Relation between Expression of APE1 Gene and Resistant to Melphalan in Multiple Myeloma Cells*

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Absract Objective To explore the relation between changes of apurinic/ apyrimidinic endonuclease(APE1) gene expressed and resistant to melphalan in multiple myeloma(MM) cells. **Methods** Forty-two bone marrow specimens were collected from 32 MM patients and 10 normal volunteers. Expression and location of APE1 protein in KM_3 cell and 42 specimens were detected by immunofluoresence double staining, cofocal laser scanning microscopy and immunocytochemical staining. After KM_3 cell was given (0~15) μmol/L of melphalan 1~2 days, the changes of expression of APE1 protein was measured by means of immunocytochemical staining and Western blot assay. **Results** APE1 and CD38 were co-expressed in bone marrow specimen of MM. Expression of APE1 gene in nucleus were significantly elevated compared to that in nucleus/cytoplasm and cytoplasm. In normal control group, untreated MM patients group, and relapse/refractory MM group, the positive degree of cytoplasmic staining were increased in turn ($P<0.05$). In KM_3 cells levels of APE1 protein were associated with the treated time and dose of melphalan. **Conclusion** Expression degree of APE1 protein is associated with therapeutic effect of MM, and its can be induced by melphalan treating. It suggest that high expression of APE1 protein may play certain roles in the resistance to melphalan in multiple myeloma.

Key words: Multiple myeloma/Hematology; APE1; Melphalan; DNA damage repair

Since the 1960s, melphalan has been used as a first-line drug for multiple myeloma (MM). But the complete remission rate it brings is as low as 5%[1]. To date, MM is still incurable. Most authors hold that multidrug resistance of MM cells is the major cause for failure in the treatment of MM[2, 3]. Recent research demonstrates that the incidence of drug resistance of MM cells is closely associated with DNA damage and repair[4,5]. Apurinic/apyrimidinic endonuclease 1(APE1), one of the essential components of the base excision repair system, is primarily responsible for repairing DNA damages at apurinic/ apyrimidinic (AP) sites caused by alkylating or oxidative agents[6]. It has been reported that APE1 is abundantly expressed in an array of tumors[7, 8]. However, its expression and localization in MM cells as well as its relationship with resistance against alkylating agents remain unclear. Here we detected APE1 protein expression in 32 MM patients by double immunofluorescent labeling in combination of confocal laser scanning microscopy and immunocytochemical staining, and investigated the changes in APE1 protein expression in KM_3 cells exposed to various doses of melphalan for different time durations by immunocytochemical and Western blotting analysis, so as to clarify the relationship between upregulated APE1 expression and resistance of MM cells to melphalan.

MATERIALS AND METHODS

Samples

The MM cell line KM_3 was a kind gift from Professor Jian Hou at Changzheng Hospital of the Second Military Medical University. The cell line was established from a multiple myeloma patient with concurrent plasmacytic leukemia. KM_3 cells resistant to 6-thioguanine and uabain were conventionally cultured and passaged in RPMI1640 containing 10% fetal calf serum, 100U/mL penicillin, 100μg/mL streptomycin

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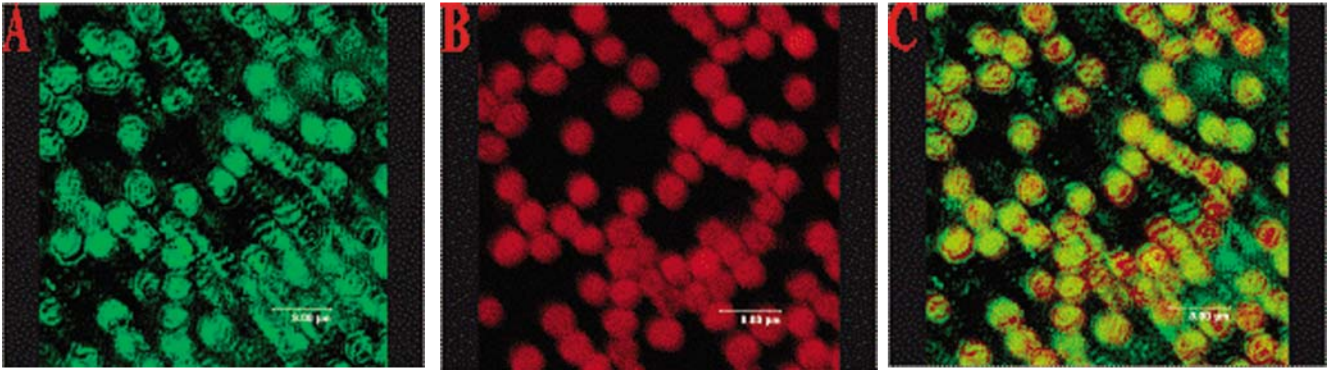


A: Expression of the CD38 protein; B: Expression of APE1 protein; C: Expression of CD38 and APE1 protein.

Fig.1 Expression of APE1 and CD38 in bone marrow cells of MM patients detedted by immunofluorecence double staining with confocal laser scanning microscopy

and 2mmol/L glutamine in air with saturated humidity and 5% CO_2 at 37℃. From January 2003 to October 2004, 18 MM patients were hospitalized at Daping Hospital and 14 MM patients at Xinqiao Hospital of the Third Military Medical University (in Shang Hai City). The 32 patients were diagnosed according to the criteria described elsewhere[9]. There were 17 males and 15 females, and their age ranged from 37 to 75 years old(median age: 53 years). Twelve of them were initially treated, and 20 were recurrent or refractory patients who had undergone chemotherapy involving melphalan. Bone marrow samples from 10 patients with idiopathic thrombocytopenic purpura or leukopenia were used as the control.

Reagents and instruments

Murine anti–human APE1 monoclonal antibody was purchased from Novus Biologicals; rabbit anti–human CD38 polyclonal antibody, TRITC–labeled sheep anti–murine IgG, and FITC–labeled goat anti–rabbit IgG from Sigma Co.; SP kits from Beijing Zhongshan Biotechnology Co. Ltd. Olympus inverted microscope and digital camera was manufactured by OLYMPUS Co. (Japan), vertical plate electrophoresis bath by Beijing 61 Factory, and MRC1024ES confocal laser scanning microscope by Bio–Rad Company (USA).

Double fluorescent antibody labeling and CLSM

Double fluorescent antibody labeling and CLSM were conducted according to Bernardini G and colleagues [10] with slight methodological modifications. MM bone marrow smears were fixated in methanol at 0℃ and then immersed in 30 ml/L H_2O_2–methanol at room temperature for 20 min. Smears were blocked with 30mL/L normal goat serum for 30min, added with the mixture of rabbit anti–human CD38 polyclonal antibody (1:200) and murine anti–human APE1 monoclonal antibody (1:200) (volume/ volume, 1:1), and incubated at 4℃ overnight. Smears were rinsed with PBS three times for 3min, added with TRITC–labeled sheep anti–murine IgG (1:100), and incubated at 37℃ for 30min. After smears were rinsed with PBS three times for 3min, they were added with FITC–labeled goat anti–rabbit IgG (1:100), incubated at 37℃ for 30min, and rinsed with PBS three times for 3min. After having been mounted with fluorescent buffer–free glycerine, smears were observed and photographed under CLSM, with an excitation wavelength of 488nm for FITC and 568nm for TRITC. The range of zoom was defined as required. For negative control, primary antibodies were replaced by PBS. Immunofluorescent results were assessed as follows: CD38–positive fluorescence is green, APE1–positive fluorescence is red, and co–expression of CD38 and APE1 results in yellow fluorescence. Immunofluorescence was localized in cell nucleus or cytoplasm.

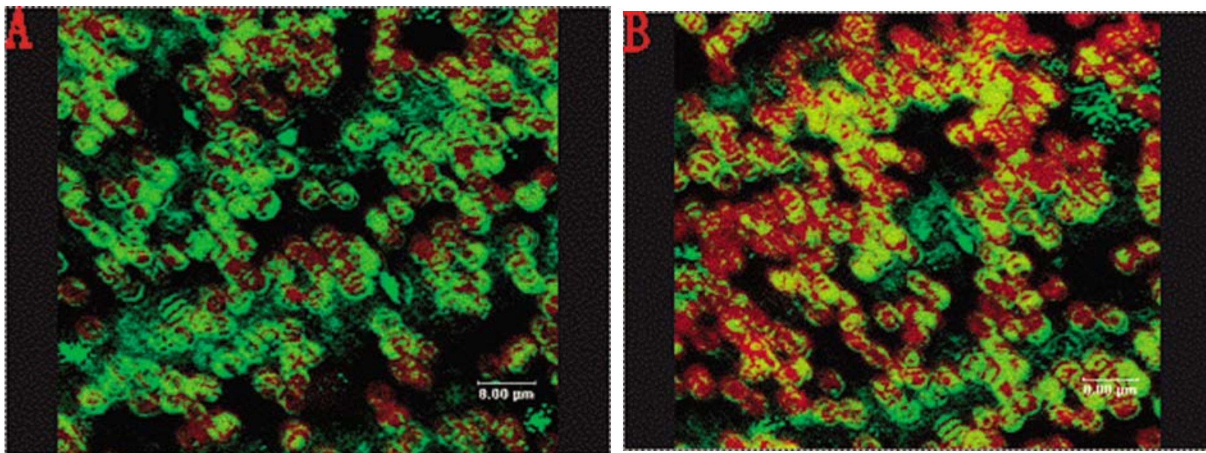
Immunocytochemical staining

Immunocytochemical staining was conducted by the streptavidin peroxidase conjunction method according to the instructions of the S–P kits. Briefly, KM_3

Tab 1 Positive degree of expression of APE1 protein in nucleated cells of MM patients

Group	N	Nucleus expression cases			Nucleus/cytoplasm expression cases		
		−	+	++	−	+	++
Normal control	10	6	4	0	10	0	0
MM Untreated patients	12	5	5	2※	10	2	0※※
MM Relapse/refractory	20	6	3	11※	6	7	7※※

※ x^2=4.57, 0.025<P<0.05. ※※ x^2=5.38, 0.01<P<0.025.



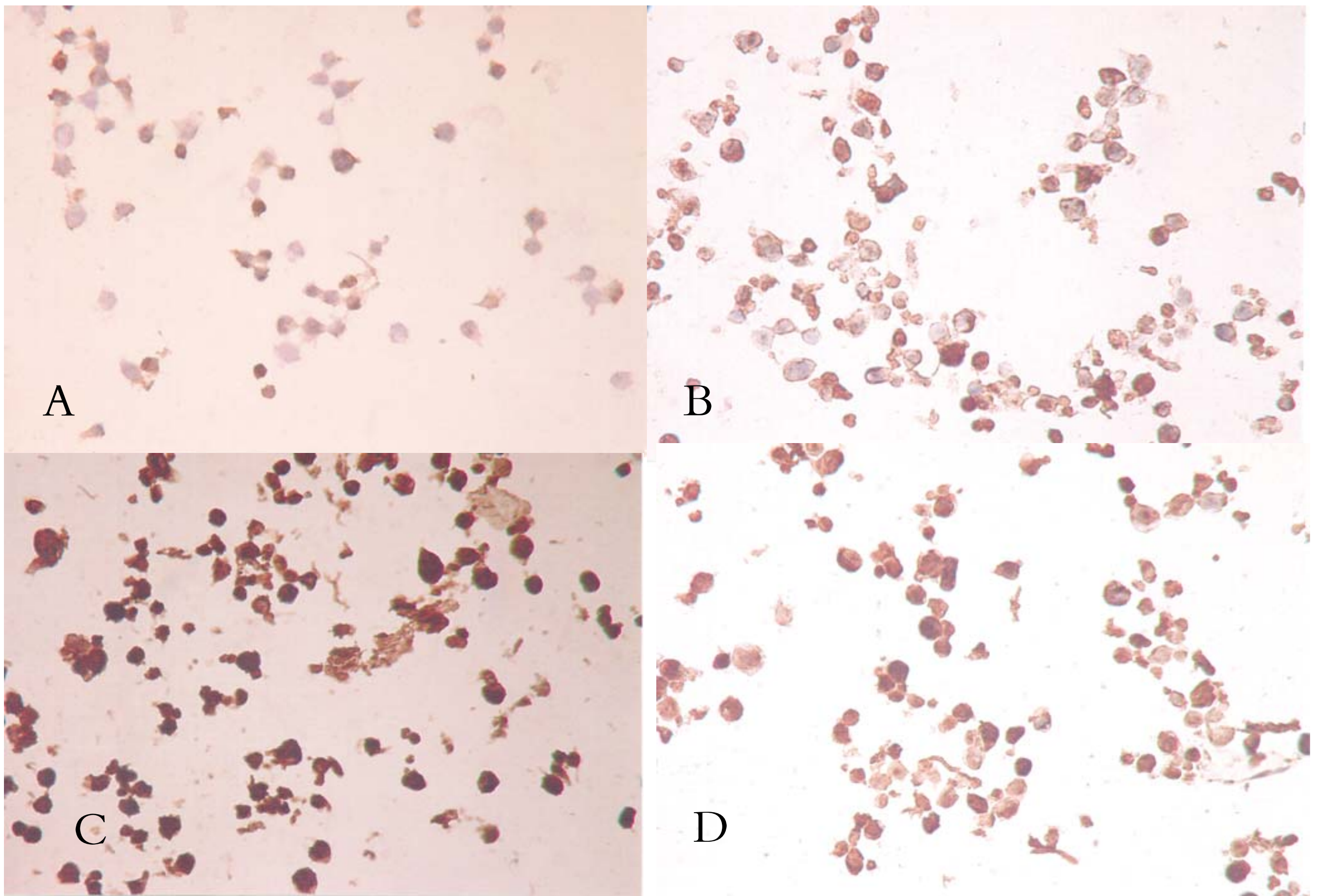
The amount of APE1 protein near the nucleus (B) is greater than that away from the nucleus (A).

Fig.2 Distribution of APE1 protein in bone marrow cells of MM patients detected by confocal laser scanning microscopy

cell suspension was dripped onto slides coated with gelatin. Slides were dried and immersed in 30ml/L H_2O_2–methanol at room temperature for 20min. 0.1% Triton–X 100 was added into H_2O_2–methanol and slides were left for 10min before primary antibody (working concentration of anti–APE1 antibody, 1:200) was added. Slides were left at 4℃ overnight, and then added with 50μL secondary antibody, and incubated at 37℃ for 30min, followed by DAB–H_2O_2 coloration and hematoxylin afterstain. For negative control, primary antibodies were replaced by PBS. Immunocytochemically positive signals for APE1 are fine, brownish yellow particles in the nucleus/ cytoplasm. Positive tumor cells were scored according to Mark Kelley and colleagues[7].

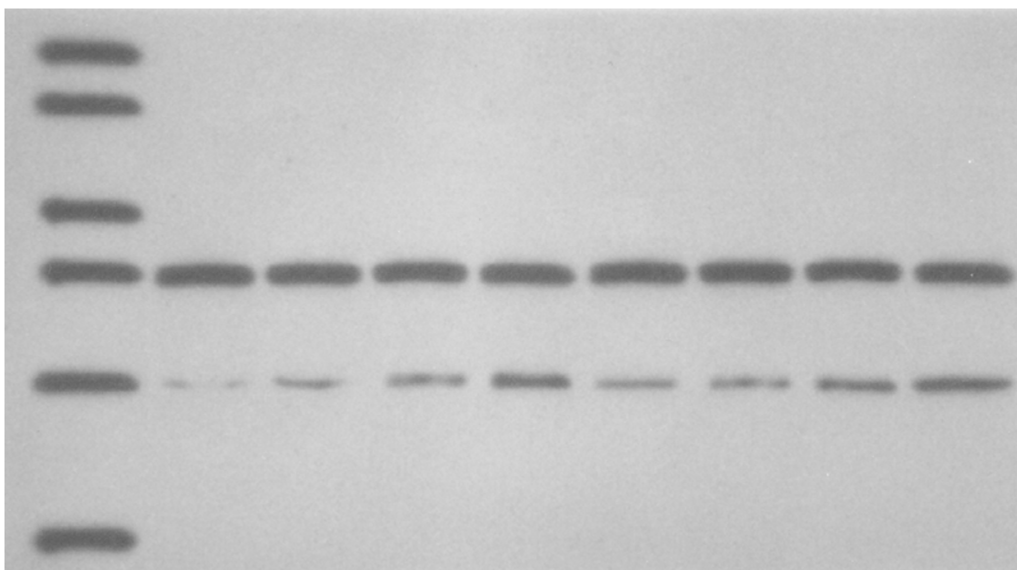
Western blot analysis

KM_3 cells were treated with 0, 5, 10, and 15μmol/L melphalan for 1–2d, and rinsed with PBS (pH7.4). 1×10^7 KM_3 cells were added with 100μL cell lysis solution pre–cooled to 0℃, left on ice for 30min, and centrifuged at 12000 r/min at room temperature for 10min. Supernatants were added with 2×SDS gel loading buffer and denatured at 100℃ for 5min. Then SDS–PAGE (separation gel concentration, 12%) and PVDF membrane transferring were conducted. PVDF membrane was blocked with 5% defatted milk and added with anti–APE1 antibody (1:500) and



A: APE1 protein expression of KM_3 cells in melphalan untreated group; B: APE1 protein expression of KM_3 cells treated by 5μmol/L melphalan; C: APE1 protein expression of KM_3 cells treated by 10μmol/L melphalan; D: APE1 protein expression of KM_3 cells treated by 15μmol/L melphalan.

Fig.3 Immunocytochemical staining of KM_3 cells treated by 0–15μmol/L malphalan 2 days (SP×200)



1~4: the group of 0、5、10、15μmol/L malphalan treated KM_3 cells 1 day; 5~8: the group of 0、5、10、15μmol/L malphalan treated KM_3 cells 2 days

Fig.4 Western blot analysis expressive change of APE1 protein in KM_3 cells treated by 0~15μm melphalan 1~2 days

horseradish peroxidase–labeled sheep anti–murine antibody (1:2000) before chemoluminescence.

Statistical analysis

Statistical analysis was conducted by using SPSS10.0. Numeration data were compared by chi–square test.

RESULTS

Observation on CD38 and APE1 expression sites in MM bone marrow cells by CLSM

Under CLSM, double fluorescent antibody labeling showed that CD38 distributed mainly on cell surface in MM bone marrow cells (Fig.1A), while APE1 distributed mainly in the nucleus/cytoplasm (Fig.1B), with higher expression near the nucleus (Fig.2B) than far from it (Fig.2A). In some MM cells, CD38 and APE1 were co–expressed in the cytoplasm (Fig.1C).

Immunocytochemical detection of positive graduation of APE1 expression in MM bone marrow nucleated cells

The positive graduation of APE1 expression in MM bone marrow nucleated cells decreased significantly from the control group, to the initially treated MM group, and still to the recurrent/refractory MM group (x^2=4.57, 0.025<P<0.05 and x^2=5.38, 0.01<P<0.025) (Tab.1).

Detection of APE1 protein expression changes in KM_3 cells following melphalan treatment

Following melphalan treatment, APE1 expression was observed in the nucleus, nucleus/cytoplasm, or cytoplasm. With increasing doses and action time of melphalan, the positive graduation of APE1 expression increased gradully (Fig.3).

Changes in APE1 protein amount in KM_3 cells following melphalan treatment

Immunoblotting analysis also confirmed that the amount of APE1 protein in KM_3 cells was positively correlated to the dose and action time of melphalan (Fig.4).

DISCUSSION

MM is a plasma cell–derived malignant tumor, and chemotherapy plays an important role in the treatment of it. It is still an incurable disease to date. Chemotherapeutic drug resistance, particularly multidrug resistance (MDR), is a major factor influencing the effect of chemotherapy [2,3]. APE1 is an essential component of the base excision repair system which is ubiquitously present in living organisms, and is the only mechanism repairing DNA damages at AP sites. In addition, APE1 has redox functions, which may activate many oncogene products, such as c–Jun, c–Fos, c–Myc and Pax proteins by regulating transcription factors [11]. It has been shown that APE1 overexpression is closely associated with poor prognoses of patients with tumor such as cancer of cervix, breast, prostate, and ovarian [12,13]. Moreover, APE1 expression is organ and tissue–specific.

Human CD38 molecule is a type II transmembrane glycoprotein. Most multipotent stem cells do not express or express low levels of CD38, while committed medullary and lymphocytic progenitors express high levels of CD38. With the maturation and differentiation of cells, CD38 expression is decreased markedly. In lymphocyte series, activated lymphocytes and plasma cells may express high levels of CD38 again. Hence, CD38 is considered a specific antibody marker of MM cells [14]. Our findings demonstrate that CD38 and APE1 proteins were co–expressed in MM bone marrow cells, confirming APE1 protein expression in MM cells. We also found that APE1 protein expression was upregulated in bone marrow nucleated cells of MM patients compared to the controls, and there were significant differences in APE1 expression between the recurrent /refractory patients and the initially treated ones, suggesting that the enhancement of APE1 protein expression is associated with poor therapeutic effects in MM patients.

Melphalan, an effective drug for MM, is a phenylalanine –based vinylamine –type alkylating agent. Alkylating agents deter cell proliferation by binding covalently to and destroy DNA. More specifically, alkyl groups in them replace hydrogen atoms in DNA

components, such as N_7 of nucleic acid guanine, N_1 of adenine and N_3, N_1 of cytosine or hydroxyl of phosphoric acid. Our findings show that following melphalan treatment, APE1 expression level was significantly elevated in KM_3 cells, and APE1 protein amount was positively correlated to the dose and action time of melphalan. We postulate that while chemotherapeutic agents are killing MM cells, APE1 gene is activated in part of dormant tumor cells, resulting in a counteraction against injuring stimuli. This might be one of the mechanisms by which MM patients develop therapeutic drug resistance. We further postulate that blockade of APE1 expression may reverse or ameliorate the susceptibility of MM cells to chemotherapeutic agents. Nevertheless, the precise mechanism by which APE1 gene plays a role in drug resistance of MM cells is still to be elucidated.

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