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Original Article

Anti-myeloma properties and mechanism of polymethyl methacrylate bone cement and allograft bone *in vitro*

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Abstract: Objective: The goal of this study was to investigate the effects of polymethyl methacrylate (PMMA) and allograft bone on apoptosis in the multiple myeloma cell line U266 and to clarify the molecular mechanism of apoptosis. Methods: Cell counting kit-8 (CCK-8) assay, flow cytometry, and Western blot were used in this study. Result: The apoptotic rate increased in U266 cells treated with PMMA bone cement extract and showed characteristic dose-dependent effects. PMMA could induce apoptosis of U266 cells via activation of Caspase-3 and inhibition of Bcl- 2 expression. Conclusion: These data support that PMMA bone cement has an anti-myeloma effect and induces apoptosis of U266 cells. Allograft bone had no significant effect on the proliferation of U266 cells. PMMA bone cement should be the first choice in surgical treatment of multiple myeloma.

Keywords: Polymethyl methacrylate, allograft bone, multiple myeloma, apoptosis

Introduction

In recent years, with development of new chemotherapy drugs and chemotherapy methods, overall survival of patients with multiple myeloma has been prolonged [1-3]. Surgical intervention is required when MM patients develop pathological fractures in the course of disease progression [4, 5]. Conventional surgical methods included foci curettage plus bone defect fillers, and plate internal fixation. PMMA bone cement and allograft bone are widely used in surgery because of their good biocompatibility [6]. In clinical surgery, myeloma cannot be completely removed because of the specificity of the tumor, which greatly increases the probability of local recurrence. Surprisingly, we found that patients filled with PMMA bone cement had a much lower probability of local recurrence than patients with other fillers such as allograft bone. Surgery may disrupt the growth environment of multiple myeloma, and PMMA bone cement may have a greater impact on tumor cells. Some researchers have reported the mechanism of apoptotic effect of antitumor bone cement on solid tumors such as osteosarcoma, but the effect of PMMA on MM has not been reported [7, 8]. Based on the above issues, experiments were designed to compare the cytotoxic effects of PMMA bone cement and allogeneic bone on U266 cells and to explore the mechanism, in order to provide the basis for filler selection in surgical treatment of multiple myeloma.

Materials and methods

Preparation of PMMA and allograft bone extract

A standard bone cement without antibiotic load was used in this trial. Mixing 5 mg PMMA clot (5 cm^3) (powder/liquid ratio of 5 mg: 2.5 ml) with 5 ml PBS and collected the 24 hour and 48 hour extract separately (PMMA clot/PBS ratio of 1 mg: 1 ml). The maximum concentration of PMMA extract was set as “1”, and a series of concentrations of extracts were prepared by stepwise dilution method. Mixing 5 cm^3 allograft bone with 5 ml PBS and collected the 24 hour and 48 hour extract separately. The concentration of allograft bone extract was “1” (MC) and a series of concentrations of extracts were prepared by stepwise dilution method.

Cells and cultures

The U266 cell lines were purchased from the ATCC and maintained in Roswell Park Memorial

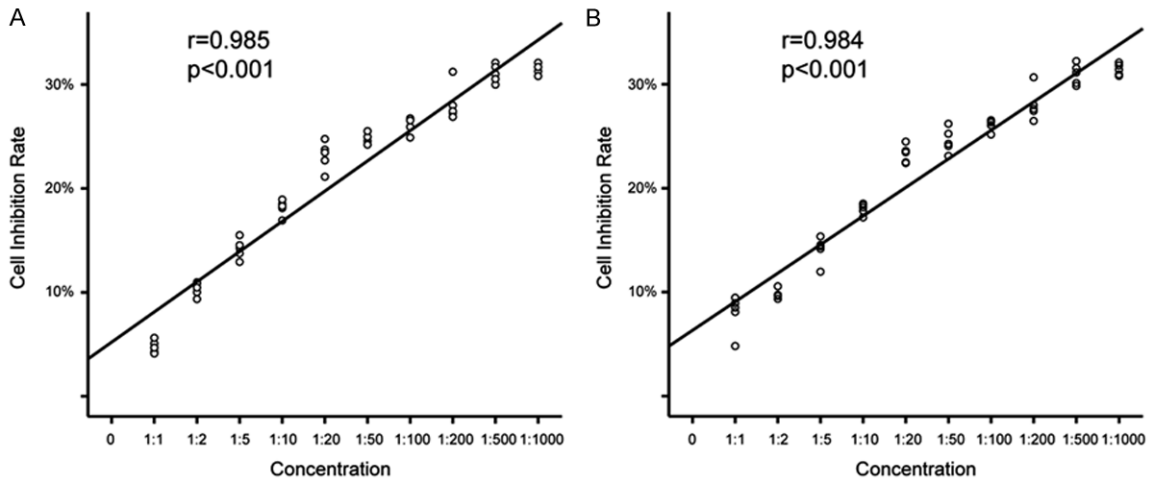

Figure 1. A. Correlation between cell inhibition rate and 24 hour PMMA extract with different concentration. The (Spearman) correlation coefficients (r) are given in the figure. B. Correlation between cell inhibition rate and 48 hour PMMA extract with different concentration. The (Spearman) correlation coefficients (r) are given in the figure.

Table 1. Cytotoxicity of PMMA extract on U266 cells

PMMA extract	Cell inhibition rate % ($\bar{X}$ ± S) (n = 5)		*P* Value
	24 hour	48 hour	
1:1	31.34 ± 0.56	31.42 ± 0.55	P > 0.05
1:2	31.06 ± 0.85	30.98 ± 0.99	P > 0.05
1:5	28.15 ± 1.76	28.04 ± 1.57	P > 0.05
1:10	26.15 ± 0.77	26.12 ± 0.56	P > 0.05
1:20	24.69 ± 0.56	24.58 ± 1.18	P > 0.05
1:50	23.15 ± 1.35	23.29 ± 0.86	P > 0.05
1:100	18.15 ± 0.76	18.02 ± 0.53	P > 0.05
1:200	14.20 ± 0.95	14.09 ± 1.27	P > 0.05
1:500	10.33 ± 0.66	9.97 ± 0.56	P > 0.05
1:1000	4.75 ± 0.62	7.96 ± 1.83	-

Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2. The medium was changed every 2-3 days.

Reagents

PMMA (a standard bone cement without antibiotic load) was purchased from Heraeus Medical GmbH, Germany. Allograft bone was purchased from Hubei Lianjie, Inc, China. Cell counting kit-8 (CCK-8) and Annexin V-FITC Apoptosis Detection Kit were purchased from Sigma-Aldrich, USA. The antibodies for active caspase-3, Bax, Bcl-2 were purchased from Abcam, UK.

Cell proliferation assay

The *in vitro* cell proliferation effects of PMMA and allograft bone extract were determined by CCK-8 assay. U266 cells were seeded onto 96-well plates at a density of 1×10^4 cells/well (100 μl) and treated with various concentrations of PMMA and allograft bone extract (25 μl) for 24 and 48 hours respectively. The blank group wells were added in 125 μL RPMI 1640 medium and PBS (25 μl) were added to control group wells. The CCK-8 solution (10 μL) was added to each well and incubated for an additional 4 hours. The absorbance was measured at 450 nm using an ELX 800 Microplate Reader (BioTek Instruments, Inc, USA). Five wells were used for each concentration. The inhibitory rate of cell proliferation was calculated by the following formula: inhibition rate (IR) = [((OD) control - (OD) treated)/((OD) control - (OD) blank) ×100%].

Apoptosis analysis

U266 cells were exposed to different concentrations of PMMA extract, allograft bone extract for 24 hours. The control group was given equal PBS. After that, 1×10^6 cells were washed twice in 4°C PBS and resuspended in 500 μL of 1×Binding Buffer. 5 μL of Annexin V-FITC and 5 μL PI (Bioscience Pharmingen, San Diego, CA,

Table 2. Cytotoxicity of allograft bone extract on U266 cells

Allograft bone extract	Cell inhibition rate % ($\bar{\chi} \pm S$) (n = 5)	
	24 hour	48 hour
1:1	5.58 ± 0.45	5.38 ± 0.57
1:2	6.89 ± 0.61	6.76 ± 0.59
1:5	5.33 ± 1.01	5.22 ± 1.03
1:10	4.78 ± 0.62	4.94 ± 1.01
1:20	5.74 ± 1.29	5.52 ± 1.17
1:50	4.45 ± 0.64	4.42 ± 0.56
1:100	5.47 ± 0.43	5.55 ± 0.55
1:200	4.86 ± 1.92	5.66 ± 1.55
1:500	4.53 ± 0.56	4.20 ± 0.48
1:1000	3.87 ± 1.60	3.46 ± 0.84

USA) were added to the cells. After incubation at room temperature for 15 minutes in the dark, cells were then analyzed by flow cytometer (Becton Dickinson, San Jose, CA, USA). Each experimental group was replicated four times.

Western blot analysis

Approximately 1×10^7 U266 cells were gathered after pretreatment for 24 hours. Equal amounts of total protein extracts from cultured cells were fractionated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred onto polyvinylidenedifluoride (PVDF) membranes. Secondary antibodies (Goat anti-Rabbit IgG (H+L) secondary antibody 1:1000) were used to detect the designated proteins. The bound secondary antibodies on the PVDF membrane were reacted with ECL detection reagents (Thermo Scientific) and exposed in Image Quant LAS 4000mini system (GE Healthcare, Buckinghamshire, UK). Results were normalized to the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each experimental group was replicated 3 times. Image J analysis software was used to measure band intensity.

Statistical analysis

Statistical analysis was performed using SPSS 23.0 software package for Windows (SPSS, Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD). The K independent samples test and Mann-Whitney U test for further comparisons between specific group pairs were used. Spearman correlation coefficients were calculated to determine the correlation between inhibition rate (IR) and concentration. Univariate analysis of independent variables with the t-test was performed for independent samples for quantitative variables. $P < 0.05$ were considered statistically significant.

Results

First, to compare the inhibitory effect of PMMA bone cement and allograft bone on proliferation of multiple myeloma cell line U266, two kinds of filler extracts were used to treat U266 cells and the inhibitory rate was evaluated by CCK-8 assay. PMMA extract after treatment was found to significantly inhibit U266 cell proliferation in a dose dependent manner (**Figure 1**). When the concentration of PMMA extract was 1:2, the inhibition rate increased to the plateau stage, and there was no significant difference in the cell inhibitory rate between the 24 hour PMMA extract group and the 48 hour PMMA extract group (**Table 1**). Both 24 and 48 hour allograft bone extract groups showed no antiproliferative activity on U266 cells (**Table 2**). The results showed that PMMA bone cement extract inhibited proliferation of U266 cells in a dose-dependent manner. Furthermore, proportions of AnnexinV FITC+/PI-as well as AnnexinV FITC+/PI+ cells were strikingly increased by 24 hour treatment of 1:1 PMMA extract (**Figure 2**). Taken together, these results indicate that PMMA extract induces apoptosis in U266 cells.

Activation of caspases plays an important role in the apoptosis-signaling pathway. Caspases-3 activation, a common effector caspase that integrates various death signals, during PMMA extract-induced apoptosis in U266 cells. Active caspase-3 appeared after treated with 1:1 and 1:50 concentrations of PMMA extract. Expression of apoptosis-associated proteins during PMMA extract-induced apoptosis in U266 cells was also examined. The results showed that protein level of Bcl-2 was clearly downregulated during 24 hours of PMMA extract-treatment, but the protein level of Bax did not change during 24 hours of PMMA extract-treatment (**Figure 3**). The results showed that protein levels of active caspase-3, Bcl-2, and Bax did not change during 24 hours of allograft bone extract-treatment. Taken together, these results suggest that PMMA extract-induced apoptosis is, at least in part, mediated through a mitochondria-dependent pathway.

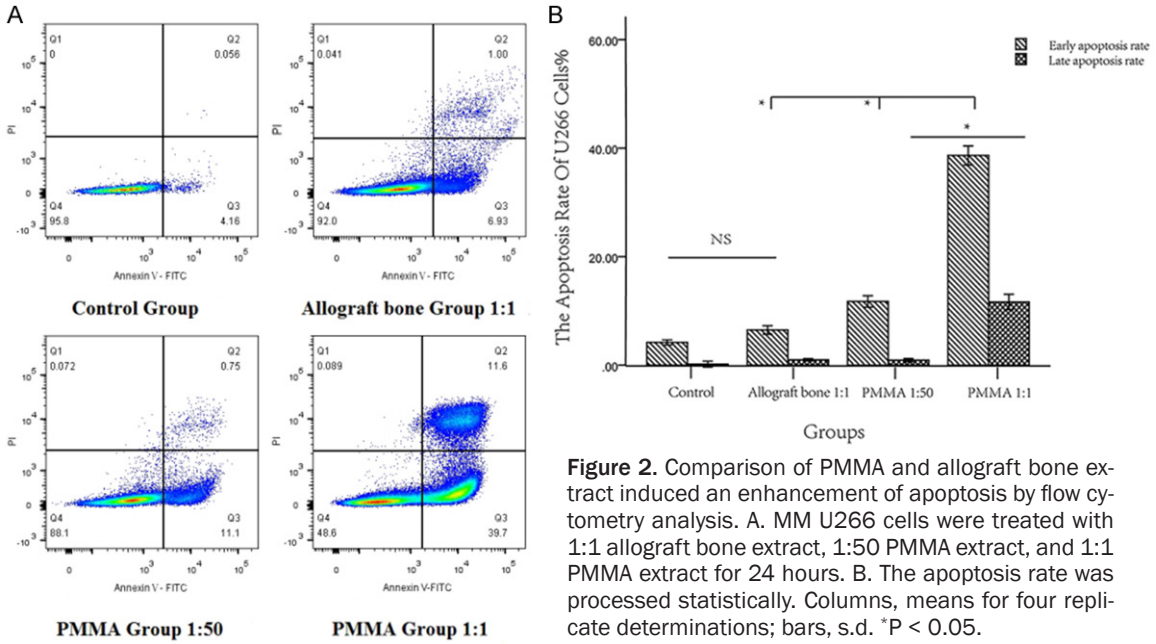

Figure 2. Comparison of PMMA and allograft bone extract induced an enhancement of apoptosis by flow cytometry analysis. A. MM U266 cells were treated with 1:1 allograft bone extract, 1:50 PMMA extract, and 1:1 PMMA extract for 24 hours. B. The apoptosis rate was processed statistically. Columns, means for four replicate determinations; bars, s.d. $^{*}P < 0.05$.

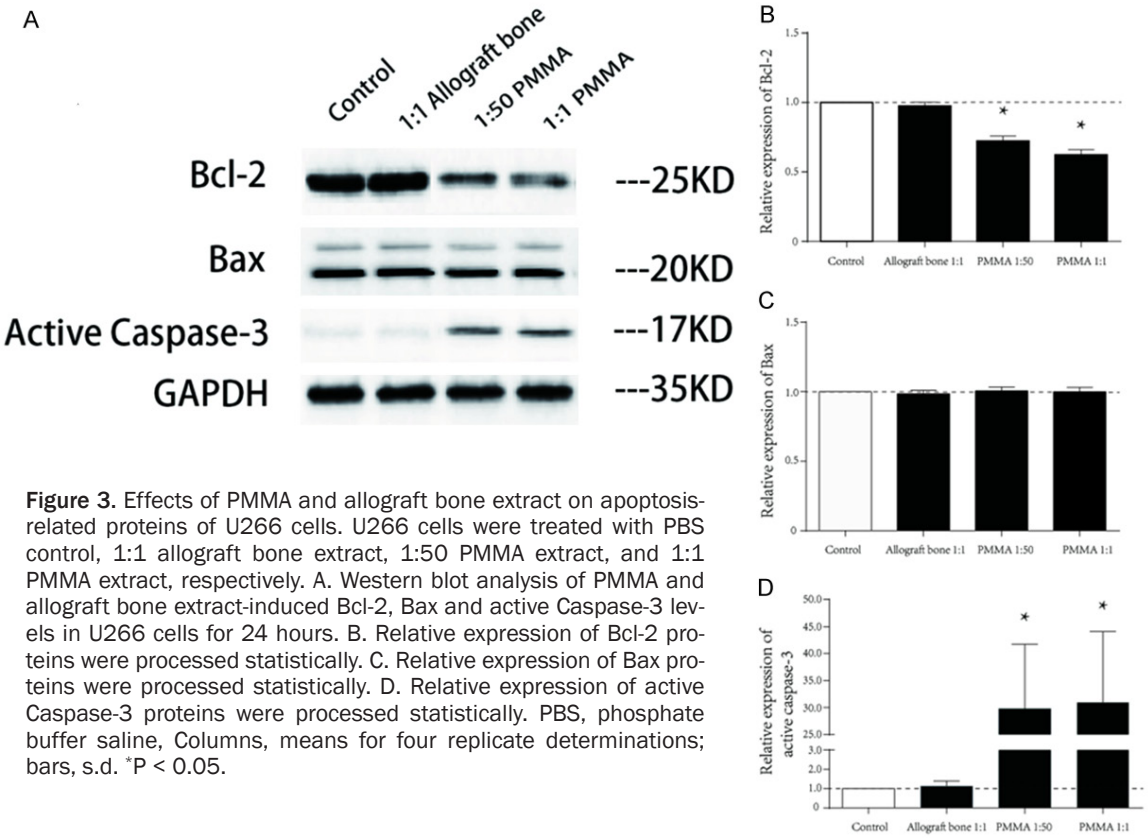

Figure 3. Effects of PMMA and allograft bone extract on apoptosis-related proteins of U266 cells. U266 cells were treated with PBS control, 1:1 allograft bone extract, 1:50 PMMA extract, and 1:1 PMMA extract, respectively. A. Western blot analysis of PMMA and allograft bone extract-induced Bcl-2, Bax and active Caspase-3 levels in U266 cells for 24 hours. B. Relative expression of Bcl-2 proteins were processed statistically. C. Relative expression of Bax proteins were processed statistically. D. Relative expression of active Caspase-3 proteins were processed statistically. PBS, phosphate buffer saline, Columns, means for four replicate determinations; bars, s.d. $^{*}P < 0.05$.

Discussion

MM is a malignant plasma cell disease. The clinical manifestation includes hyperglobulinemia, renal dysfunction, bone damage, and cytopenia. Surgical intervention is required when a patient has a pathological fracture. The significance of surgical intervention for MM

patients is more important in improving the quality of life. Implants have developed rapidly and have been widely used in clinical operations in recent years, such as autologous bone, allograft bone and bone cement. Since 1950s, PMMA bone cement has been widely used in orthopedic surgery because of its good biocompatibility and mechanical stability. With its further study, drugs loaded bone cement have been put forward as a hot topic, including antineoplastic bone cement. Marcus Tonak [8] found that HDAC inhibitor-loaded bone cement could induce the apoptosis in SaOs-2 and SW1353 cells but did not change the mechanical properties of the bone cement. Özben H [7] found that cisplatin loaded PMMA could induce apoptosis in SaOs-2 cells and help local control of tumor growth and PMMA also had direct cytotoxicity to SaOs-2 cells. However, most of the previous work were based on solid tumors. In the operation of multiple myeloma patients, there was little local recurrence in patients who filled PMMA bone cement. Does PMMA cement have a cytotoxic effect on multiple myeloma cells? To test this, PMMA cement was chosen and allografted bone was observe for cytotoxic effects in U266 cells.

In this study, CCK-8 assay indicated that PMMA extract exhibited evident cytotoxicity to multiple myeloma U266 cells in dose-dependent manner. Apoptosis, is one of the most important anticancer mechanisms. In this study, apoptosis was observed by flow cytometry assay after U266 cells were treated by PMMA and allograft bone extract. The results demonstrate that PMAA extract induced U266 cells apoptosis.

Bcl-2, widely expressed in several B/T-cell lymphomas such as lymphoma, chronic lymphocytic leukemia, MM [9-11]. The study from Pettersson showed that Bcl-2 was expressed in most MM cell lines such as U266, U1958, L363, Karpas 707 [9]. This study found that U266 cells expressed a high level of Bcl-2 and Bax in both control and allograft bone group. The results are consistent with previous findings. Bcl-2 expression was also downregulated in the process of MM cell apoptosis induced by PMMA bone cement.

The mitochondrial pathway is a key pathway in cell apoptosis. DNA-damaging agents signal cell death by altering the mitochondrial transmembrane protein, activating Bcl-2 family members with subsequent cytochrome c release, and activating the caspase family of proteins [12, 13]. Caspase-3 is a central component in cell apoptosis caused by exogenous or endogenous apoptotic signal. This study show that active caspase-3 expression was increased, and Bcl-2/Bax expression was downregulated in the process of MM cell apoptosis induced by PMMA bone cement. Therefore, induction of MM cell apoptosis by PMMA bone cement may be through the mitochondrial pathway, inhibiting Bcl-2/Bax expression, and activating caspase-3.

However the experiment still has some limitations. First, different from the past studies of PMMA and human osteosarcoma cells, U266 are a suspension cell line. We could not determine whether all cells had an effective interaction when adding PMMA clots. In order to ensure the reliability of the experiment, we choose PMMA bone cement extract instead of the PMMA clots, which may weaken the cytotoxic effect. Second, PMMA polymerization reaction is a complicated process which is heat releasing. Deramond's [14] studies have shown that after the injection of PMMA 10 ml into the vertebral body, the central peak temperature reached 52-93°C, average 61.8°C. Burd R [15] found that when cells were heated to 43°C for 30 minutes, apoptosis could be induced. Therefore, the cell apoptosis promoting effect of PMMA may be stronger in practical applications.

In addition, cell experiments can't simulated the surgical environment, the effect of PMMA bone cement should be confirmed *in vivo*. We are also looking forward to the emergence of MM skeleton lesion animal models so that further studies can be made.

Conclusion

In the present study, PMMA bone cement had an anti-proliferative effect and induced apoptosis of U266 cells. The mechanisms might involve downregulation of Bcl-2 expression and activating the caspase-3 protein. Allograft bone had no significant effect on the proliferation of U266 cells. PMMA bone cement should be the first choice in surgical treatment of multiple myeloma.

Future studies will focus on the effect of PMMA bone cement combined with other anti-myeloma drugs, such as bortezomib, thalidomide, and velcade. It would help improve the outcome of therapy if using anti-myeloma bone cement after resection, but this requires further investigation.

Acknowledgements

Informed consent was obtained from all of the participants included in the study.

Disclosure of conflict of interest

None.

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