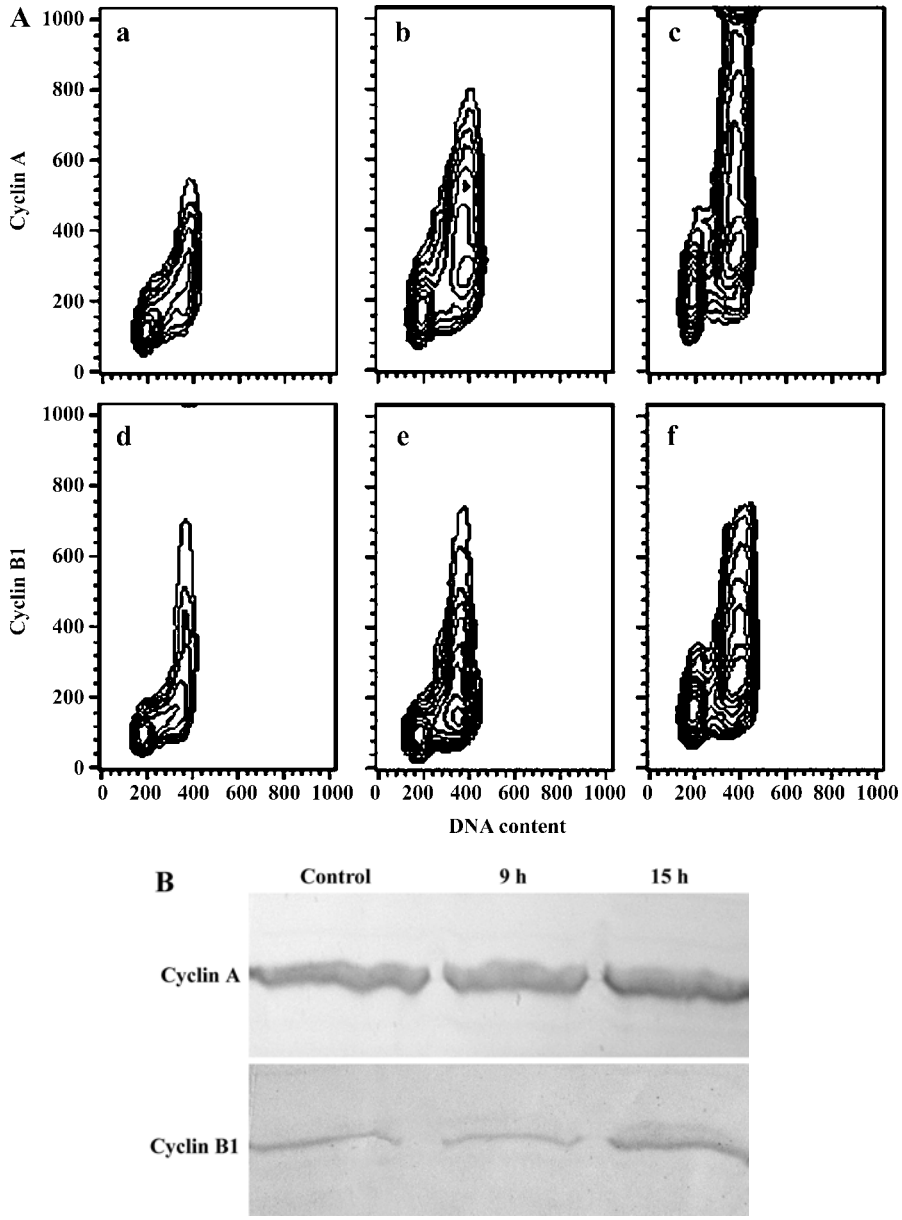

Fig 5. Effect of PEP 50 μmol/L treatment on cyclin A and Cylin B1 levels. (A) represents the results of flow cytometric. (a)-(c) Cyclin A expression:(a) untreated, (b) treated for 9 h, (c) treated for15 h. (d)-(f) Cyclin B1 expression: (d) untreated, (e) treated for 9 h, (f) treated for 15 h. (B) represents the results of Western blot. Results were from one representative experiment of three.

knotic nucleus and condensed cytoplasm. For the reasons that remain to be elucidated, Annexin V preferably binds to apoptotic cells even in the conditions of excess necrosis. However, only Annexin V labeling does not provide information of the cell cycle specificity of apoptosis. Annexin V/PI labeling can discriminate between apoptosis and necrosis and pinpoint the special phase of the cell cycle in which cells undergo apoptosis. The result from this study showed that Annexin V assay positive cells were cells in G_1 phase. It is reported that the apoptotic-signaling pathway is related to the arrested phase of the cell cycle[19]. In this study, the Bel-7402 cells treated with PEP were arrested in G_2 phase, which was also related to cyclin A and B1 accumulation, and we considered this step was necessary for apoptosis in this mode. This phenomenon could be explained according to Halicka *et al*[20]. Three types of apoptosis could be distinguished in relation to the initial damage to the cell: (1) homo-phase apoptosis, where the cells underwent apoptosis during the same phase in which they were initially affected; (2) homo-cycle apoptosis, where the cells underwent apoptosis

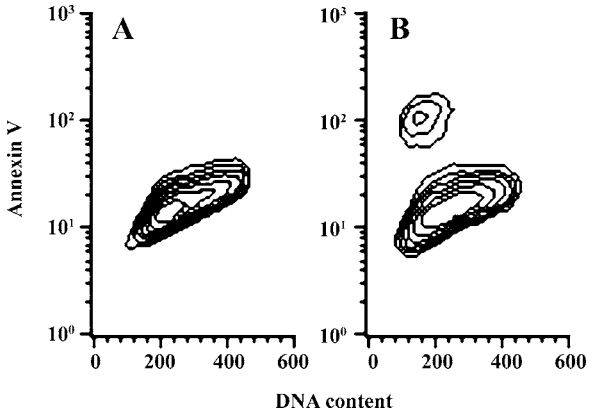

Fig 6. Detection of Annexin V-FITC based on the analysis of DNA content. The data showed the G_1-phase specificity of apoptosis (Annexin V positive) induced by PEP. (A) untreated, (B) treated with PEP 50 μmol/L for 24 h. Results were from one representative experiment of three.

during the same cell cycle in which they were initially affected, ie, prior to or during the first mitosis, and (3) post-mitotic apoptosis, where cells underwent apoptosis during the cell cycle(s) subsequent to that in which the cell was initially affected, most likely at the G_1 or G_2 checkpoints of these cycle (s).

Cell cycle specificity analysis of apoptosis is very important for the study of molecular mechanism of cell death. From all the results of our experiments, it was clear that PEP could induce G_2 arrest and post-mitotic (G_1-phase specific) apoptosis in Bel-7402 cell line.

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