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Adhesion of Beta1 Integrin to Fibronectin Regulates CAM-DR Phenotype via p21$^{WAF1/cip1}$ in HL60 Acute Myeloid Leukemia (AML) Cells

Hande CANPINAR[1]
Güneş ESENDAĞLI[1]
Emin KANSU[1]
Ayşen GÜNEL ÖZCAN[2]
Dicle GÜÇ[1]

[1] Department of Basic Oncology, Institute of Oncology, Hacettepe University, Sihhiye, Ankara - TURKEY

[2] Department of Medical Biology and Genetics, Faculty of Medicine, Kırıkkale University, Kırıkkale - TURKEY

Aims: Drug resistance is a major obstacle for a successful cancer therapy. Cell adhesion mediated drug resistance (CAM-DR) is a novel type of drug resistance and generated via interaction of cancer cells with the microenvironment. In this study, CAM-DR phenotype was analyzed in HL60 acute myeloid leukemia (AML) cells.

Materials and Methods: Fibronectin (FN) adherence of HL60 cells was tested by a colorimetric adhesion assay. Flow cytometry analyses were performed to evaluate doxorubicin-induced apoptosis and to determine cell cycle status. Proliferation rate was evaluated by [^{3}H]-thymidine incorporation assay. Western blot and RT-PCR were used for analysis of the factors involved in cell cycle control.

Results: Binding of HL60 to FN via $\alpha4\beta1$ and $\alpha5\beta1$ integrins exerted a CAM-DR phenotype, which shows resistance to apoptosis triggered by doxorubicin. FN-adherent HL60 cells accumulated in the G_0/G_1 phase of cell cycle and stopped proliferation. However, after detachment from FN, cells entered S phase, proliferated, and became sensitive to apoptosis. The analysis of the factors involved in the G_0/G_1 cell cycle checkpoint showed that CAM-DR phenotype might be regulated mainly by p21$^{waf/cip}$.

Conclusions: Here we showed that CAM-DR may also represent a reversible drug resistance mechanism that decreases apoptosis and causes growth arrest in AML blasts.

Key Words: Apoptosis, CAM-DR, fibronectin, cell cycle

Beta1 İntegrinin Fibronektine Adezyonu HL60 Akut Myeloid Lösemi (AML) Hücrelerinde p21$^{WAF1/cip1}$ Aracılığı ile CAM-DR Fenotipini Düzenler

Genel Bilgiler: İlaç direnci, kanser tedavisinin başarısında büyük zorluktur. Hücre adezyon aracılı ilaç direnci (CAM-DR), kanser hücrelerinin mikroçevre ile ilişkisiyle gelişen, çeşitli tümörlerde gösterilen yeni bir ilaç direncidir. Bu çalışma da, HL60 AML hücrelerinde CAM-DR fenotipi analiz edildi.

Yöntem: HL60 hücrelerin fibronektine bağlanması kolorimetrik adezyon deneyi ile yapıldı. Doksorubisin ile indüklenen apoptozis ve hücre siklusu akım sitometrik analiz ile belirlendi. [^{3}H]-timidin inkorporasyon deneyi ile proliferasyon hızı değerlendirildi. Hücre siklusunu kontrol eden proteinler, western blot ve RT-PCR analizi ile belirlendi.

Bulgular: HL60 hücrelerinin $\alpha4\beta1$ and $\alpha5\beta1$ integrin aracılığı ile fibronektine bağlanmasıyla CAM-DR fenotipi oluşmuştur, aderent HL60 hücreleri doksorubisin ile tetiklenen apoptozise dirençli hale geçmiştir. Fibronektine aderan/yapışan HL60 hücrelerinde hücre siklusunun G_0/G_1 fazında birikimi ile hücre proliferasyonu durmuştur. Buna karşılık, fibronektinden sökülen hücreler 8 saat sonra tekrar sentez fazına girerek apoptozise duyarlı hale gelmiştir. Hücre siklusunun G_0/G_1 kontrol noktalarının analizi, CAM-DR fenotipinin p21$^{waf/cip}$ proteini ile düzenlendiğini göstermiştir.

Sonuç: Bu çalışmada, CAM-DR fenotipininin AML blastlarında apoptozisi azaltan ve proliferasyonu durduran geri dönüşümlü bir ilaç direnci mekanizması olabileceğini gösterdik.

Anahtar Sözcükler: Apoptozis, CAM-DR, Fibronektin, Hücre Siklusu

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Correspondence
Hande CANPINAR
Department of Basic Oncology, Institute of Oncology, Hacettepe University, Ankara - TURKEY

handecan@ada.net.tr

Introduction

Acute myeloid leukemia (AML) represents a group of clonal hematopoietic stem cell disorders that result in accumulation of non-functional cells in the stem cell compartment (1). Although chemotherapy may lead to an initial tumor response in up to 85% of AML patients, clinical remission is often followed by a relapse made up of chemo-resistant tumor cells (2). Classical multi-drug resistance (MDR) has been

identified as an important cause of refractory disease in AML (3). It has also been demonstrated that environmental factors contribute to the survival of tumor cells after initial therapy (4,5) and influence the response to chemotherapeutic agents (6). Leukemia cells could become less sensitive to radiation exposure upon contact with the microenvironment (7). Recently, a new phenomenon was described in myeloma cells and termed as cell adhesion mediated drug resistance (CAM-DR). Adhesion of myeloma cells to the extracellular matrix component fibronectin (FN) is proposed as an important determinant of drug response and contributes to the survival of tumor cells (8).

Cell adhesion to FN is mediated by integrins, which are heterodimeric cell surface glycoproteins. They are composed of an α-subunit and a β-chain non-covalently linked to each other. Integrins are reported to modulate cellular processes such as activation, proliferation, and apoptosis (9). The behavior of tumor cells is also strongly influenced by FN-integrin interactions (10,11). In particular, apoptotic response triggered by DNA damaging drugs, anthracyclines and alkylating agents is reduced in cells of CAM-DR phenotype and therefore they become resistant. Furthermore, the activation of cell signalling pathways that increase the expression of cell cycle control proteins may increase survival of multiple myeloma cells, which results in drug resistance (12).

Besides the classical MDR and MDR-related resistance mechanisms, CAM-DR phenotype observed in multiple myeloma may also play an important role in other hematologic malignancies such as AML. In the present study, we aimed to investigate the presence of CAM-DR phenotype in HL60 AML cell line and to evaluate its effect on drug-induced apoptosis and cell cycle regulation.

Materials and Methods

Cell Lines and Culture Conditions

Human acute promyelocytic cell line HL60, human chronic myelogenous leukemia cell line K562 and human acute myeloblastic leukemia cell line KG1 were obtained from the American Type Culture Collection (USA). Cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) (Biochrom, Germany), 1% penicillin (100 U/ml) and streptomycin (100 U/ml) (Biochrom, Germany) at 37 °C in a humidified atmosphere with 5% CO_2, and were passaged twice weekly.

Antibodies and Other Reagents

Doxorubicin HCL (Applichem, Germany) was dissolved in ddH_2O. Blocking integrin antibodies α4 (P4G9, Dako), α5 (P1D6, Dako), β1 (MAB1987Z, Chemicon, USA), isotypic mouse IgG1 (X0931, Dako), isotypic mouse IgG3 (MAB1311, Chemicon, USA), and human plasma FN (33016-015 Invitrogen, USA), were used in cell adhesion assays. Monoclonal antibody p27 (sc-1641, Santa Cruz, USA), p21 (Santa Cruz, sc-817, USA), cyclin A (sc-751, Santa Cruz, USA), and cyclin E (sc-198, USA), were used in Western blot analysis. Flow cytometric analysis was performed by using Annexin V FITC kit (IM 2375, Immunotech) and anti bromodeoxyuridine (BrdU)- FITC antibody (F7210, Dako).

Cell Adhesion Assay

Cell adhesion assay was performed as described previously (13). Briefly, 96 well plates (Nunc, Denmark) were coated with either 50 µl (40 µg/ml) FN or bovine serum albumin (BSA) (10 mg/ml) and allowed to evaporate overnight. Cells were washed in serum free medium, resuspended (10^6 cells/ml), and incubated for 30 min with α4, α5, and β1 blocking antibodies and isotypic controls. After 2 h of adhesion, unattached cells were removed by serum free RPMI medium. Adherent cells were fixed in 70% methanol and stained with 2% crystal violet. After solubilization of the adherent cells with Sorenson solution, the absorbance at 540 nm was read by microplate reader (Spectra Max, Molecular Devices, UK). Experiments were repeated three times.

Proliferation Assay

The effects of FN adhesion on DNA synthesis were measured by ^{3}H thymidine incorporation assay. 96 well plates were coated with FN as described in cell adhesion assay. Fifty thousand cells per well were added to FN coated wells for 24 h. Suspension cells were also incubated in serum free medium for 24 h. Then, cells were incubated with ^{3}H thymidine (1 µCi/well) for 3 h and DNA was collected on a filter paper using a cell harvester. Incorporated [^{3}H]-thymidine was detected by beta counter (Packard, USA). Each experiment was performed in quadruplicate.

Analysis of Cell Cycle

Different phases of the cell cycle were analyzed by two-color flow cytometry method (14). S phase was detected by anti-BrdU FITC conjugated antibody (Dako, Denmark), whereas G_0/G_1 and G_2/M phases were identified by propidium iodide (PI). FN-adherent cells and suspension cells were incubated with 30 µg/ml BrdU for 1 h in a CO_2 incubator at 37 °C. Unattached cells were removed by gentle washes with RPMI 1640 medium, and cells were incubated with 5 mM EDTA in phosphate buffered saline (PBS) in order to remove adherent cells. Cells were added drop wise into 5 ml of –20 °C cold 70% ethanol and incubated on ice for 30 min and digested with 0.04% pepsin solution containing 0.1% HCl for 1 h at 37 °C. Following the centrifugation and acid denaturation by 2 N HCl solution at 37 °C, samples were washed with sodium borate and the supernatant was aspirated and resuspended in PBS tween (PBST) then incubated in 10 ml anti BrdU FITC antibody for 60 min in the dark. After washing with PBST, cells were resuspended in PBST containing 5 µg/ml PI (10 µg/ml) and 25 µl RNase (10 mg/ml, Boehringer Mannheim, Germany) and 10,000 events were analyzed by flow cytometry (EPICS XL-MCL Beckman Coulter, USA). Experiments were repeated three times.

Analysis of Apoptosis by Annexin V

Cells (5×10^5) were adhered to FN-coated 96 well plates. Nonadherent cells were removed from the plates by washing twice with serum free medium after 2 h of incubation. Cells were allowed to adhere to FN for another 48 h in RPMI medium containing 10% FBS then were exposed 0.02 nM doxorubicin for 1 h, 24 h and 48 h and apoptotic cell population was detected by Annexin V-FITC (Immunotech, Beckman Coulter) staining and analyzed by flow cytometry. In order to analyze the reversible effect of FN adhesion, adherent cells were detached from FN and suspended in 5% FBS containing RPMI 1640 medium. After a further 8 h of incubation of detached cells, the amount of apoptotic cell population was measured as described above. All the assays were performed in quadruplicate.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated by using TriReagent (Sigma, St. Louis, USA) based on the method described by Chomczynski and Sacchi (15). cDNA was synthesized from 2 µg of RNA, using oligo(dT) primers and RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Lithuania) according to manufacturer's instructions. Previously reported primer oligonucleotide sequences and PCR conditions were used to amplify $p21^{waf/cip}$, $p27^{kip1}$ and $p57^{kip2}$ genes (16). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified as house keeping gene. Preliminary reactions had been set to determine PCR cycle number that meets logarithmic amplification phase for each primer pair. Three sequential independent PCR amplifications had been performed for each gene and reactions were terminated at the predetermined cycle. Products were resolved on 1% agarose gels and visualized with ethidium bromide staining under UV light.

Western Blot Analysis

One million cells per ml were plated in FN-coated 24 well plates. Cells were allowed to adhere to FN for 2 h and unattached cells were removed with RPMI medium. Cells were detached, washed with ice-cold PBS and resuspended in 200 µl lysis buffer containing (Tris HCl (50 mM) pH 7.5, NaCl (150 mM), glycerol (10%), Triton X 100 1%), EDTA (2 mM), NaF (100 mM), Na_3VO_4 (1 mM), PMSF (1 mM), leupeptin (10 mg/ml) and 1 mM dithiothreitol. After 30 min on ice, the nuclei were removed by centrifugation at 12,000 g, 4 °C, 15 min. The supernatant was collected and cell extracts were quantified by BCA protein assay (BioRad). Fifty µg of cell lysate was dissolved in Laemnli SDS-PAGE. Aliquots of 25 µl were mixed with 8 µl sample buffer with 0.4% β-mercaptoethanol, heated for 5 min at 99 °C and then separated on 12% SDS –PAGE for detection of the band of interest. Proteins were transferred to polyvinylidene (PVDF) membrane and incubated with 4% BSA in Tris buffered saline+Tween20 overnight at +4 °C. Monoclonal antibodies against p21, p27, cyclin A or cyclin E were used as probe. Detection was performed using horseradish peroxidase-conjugated antibodies (Santa Cruz, USA) and ECL chemiluminescence (Amersham, Oslo, Norway). Experiments were repeated three times.

Statistical Analysis

All values are expressed by arithmetic mean ± standard deviation (SD). Statistical difference between experimental groups was determined using Student's *t*-test where appropriate (GraphPad Instat Dr. Granger, LSU Medical Center, 1993). Differences were regarded as statistically significant when $P \leq 0.05$.

Results

HL60 cells adhere to FN via α4β1 and α5β1 integrins

HL60 cells were pre-incubated with blocking antibodies against α4, α5 and β1 integrins and isotypic controls. As a control, an equal number of cells were added to wells coated with BSA. After 2 h of incubation, HL60 cell line was observed to be adhered to FN. Adhesion of HL60 cells to FN was more than 1.5 fold as compared to control wells. Blocking antibodies against α4, α5 and β1 integrins inhibited adhesion to FN compared to IgG3 and IgG1 isotypic controls, respectively (Figure 1). Adhesion assay was also done with KG1 cell line as a negative control and K562 cell line as positive control. The blocking antibodies that diminished adhesion of HL60 cells also reduced adhesion of K562 cells to levels comparable to those in BSA. In contrast, α4 and α5 or β1 blocking antibodies did not have any effect on cell adhesion to FN in KG1 cell line since this cell line does not express β1 integrin (data not shown). These experiments showed that adhesion of HL60 cells to FN is mediated through α4β1 and α5β1 integrin molecules.

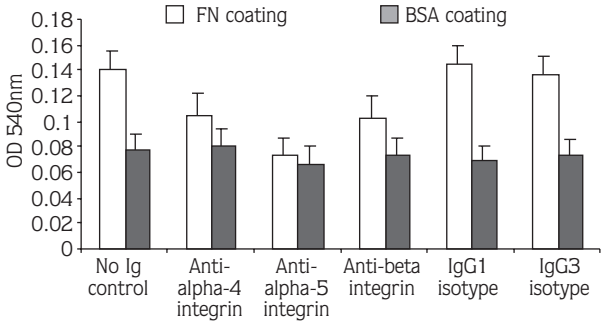

Figure 1. Adhesion of HL60 cells in the presence or absence of integrin blocking antibodies or isotype controls. The presence of the α4 and α5 or β1 blocking antibodies reduced FN adhesion of HL60 cells to levels comparable to those with BSA. In contrast, IgG1 and IgG3 isotype antibodies did not reduce adhesion of HL60 cells.

Adhesion of HL60 cells to FN inhibits drug-induced apoptosis

For the evaluation of *in vitro* chemosensitivity of adherent and suspension HL60 cells to doxorubicin, a MTT assay was used. LD50 value was determined using the dose-response curve preliminarily calculated and further experiments on adherent and suspension cells were performed with 0.02 nM doxorubicin (data not shown). Analysis of 0.02 nM doxorubicin-induced apoptosis was performed by Annexin V staining, which is an early stage apoptotic marker. Percentage of apoptotic cells in FN-adherent cells at different time intervals of doxorubicin incubation was significantly lower than in suspension cells, at 1 h ($P < 0.0001$), 24 h ($P < 0.01$) and 48 h ($P < 0.01$) (Figure 2A).

Detachment of cells from FN and further incubation in the medium without doxorubicin for 8 h induced apoptosis in HL60 cells (Figure 2B). These results indicated that doxorubicin resistance developed by CAM-DR phenotype is reversible. We also confirmed apoptotic changes morphologically by acridine orange staining and by DNA fragmentation assay (data not shown).

CAM-DR phenotype in HL60 cells results in cell cycle arrest

Two-color (BrdU FITC/PI) flow cytometry analysis was performed to examine the effects of FN adhesion on cell cycle. Adhesion of HL60 cells to FN for 24 h resulted in accumulation in the G_0/G_1 phase. According to the cell cycle analysis, 72.3% of the FN-adherent cells and 29.7% of suspension cells were in the G_0/G_1 phase whereas 27.7% of the FN-adherent cells and 58.6% of suspension cells were in S phase (Figure 3A). S phase in FN-adherent cells was significantly decreased ($P < 0.001$, $n = 3$). To this end, CAM-DR phenotype in HL60 cells led to accumulation in the G_0/G_1 phase and to growth arrest. Correspondingly, when the proliferation rate of adherent and suspension HL60 cells was determined, a decreased DNA synthesis was observed in FN0adherent cells ($P < 0.01$, $n = 6$) (Figure 3B).

On the other hand, as HL60 cells were detached from FN, they entered rapidly into the S phase. Therefore, growth arrest in the G_0/G_1 phase induced by FN adherence was reversible (Figure 3C).

Analysis of G_0/G_1 cell cycle checkpoint in HL60 cells with CAM-DR phenotype

The expression of $p21^{waf/cip}$, $p27^{kip1}$, and $p57^{kip2}$ cyclin dependent kinase (CDK) inhibitor molecules involved in the G_0/G_1 cell cycle checkpoint was analyzed in HL60 cells adhered to FN for 24 h or grown in suspension. $p21^{waf/cip}$ and $p27^{kip1}$ gene expression was found to be higher in FN-adherent cells (Figure 4A). There was no $p57^{kip2}$ expression in either group (data not shown). On the other hand, when the Western blot analyses were performed on $p21^{waf/cip}$ and $p27^{kip1}$ molecules, $p21^{waf/cip}$ protein was only

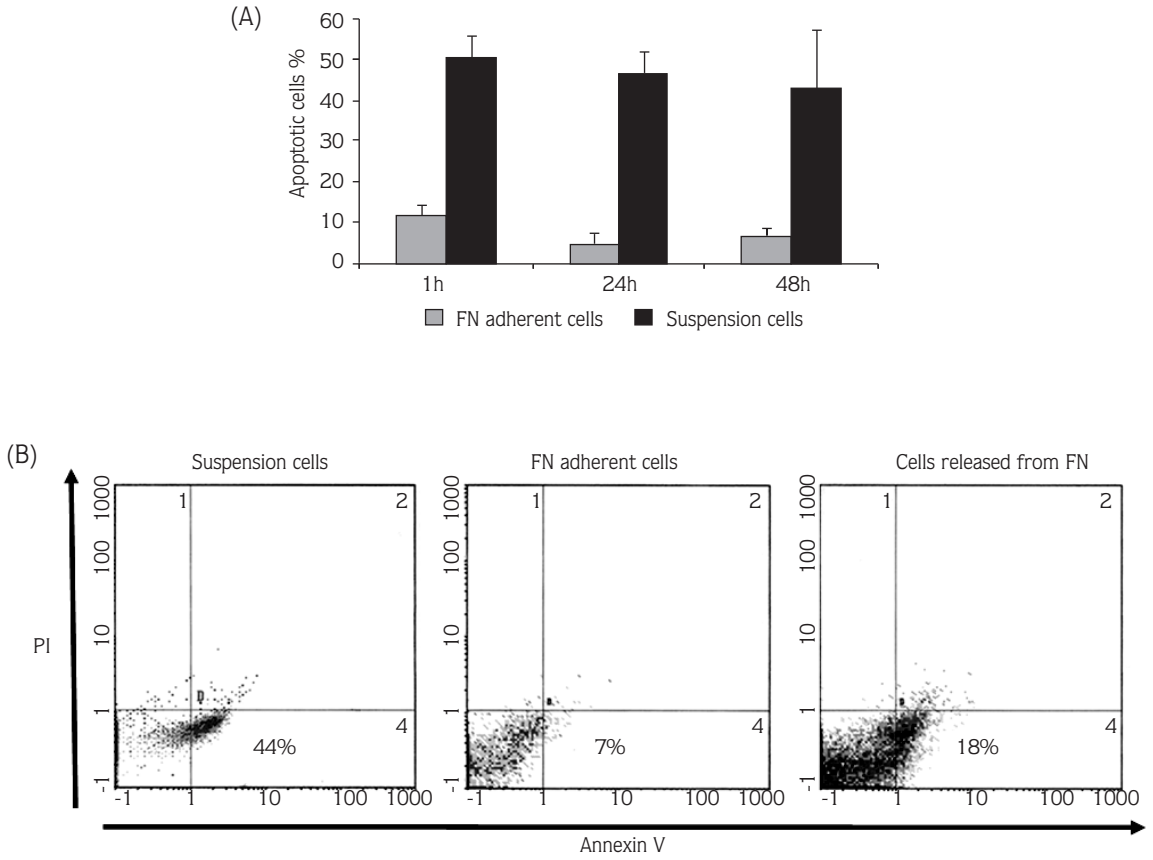

Figure 2. Analysis of doxorubicin-induced apoptosis. A) Flow cytometry results of annexin V staining was significantly low in FN-adherent HL60 cells for 1 h (**P < 0.0001), 24 h, and 48 h (*P < 0.01) compared to cells grown in suspension. B) In order to analyze the reversible effect of FN adhesion, adherent cells were detached from FN and re-suspended and incubated for 8 h in culture medium. Representative flow cytometry results of annexin V-PI staining of suspension cells, FN-adherent cells, and cells detached from FN are shown in the dot-plots. Non-apoptotic (lower left quadrants), early apoptotic (lower right quadrants), and late apoptotic (upper right quadrants) cells can be seen. Percentages of early apoptotic cells are indicated.

present in adherent cells and $p27^{kip1}$ was not detectable in either group at the protein level. Lysate from rat liver was used as positive controls (Figure 4B). Cell cycle associated proteins, cyclin A and cyclin E, were present both in suspension and in adherent HL60 cells to the same extent (data not shown).

Discussion

Drug resistance is a major obstacle for a successful cancer therapy (17). CAM-DR represents a novel type of drug resistance mechanism that has been demonstrated in several tumors like multiple myeloma (18,19), chronic myeloblastic leukemia, and small cell lung cancer (20). In this study, we have shown that adhesion to FN via $\alpha4\beta1$ and $\alpha5\beta1$ integrins exerted a CAM-DR phenotype in the acute promyelocytic cell line, HL60. HL60 cells with CAM-DR phenotype are resistant to apoptosis triggered by doxorubicin. CAM-DR caused an accumulation in the G_0/G_1 phase of the cell cycle, inhibition of DNA synthesis, and growth arrest in adherent cells. Our findings are well-matched with Hazlehurst's (18) report, in which they showed that adhesion of multiple myeloma cells to FN resulted in a reversible reduction in cell proliferation and accumulation of cells in the G_0/G_1 phase of the cell cycle.

Cell cycle progression is regulated by balanced interaction between cyclins and CDKs. CDK inhibitors (CDKIs), $p21^{waf/cip}$, $p27^{kip1}$ and $p57^{kip}$, also play a key role in the regulation of the cell cycle. The role of $p21^{waf/cip}$ in human cancer cells is complex. Initially, $p21^{waf/cip}$ was identified as an inhibitor of cell proliferation. However, a number of recent studies have shown that $p21^{waf/cip}$

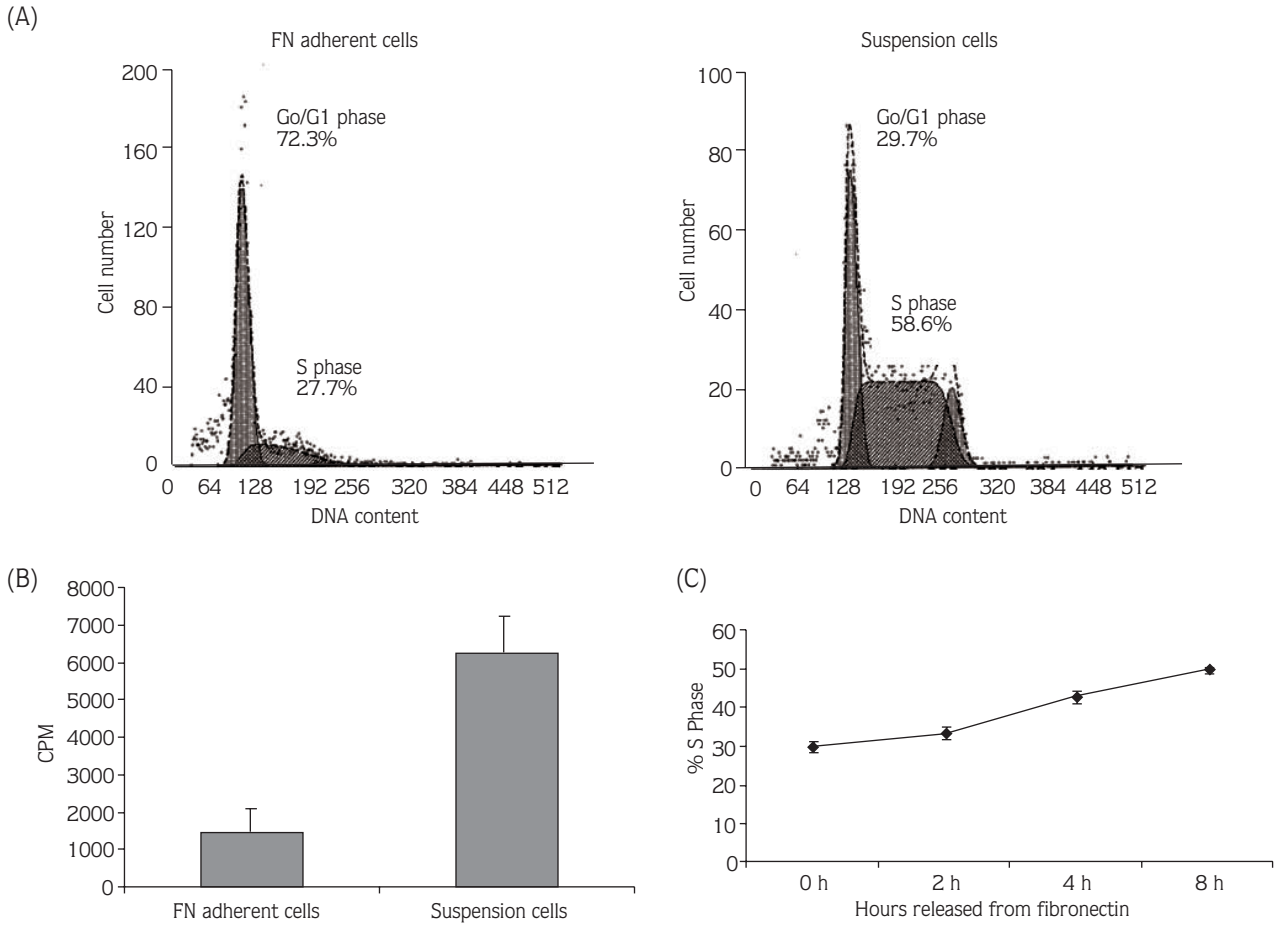

Figure 3. Cell cycle analysis of HL60 with CAM-DR phenotype. A) Cells adhered to FN for 24 h or cells grown in suspension were stained with PI and cell cycle status was determined by flow cytometry. A representative experiment is shown. The cell cycle arrest in the G_0/G_1 phase can be clearly seen in FN-adherent cells. B) Proliferation rate was analyzed by ^{3}H-thymidine incorporation assay. Proliferation was significantly decreased in FN-adherent cells ($n = 3$, $P < 0.01$). C) Following 24 h of adhesion, cells were detached and cultured as suspension for 2, 4 and 8 h before BrdU flow cytometry analysis. Increased percentage in S phase indicated the reversibility of CAM-DR induced growth arrest.

possesses pro- and anti-apoptotic capacities in response to anti-tumor agent depending on cell type and oncogene expression (21,22). Chang and colleagues (21) also revealed that p21[waf/cip] activated both anti-apoptotic and pro-apoptotic genes (23). Our observations are consistent with the anti-apoptotic role of p21[waf/cip] in CAM-DR phenotype in HL60 cells, since the percentage of cell death via drug-induced apoptosis was significantly lower in adherent HL60 cells compared to suspension cells. It has also been reported that the production of anti-apoptotic proteins in p21-expressing cells might influence the survival of adjacent cells through a paracrine effect (23). Recently, Matsunaga et al. (24) found that $\alpha4\beta1$ (VLA-4)-positive cells acquired resistance to drug-induced apoptosis through the phosphatidylinositol-3-kinase (PI-3K)/AKT/Bcl-2 signalling pathway, which is activated by the interaction of $\alpha4\beta1$-FN. Next to the effect on prevention of apoptosis, PI-3K/AKT pathway also plays a role in the regulation of cell cycle progression (25, 26). Mitsuuchi et al. (27) demonstrated a direct link between the PI3K/AKT signalling pathway and p21 expression in the human ovarian carcinoma cell line A2780. In addition, expression of constitutively active or wild-type AKT enhanced p21 expression and sustained p21 induction in response to drug treatment (28). The detected increased expression of p21[cip] in adherent HL60 cells in our study may be correlated with the reported activation of the PI-3K/AKT signalling pathway.

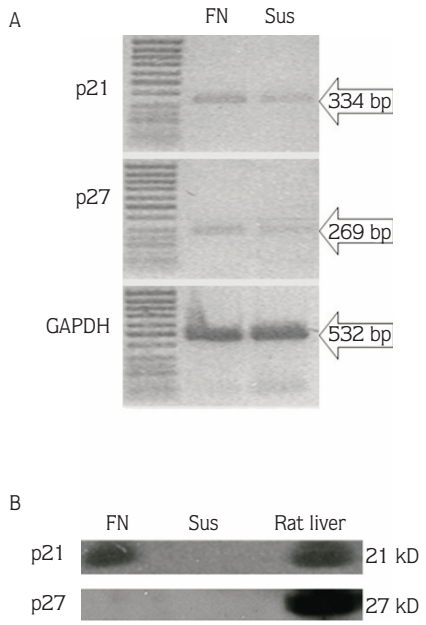

Figure 4. Analysis of the factors at the G_0/G_1 cell cycle checkpoint. A) Expression of p21$^{waf/cip}$ and p27^{kip1} genes in suspension and FN-adhered cells was analyzed by RT-PCR. GAPDH gene was amplified as house keeping gene. p21$^{waf/cip}$ and p27^{kip1} expression were found to be increased in FN-adherent HL60 cells when compared to suspension cultures. B) Western blot results of p21$^{waf/cip}$ (21 kD) and p27^{kip1} (27 kD) are shown. Only p21$^{waf/cip}$ could be detected at the protein level in FN-adherent HL60 cells. p27^{kip1} protein was not detectable in either suspension or adherent cells. Lysate prepared from rat liver was used as a positive control.

Cell cycle regulatory function of p21^{cip} is also complex. From early G_1 until the middle of the S phase, p21^{cip} can bind to Cdk4/cyclin D and Cdk6/cyclin D complexes, and induce their kinase activities. From late G_1 until the S phase, p21^{cip} can bind to Cdk2/cyclin A and Cdk2/cyclin E complexes, and inhibit their kinase activities (28, 29). The role of p21$^{waf/cip}$ in adherent HL60 cells in our study appears to be related with the inhibitory function of the protein since we observed a decrease in DNA synthesis and cell cycle arrest.

In different tumor cell lines, the anti-apoptotic effect of adhesion to FN has been correlated with p21$^{waf/cip}$ protein and p27^{kip1} protein levels (30,31). We also detected an induction of p27^{kip1} gene expression in adherent HL60 cells, although p27^{kip1} protein level was undetectable by Western blot analysis. However, p27^{kip1}-dependent growth arrest in EMT-6 mammary tumor spheroids (32) and protection from the drug-induced apoptosis by the overexpression of p27^{kip1} protein were reported previously (30). Besides p27^{kip1}, β1 integrin-mediated adhesion to FN has been shown to be associated with reduced CD95 (Apo/Fas)-mediated cell death (33). There might be multiple signalling pathways for the induction of anti-apoptotic activity and/or cell cycle arrest in adherent AML cells that may result in the drug resistance.

We did not detect the expression of p57 transcript in either adherent or suspension cells. In accordance with our results, the down-regulation of p57^{kip2} expression in hematologic malignancy has been published recently (34). These results indicate that cell cycle regulatory proteins p21$^{waf/cip}$ and p27^{kip1} may play an important role in the CAM-DR phenotype of AML. Increased levels of p21$^{waf/cip}$ and p27^{kip1} corresponded with resistance to cytotoxic insult and cell cycle arrest.

In conclusion, adhesion to FN via β1 integrins may protect AML cells from drug-induced cytotoxicity, enhance tumor cell survival, and prevent apoptosis. Interruption of the adhesion may represent a new target and may improve survival when used in combination with chemotherapy in AML patients.

Acknowledgements

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