Studies on apoptosis in bone tumor cells induced by ^{153}Sm

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Abstract The apoptosis in human bone tumor cells induced by internal irradiation with ^{153}Sm was studied. The morphological changes in bone tumor cells were observed by electronic and fluorescent microscopy, as well as DNA agarose gel eletrophoresis. DNA chain fragmentation, microautoradiographic tracing and the inhibition rate of proliferation in bone tumor cells exposed to ^{153}Sm with different duration time were examined. It was demonstrated that the bone tumor cells exposed to ^{153}Sm displayed nuclear fragmentation, pyknosis, margination of condensed chromatin, and formation of membrane bounded apoptotic bodies, whereas the percentage of DNA chain fragmentation of bone tumor cells increases in direct proportion to the duration of irradiation with ^{153}Sm, as well as DNA ladder formation in apoptotic cells. Also a marked inhibition effect of proliferation in bone tumor cells after exposure with ^{153}Sm was observed.

Keywords Apoptosis, Bone tumer cells, ^{153}Sm, Nuclear fragmentation, DNA ladder formation

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1 Introduction

The favourable characteristics of radionuclide ^{153}Sm-EDTMP (ethylene diaminetetramethylene phosphonate) permit optimal internal radiotherapy for clinical application.[1] Now ^{153}Sm-EDTMP has been used in patients requiring analgesia for pain arising from disseminated skeletal metastasis unresponsive to all appropriate conventional treatment modalities.[2] ^{153}Sm-EDTMP is effective not only in alleviating the pain of disseminated skeletal metastasis, but also in treatment of recurrent pain.[3] But up to date, its mechanism in the treatment of painful skeletal metastasis is still unknown.[4] No such studies were reported yet. Only studies on clinical and clinicopathologic responses to treatment with ^{153}Sm-EDTMP showed that the major adverse side effects of the treatment were platelet and white blood count depression.[5] So we observed apoptosis in bone tumor cells induced by ^{153}Sm-EDTMP internal irradiation.

2 Experimental

2.1 Cell culture conditions

Bone tumor cells were harvested and maintained in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum, 100 u/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L l-glutamine as well as 5×10^{-5} mol of 2-mercaptoethanol, which was known as the complete RPMI 1640 medium. The tumor cells were kept in an atmosphere containing 5% CO_2 at 37 °C and used when in exponential growth.[6] Cells were harvested from exponential-phase maintenance cultures using trypsin:versene (0.05%:0.02%) treatment of monolayer cultures. Thereafter, the bone tumor cells were washed three times with Ca^{2+} and Mg^{2+} free Hanks solution. Single cell suspensions were prepared with complete RPMI 1640 medium and the cells were counted using a hemocytometer. Finally, the suspensions were adjusted to a concentration of 2×10^6 cells/mL.

^{153}Sm-EDTMP with radioactive and chemical purity was used in this study. The bone tumor cell suspensions at 2×10^6 cells/mL were added to 1 mL of ^{153}Sm-EDTMP solution with radioactivity of 3.7×10^2 kBq/mL in complete RPMI 1640 medium in a 24-well microtitration plate. Then the cells were incubated in a 5% CO_2 atmosphere at 37 °C for different intervals with ^{153}Sm-EDTMP.

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2.2 Fluorescent microscopic observations

The bone tumor cells were suspended in complete RPMI 1640 medium to form suspensions at 2×10^2 cells/mL. 1 mL of the cell suspension was added to 24-well microtitration plate. Then 1 mL of ^{153}Sm-EDTMP with radioactivity of 3.7×10^2 kBq/mL also in complete RPMI 1640 medium was added to each experimental well. To control wells, only 1 mL RPMI 1640 medium was added. The microtitration plates were then incubated in 5% CO_2 at 37 ºC for 3, 6, 9, 12 and 24 h. After internal irradiation with ^{153}Sm for different intervals, the tumor cells were harvested, and washed 5 times with Hanks solution in order to remove the free radioactivity of ^{153}Sm-EDTMP. After a while, the bone tumor cells were suspended in hypotonic fluorescent solution which consists of 50 mg/mL pyridine iodide and 0.1% sodium acetate as well as 0.1% triton X-100,[7] in order to examine the morphology of nuclei of bone tumor cells.

2.3 Electron microscopic observations

Experimental bone tumor cells were suspended in complete RPMI 1640 medium to form suspension at 2×10^6 cells/mL. The tumor cell suspensions of 1 mL were added to 24-well microtitration plate. Then 1 mL of ^{153}Sm-EDTMP with 3.7×10^2 kBq/mL also in complete RPMI 1640 medium was added to each experimental well. To control wells, 1 mL RPMI 1640 medium was added only. The microtitration plates were then incubated in a 5% CO_2 at 37 ºC through 3, 6, 9, 12 and 24 h, respectively. After internal irradiation, tumor cells were harvested and washed 5 times with Hanks solution, in order to take away the free radioactivity of ^{153}Sm-EDTMP. Thereafter, the tumor cells were fixed for 1 h in 3% (mass fraction) glutaraldehyde solution. Then experimental cells were washed three times with 0.1 mol/L of PBS (pH 7.2). Again the cells were fixed in 1% (mass fraction) osmium acid solution for 1 h. The specimens were dehydrated with 30, 50, 70, 80, 90 and 100% (volume fraction) acetone one by one.[8] Next, the tumor cells were infiltrated with a miscible liquid with both dehydrating agent and embedding agent, so that the embedding agent could replace dehydrating agent fully. Epoxy resin 618 was exploited as embedding agent. The embedded tissues were cut into ultrathin sections with 0.05 μm thick by an ultramicrotome. The sections were mounted on the copper grids which were previously covered with support film of collodion.

The specimens were stained as follows. First of all, the experimental copper grids were immersed in 2.5% (mass fraction) aqueous uranyl acetate for 3 min at room temperature in a darkroom, then rinsed with distilled water thoroughly. After drying, they were stained in lead citrate solution for 30 min, rinsed with distilled water again. After drying in dust free air, the grids were stored in a dust free container and ready for viewing by the electron microscope H-600.

2.4 DNA chain fragmentation

Bone tumor cells were harvested, washed with Hanks solution and centrifuged. Thereafter, the suspensions of cells were adjusted to a concentration of 1×10^6 cells/mL in complete RPMI 1640 medium. Then 37 kBq ^3H-TdR[9] were added to each 1×10^6 cells/mL bone tumor cells and were incubated in a 5% CO_2 atmosphere at 37 ºC through 6 h. The labelled bone tumor cells were harvested again and washed with Hanks solution in order to remove the free ^3H-TdR. 1 mL of the bone tumor cells suspension in complete RPMI 1640 medium of 1×10^6 cells/mL was added to each well of 24-well microtitration plate.[10] Then, 1 mL of ^{153}Sm-EDTMP with 3.7×10^2 kBq/mL in complete RPMI 1640 medium was added to each experimental well. To control wells, 1 mL RPMI 1640 medium was added only. The microtitration plates were then incubated in humidified atmosphere of 5% CO_2 incubator at 37 ºC through 3, 6, 9, 12 and 24 h, respectively. In order to detect the cpm caused by ^{153}Sm remaining in the cells, parallel samples were cultured without ^3H-TdR. After each interval culture, the cells of each sample were gathered by multicanal cell collector apparatus onto a No.49 glass fibre filter, dried at 50 ºC, dropped into 5 mL scintillation cocktail with 0.4% (mass fraction) PPO and 0.04% POPOP in xylene. Their radioactivities were determined with a Beckman LS 6800 liquid scintillation counter. Experimental results are expressed as f (%), fraction of DNA chain fragmentation:

$$f(\%) = \frac{\text{Control cpm} - \text{Experimental cpm}}{\text{Control cpm}}$$

2.5 Cellular DNA extraction and agarose gel electrophoretic observations

Cellular DNA of 2×10^6 bone tumor cells after 3, 6, 9 and 12 h irradiation with ^{153}Sm-EDTMP was obtained by extraction with 1 mL buffer solution containing 10 mmol tris·HCl (pH 8.0), 1 mmol EDTA, 10 mmol NaCl, 1% SDS, 20μg RNAase and 100μg proteinase K (Merck, Germany). Cells were kept in 37 °C through 12 h and cooled down to room temperature. Then saturated phenol solution was added with the same volume. The mixed solution was isolated centrifugally with 12,000 g in 10 min. Thereafter, the supernatant liquor was drawn apart and put into the Eppendorf centrifuge tube. Specimens were extracted again with phenol:chloroform=1:1 solution, then added to 0.3 mol/L sodium acetate with dehydrated ethanol at 4 °C for centrifugal extraction in 10 min. Extracted DNA was washed twice in 70% ethanol, and dried at routine temperature. Finally, DNA was dissolved in 1 mmol EDTA with 10 mmol tris·HCl (pH 7.8) TE buffer solution, and was kept at 4 °C.

Agarose gel electrophoresis made by 1.5% agarose gel containing 0.5 mg/L ethidium bromide and 20 μg/L extracted sample containing 20 μg DNA mixed with 0.25% bromophenol blue and 40% sucrose were then put into gel electrophoretic slot at 50 V for 1.5 h electrophoretic analysis. The gel sample was photographed under UV light.

2.6 Microautoradiographic observations

A microautoradiographic study was made on bone tumor cells internally irradiated with ^{153}Sm-EDTMP for 3, 6, 9, 12 and 24 h, respectively, in a 5% CO_2 atmosphere at 37 °C. Microautoradiographic procedures were as follows: 20 μL each of control and experimental tumor cells were mounted separately on microscopic slides, which were then coated with thin collodion membrane. Thereafter, the slides were smeared with type N-4 liquid nuclear emulsion, which was 1:1 diluted with double distilled ion-free water, and put into 10% stable reagent 6-nitrobenzene miazol. The slides were then allowed to be exposed to dry nitrogen sphere condition for 3 d at 0 °C. After 3 d of exposure, the emulsion-coated sections were developed and fixed at 18 °C for 12 min. Soon afterwards, the slides were washed with running water and dipped in 5% glycerol solution. The sections were soon double stained with hematoxylin and eosin with reformed method.[11] Relative regional autoradiographic activity was then determined by visualization of autoradiographic activity tracks in internally irradiated tumor cells.

2.7 Inhibition rate of proliferation

The inhibition rate of proliferation of bone tumor cells after internal irradiation with ^{153}Sm-EDTMP was measured by colorimetric MTT assay.[12] 1 mL of the tumor cell suspensions at 2×10^6 cells/mL were added to 24-well sterile microtitration plates. Then 1 mL of ^{153}Sm-EDTMP with radioactivity of 3.7×10^2 kBq/mL also in complete RPMI 1640 medium was added to each experimental well. To control wells, only 1 mL RPMI 1640 medium was added. Thereafter, the microtitration plates were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 3, 6, 9, 12 and 24 h, respectively. After internal irradiation with ^{153}Sm-EDTMP for different intervals, the tumor cells were harvested and washed with Hanks solution. Cell suspensions were adjusted to 5×10^5 cells/mL and put into 55-well Elisa microplate of a volume of 100 μL/well in replicate. Then 10 μL of MTT solution (5 mg/mL in sterile PBS, filtered through a 0.22 μm filter) was added and the microplates were incubated in 5% CO_2 atmosphere at 37 °C for 4 h. Finally, 100 μL of SDS was added to each well and the microplates were incubated over night. The absorbance (*A*) was measured on DG-3022A Elisa microplate reader at wavelength of 540 nm.

3 Results

3.1 Fluorescence microscopic observations

The fluorescence microscopic appearance of PI-stained nuclei of the control bone tumor cells after 12-h incubation in RPMI 1640 medium alone indicated that the tumor cells showed heterogeneous nuclear chromatin. Bone tumor cells internally irradiated with ^{153}Sm-EDTMP displayed marked nuclear fragmentation as well as showed marked pyknosis in nuclei. A tremendous fragmentation and the apoptotic bodies formation were observed in the nuclei of tumor cells

as shown in specimens.

3.2 Electron microscopic observations

Control bone tumor cells have ultramicrostructural features of heterogeneous nuclear chromatin. After internal irradiation with ^{153}Sm-EDTMP, tumor cells could show the fragmentation of chromatin, the margination of condensed chromatin and the membrane-bounded apoptotic bodies.

3.3 DNA chain fragmentation

It can be seen from Table 1 that the fraction of DNA chain fragmentation in bone tumor cells increased progressively while the interval of ^{153}Sm-EDTMP internal irradiation prolonged gradually. Especially in the case of 9 to 24 h after ^{153}Sm-EDTMP internal irradiation, fraction of DNA fragmentation for bone tumor cells increased significantly.

Table 1 Quantitative changes of DNA fragmentation for bone tumor cells treated with ^{153}Sm-EDTMP

t (h)	Percent of DNA chain fragmentation	
	Control	^{153}Sm-EDTMP
3	5.9±0.5	6.2±0.8
6	6.5±0.8	8.9±1.6
9	10.7±1.2	34.9±2.5*
12	13.1±4.8	44.6±3.8*
24	14.5±5.3	77.2±7.8*

* $p<0.01$

3.4 Agarose gel electrophoretic observations

Agarose gel electrophoresis illustrated the DNA fragmentation such as ladder pattern formation in bone tumor cells, irradiated by ^{153}Sm-EDTMP after different intervals. The internucleosomal DNA damage in bone tumor cells were readily detected 9 h after exposure with ^{153}Sm-EDTMP, in which a typical ladder of DNA was seen. In comparison, no obvious DNA ladder in bone tumor cells treated with ^{153}Sm-EDTMP less than 6 h was observed.

3.5 Microautoradiographic observations

The microautoradiographic study showed that ^{153}Sm-EDTMP could penetrate through cell membrane.

Observation displayed membrane-seeking condensation with tracks of ^{153}Sm-EDTMP in bone tumor cells. The autoradiographic tracks indicated that ^{153}Sm-EDTMP could be phagocytized by bone tumor cells. The tracks of ^{153}Sm-EDTMP were distributed in cytoplasm and nucleus in the form of phagosome. The autoradiographic tracks of ^{153}Sm-EDTMP were also observed in membrane-bounded apoptotic bodies.

3.6 Inhibition rate of proliferation

From experimental results, the inhibition rate of proliferation of bone tumor cells after internal irradiation with ^{153}Sm-EDTMP for different intervals is shown in Table 2. The inhibition rate of proliferation of bone tumor cells increased progressively with prolonging the time of ^{153}Sm-EDTMP internal irradiation. Especially, the inhibition rate of proliferation of bone tumor cells increased significantly after treatment with ^{153}Sm-EDTMP for 6 h to 24 h.

Table 2 The inhibition rate of proliferation of bone tumor cells after internal irradiation with ^{153}Sm-EDTMP for different intervals

t (h)	MTT, A_{540}		Inhibition rate (%)
	Control	^{153}Sm-EDTMP	
0	0.45±0.07	0.45±0.07	—
3	0.57±0.01	0.53±0.02	7.1
6	0.62±0.02	0.54±0.08	12.9*
9	0.65±0.03	0.50±0.14	23.1**
12	0.71±0.05	0.43±0.12	39.4**
24	0.78±0.03	0.29±0.04	62.8**

* $p<0.05$, ** $p<0.01$

4 Discussion

Previous study was directed towards the external radiation induced apoptosis in cells.[13] In present study, bone tumor cells were internally irradiated with ^{153}Sm-EDTMP. After irradiations the apoptotic changes in these cells displayed significant nuclear fragmentation, and marked pyknosis in nuclei, as well as apoptotic bodies formation. The internucleosomal fragmentation of DNA in bone tumor cells, which re-

sulted in a ladder type pattern comprising 180 base pair intervals in agarose gel electrophoresis, was a key molecular event in apoptosis. Recent data provided strong evidence that bone tumor cells underwent apoptosis when encountered ^{153}Sm-EDTMP. In the meantime, the microautoradiographic study showed that ^{153}Sm-EDTMP could permeate through cell membrane and displayed membrane-seeking condensation in bone tumor cells. Thereafter, ^{153}Sm-EDTMP could be phagocytized and distributed in cytoplasm and nucleus in the form of phagosome. At the same time, the membrane-bounded apoptotic bodies were observed. Our experimental study indicated that progression of apoptosis of bone tumor cells induced by ^{153}Sm-EDTMP was dependent on the ^{153}Sm-EDTMP exposure time.

As a rule, the longer the irradiation time, the greater the inhibition rate of cell proliferation. The present study exactly showed that with the prolongation of internal irradiation time by ^{153}Sm-EDTMP, the inhibition rate of proliferation of bone tumor cells increased progressively.

References

1 Billings E, Hosain F. Clin Nucl Med, 1990, **15**(2): 364-370

2 Nielsen O S, Munro A J, Tannock I F. J Clin Oncol, 1991, **9**(3): 509-512

3 Coyle N, Adelhardt J, Foley K M. J Pain Sympt Manage, 1990, **5**(1): 83-89

4 Zhu S P. Radiotoxicology, 2nd ed, Beijing: Atomic Energy Press, 1992, 57-63

5 Lattimer J C, Corwin L A, Stapleton J. J Nucl Med, 1990, **31**(8): 1316-1321

6 Zhu S P, Lai G H, Wang L Y. Nucl Sci Tech, 1994, **5**(2): 93-98

7 Nicoletti I, Migliorati G, Pagliacci M C. J Immunol Methods, 1991, **139**(1): 271-276

8 Zhu S P. Autoradiographic tracing, Beijing: Atomic Energy Press, 1995, 100-126

9 Matzinger P. J Immunol Method, 1991, **145**(1): 185-191

10 Zhu S P, Wang L Y. Nucl Sci Tech, 1995, **6**(1): 18-21

11 Zhu S P. Radiotoxicology of Promethium, Beijing: Atomic Energy Press, 1994, 15-42

12 Price P, McMillan T J. Cancer Res, 1990, **50**(4): 1392-1399

13 Akagi Y, Ito K, Sawada S. Int J Radiat Biol, 1993, **64**(1): 47-53