Initial Approach to the Cellular Irradiation Injury of Human Pancreatic Carcinoma Cell Line MIA PaCa-2 by High Dose per Fraction

**Zhi-zhen WANG**
**Hai-li YU**
**Zhi-yong YUAN**
**Ping WANG**

Department of Radiation Therapy, Tianjin Medical University Cancer Institute and Hospital; Tianjin Key Laboratory of Cancer Prevention and Therapy, Tianjin 300060, China.

**OBJECTIVE**
To investigate an initial approach of radiotherapy, which produces cellular radiation injury by high dose in one fraction.

**METHODS**
Human pancreatic carcinoma cell line MIA PaCa-2, was cultivated and divided into 5 groups: 0, 2, 5, 10, 17 Gy. Cultivated cells were irradiated by 6MV-X ray in one fraction. Analysis were done as follows: comet assay, which assessed the level of DNA damage in the treated cells right after the cell was irradiated, flow cytometry, which was performed at 0, 6, 12, 24, 36 h after the cell line treated to asses changes of its cell cycle, DNA ladder, which quantitatively assessed the degree of DNA injury after 6 and 12 h, and histological examination, which analyzed cellular morphology after 24 h.

**RESULTS**
(1) After X-ray irradiated, the morphological change of human pancreatic carcinoma cell line (MIA PaCa-2) was mainly swelling. (2) When the dose of radiation was lower than 10 Gy, increasing the dose could greatly improve cell necrosis, apoptosis and blockage of cell cycle in G2/M phase, which was consistent with the theory of radiation biology. (3) When radiation dose was more than the 10 Gy, the peak of apoptotic necrosis appeared strong and early. (4) The degree of DNA injury was also related to the dose of radiation therapy and most obvious in the 10 Gy group and not so obvious in the 17 Gy group. (5) When dose was less than 10 Gy, DNA ladder was a single electrophoretic band; in the 10 Gy group, the electrophoresis showed a multiple ladder band; when dose was more than 10 Gy, a vague and irregular band appeared on the electrophoresis.

**CONCLUSION**
Oncotic necrosis may be the main cell death style when dose per fraction is high, which differs from conventional dose fraction radiation therapy.

**KEY WORDS:** dose-response relationship, drug, necrosis, irradiation, injury.

**Copyright © 2010 by Tianjin Medical University Cancer Institute & Hospital and Springer**

**Introduction**

In cancer therapy, prognosis of the patients is directly related to accumulated dose of radiation in cancer cells. Data from animal and clinical research offer an S-curve showing the relationship between tumor control rate and accumulated dose in many solid tumors. This means that an increased total dosage doesn’t result in an increased tumor...
control rate when the total dosage is only considered.

Because of acute and subacute injury, the mode of short-time high dose per fraction radiation therapy had excellent efficacy\(^1\). As dose per fraction is increased, clinical effectiveness is also increased. It is absolutely important to approach the suitable value of dose per fraction and to explore the injury mechanism to tumor injury of this mode. In this study, human pancreatic carcinoma cell line, MIA PaCa-2, was cultivated and divided into 5 groups, respectively treated with 0, 2, 5, 10, 17 Gy to find out an initial approach, producing biological injury, which high dose per fraction of radiation can achieve.

Materials and Methods

Cell culture

MIA PaCa-2 was cultured in RPMI 1640 medium, containing 10% fetal bovine serum (Gibco, Co.). When cell density reached to 5 × 10^5/mL in the exponential phase of growth, cultivated cells were irradiated by 6MV-X ray in 1 fraction produced by medical processing accelerator and then divided into 5 groups: 0, 2, 5, 10, 17 Gy. Continuously, the cells in the 5 groups were cultured. At 0, 6, 12, 24, 36 h after irradiated, the cells were harvested.

Observation under light microscope

Twenty-four hours after the radiation, cell morphologic change was observed and pictures were taken by inverted microscope.

DNA staining

Fixed cells were washed 2 times with phosphate buffered saline (PBS), and then incubated with phosphocitrate balanced solution (PC) in an incubator for 30 min. After being washed again, the incubated cells were added 400 μL solution (PI 50 μg/mL, RNase A 50 μg/mL) and then stained in the dark, at room temperature for 30 min followed by being analyzed.

Flow cytometry

After successfully fluorescent labeling, the flow cytometry of the cultured cells was performed using FACSort (Becton Dickinson, Co.) equipped with laser excitation at 488 nm, and then cellular fluorescence intensity was quantitatively analyzed by Cellquest.

Alkaline singlet-cell gel electrophoresis

On the basis of Oliver comet analytic process, single-strand break in the cellular DNA was analyzed. MIA PaCa-2 in each group was collected instantly after irradiation and then digested by trypsin. They were continuously washed 3 times with precooling PBS, and the supernatant was removed. After that the cells were suspended again with precooling PBS and cell density was modulated to 5 × 10^6/mL with a counting plate under a microscope. Two-layer gelatum (first layer: 100 μL of 0.75% normal melting point gelatum; second layer: 75 μL of 0.75% low melting point gelatum) was mixed with 25 μL of MIA PaCa-2 and then was cooled at 4°C in a refrigerator for electrophoresis. Then the slides were dipped into fresh alkaline lysate for 1.5 h. The alkaline lysate was prepared as follows: 58.40 g NaCl + 5.58 g Na2EDTA + 5.00 g sarcosine + 0.79 g Tris-HCL + 5 mL TritonX-100 + 50 g dimethyl sulfoxide before applying, solution end-volume to 500 ml with double distilled water, pH = 10.0, kept at 4°C. Then the slides were moved to dip again for 20 min in electrophoretic solution, including 0.37 g Na2EDTA, 7.10 g Tris-HCL, 2.78 g boric acid, and double distilled water made the solution end volume of 500 ml, with pH = 13.5, which was precooled at 4°C before application. After the process, electrophoresis continued for 25 min (voltage 20 V, electric current 200 mA). When electrophoresis finished, superfluous electrophoretic solution was washed out with distilled water. It was neutralized every 10 min for 2-3 times with precooling Tris balanced solution (0.4 mmol/L, pH = 7.5), and then stained with 2 μg/mL bromination in the dark followed by immediate observation under the fluorescence microscope (excitation wave 515-560 nm). Pictures were collected to be analyzed with CASP software.

DNA ladder

At 0 and 6 h after irradiation, MIA PaCa-2 in each group was collected to be digested by Tryspin, and then centrifuged for 8 min, at 800 rpm, and continuously washed 3 times with precooling PBS buffer solution. The supernatant was removed. DNA was extracted with E.Z.N.A. blood DNA extraction kit D3392-01 (50 prep), and then kept at -20°C. The extracted DNA was mixed with TE buffer solution and then 5 μL of the mixture was added to 1.5% agarose gel for electrophoresis (voltage drop 1-5V/cm), which showed ladder in proper distance on agarose gel. It was then observed under ultraviolet lamp and the pictures were taken with it.

Statistical analysis

Two-sample t test was performed with SPSS11.5 software to determine the 2 population means. A P value less than 0.05 was considered statistically significant.

Results

Observation under light microscope

MIA PaCa-2 morphological change

MIA PaCa-2 in the 0 Gy group grew very well observed under inverted microscope. After MIA PaCa-2 was irradiated with 2-17 Gy in 1 fraction and then cultured for 24 h, cell volume increased with obvious
morphologic changes: the morphology of many cells changed from spherical to fusiform or irregular, which looked like network structure of neurocytes. Others presented obvious swelling (Fig.1).

**Flow cytometry**

**Analysis of apoptotic percentage of MIA PaCa-2**

Flow cytometry was done with PI method when MIA PaCa-2 was irradiated by different doses and after irradiated, MIA PaCa-2 was continuously cultured for different time frames. After the irradiation, different groups showed different apoptotic peak values at different time. The percentages of spontaneous apoptosis of MIA PaCa-2 in the 0 Gy group was 0%, however, the percentage in 2, 5, 10, 17 Gy groups respectively increased with the increased doses. This demonstrated the relationship of effect-dose dependence.

As radiation dose increased, appearance of the peak of MIA PaCa-2 apoptotic percentage was delayed. Particularly at 12 h, the peak of MIA PaCa-2 apoptotic percentage in 17 Gy group appeared earlier than those in the other groups (Table 1).

**Non-apoptotic cell phase analysis in cell cycle**

All the groups showed that the MIA PaCa-2 cells in G1 phase kept decreasing, and those in G2/M and S phases increased (Table 2, Fig.2). After the irradiation, the blockage of MIA PaCa-2 cells in G2/M phase in 2 Gy and 5 Gy groups occurred approximately at 12 h, and that in 10 Gy group occurred later than 36 h. It was abnormal that the blockage in 17 Gy group occurred approximately at 24 h.

As radiation dose increased, the blockage of G2/M phase delayed in the lower dose groups. But the blockage was earlier than the MIA PaCa-2 apoptotic peak in

![Morphologic change of MIA PaCa-2 cell necrosis was on the base of swelling and more obvious followed larger dosage in one fraction. That means effect dose dependence.](image)

*Fig.1. MIA PaCa-2 cell morphologic change after MIA PaCa-2 was irradiated 2-17 Gy in one fraction and cultured for 24 h (× 100).*

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0 Gy</th>
<th>2 Gy</th>
<th>5 Gy</th>
<th>10 Gy</th>
<th>17 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>1.1 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>0.5 ± 0.12*</td>
<td>0.1 ± 0.02</td>
<td>4.6 ± 0.9**</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>1.2 ± 0.35**</td>
<td>2.6 ± 0.7**</td>
<td>6.0 ± 1.2**</td>
<td>1.0 ± 0.3**</td>
</tr>
<tr>
<td>36</td>
<td>0</td>
<td>0.5 ± 0.13**</td>
<td>1.9 ± 0.5**</td>
<td>8.6 ± 1.6**</td>
<td>0.6 ± 0.1**</td>
</tr>
</tbody>
</table>

Note: compare with control group *P < 0.05, **P < 0.01

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0 Gy</th>
<th>2 Gy</th>
<th>5 Gy</th>
<th>10 Gy</th>
<th>17 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>11.7 ± 1.6</td>
<td>16.8 ± 2.7*</td>
<td>17.3 ± 2.9*</td>
<td>12.4 ± 2.0</td>
<td>10.4 ± 0.9</td>
</tr>
<tr>
<td>12</td>
<td>15.2 ± 6.5</td>
<td>31.8 ± 0.4*</td>
<td>35.3 ± 1.5**</td>
<td>36.6 ± 5.2*</td>
<td>30.4 ± 2.9*</td>
</tr>
<tr>
<td>24</td>
<td>16.5 ± 0.4</td>
<td>14.5 ± 0.8*</td>
<td>26.3 ± 0.5**</td>
<td>69.8 ± 0.9**</td>
<td>71.7 ± 1.2**</td>
</tr>
<tr>
<td>36</td>
<td>10.4 ± 1.1</td>
<td>18.8 ± 4.9*</td>
<td>25.6 ± 3.2**</td>
<td>76.2 ± 6.4**</td>
<td>42.5 ± 0.6**</td>
</tr>
</tbody>
</table>

Note: compare with control group *P < 0.05, **P < 0.01
all groups. In the 17 Gy group, the blockage of G2/M phase occurred at approximately 24 h which was later than the MIA PaCa-2 apoptotic peak.

Alkaline single cell gel electrophoresis
Example pictures of Comet, see Fig.2.

DNA injury in MIA PaCa-2 after irradiated with different doses
Fig.3. shows the change of tail moments after irradiated with different doses. As radiation dose increased, DNA injury in MIA PaCa-2 gradually aggravated in 0-10 Gy groups and extenuated in 17 Gy group. The difference was significant, when comparing tail moment in 0 Gy with that in each of the other groups ($P < 0.05$). When comparing the tail moment in the 10 group with that in each of the other groups, the difference was significant. Data of DNA injury is shown in Table 3.

DNA-ladder
Fig.4 (electropherogram) shows biochemistry detection of DNA in apoptotic cells. With the radiation dose increased, lytic fragments of DNA were also increased. Apoptotic cells presented typical DNA ladder bands and were analogous with standard DNA markers in 10 Gy group. When radiation dose continued to increase, many bands were vague and not obvious compared with that of cellular necroses.

Discussion
In 1995, Majno and Joris first introduced oncosis, a cell injury process with caryolysis and cellular swelling. Oncosis was defined by Majno as a cellular death process composed of increased cellular membrane permeability, destruction of integrated cellular membrane, DNA schizolysis into non-specific fragments, and final cytolysis, and its whole process was accompanied with an inflammatory reaction[1].

In 1997, the Cell Death Nomenclature Committee of American Society of Toxicology Pathologists suggested

<table>
<thead>
<tr>
<th>Dose</th>
<th>0 Gy</th>
<th>2 Gy</th>
<th>5 Gy</th>
<th>10 Gy</th>
<th>17 Gy</th>
</tr>
</thead>
</table>

Fig.2. Example picture of Comet.

Fig.3. Tail moment in MIA PaCa-2 after irradiated with different doses.

Fig.4. DNA-ladder electropherogram.
Deng et al. [2] observed death modes and ultrastructural changes of the brain neurons of heroin-addicted rats with electron microscopy. They found that apoptosis and oncosis both occurred. Cornelissen et al. [3] confirmed that oncosis was the main cellular death mode experienced after high dose irradiation.

The morphological changes of cellular oncosis are swelling, increased cellular volume, cytoplasm vacuolization, dense granules in the cytoplasm, destruction of the integrated cellular membrane, and finally lytic necrosis. Because the content of the cytoplasm dissolves and extends, an inflammatory reaction must occur outside of the cell. In contrast, the morphological changes of cellular necrosis are the scattering of cellular arrangement, no normal cellular connection, condensed cytoplasm, decreased cellular volume, karyopycnosis, chromatin agglutination and side-collection, nuclear fragmentation, and finally formation of an apoptotic body. The cellular membrane of the apoptotic body is integrated and organelles inside are not accompanied with a surrounding inflammatory reaction. Oncosis is usually a passive cellular death mode originated by ischemia, hypoxia, heat shock and toxic stimulus. Its morphological change resembles cloudy swelling of the cells, hydro-degeneration or ballooning degeneration. Then lytic necrosis finally occurs [4-9].

Apoptosis and oncosis are two different modes of cellular death during the earlier period. However, they both eventually develop into necrosis.

After MIA PaCa-2 was irradiated with 2-17 Gy in 1 fraction and then cultured for 24 h, cell volume increased with obvious morphologic changes: the morphology of many cells changed from spherical to fusiform or irregular, which looked like network structure of neurocytes. Others presented obvious swelling. Morphological cellular change was mainly necrosis on the base of swelling. There is an inverse relationship between necrosis and the radiation dose.

Gao et al. [10] studied morphological change of cell differentiation in rat adrenal medullosuprarenoma (PC12) and survival of the studied mice after irradiated with high doses (0, 8, 16, 32 Gy) of γ-ray with light microscope and electron microscopy. Following the radiation dose increased, cellular necrosis increased but apoptosis decreased. Cellular necrosis occurred on the base of swelling. It was demonstrated by electron microscopy that the characteristic morphologic changes (caryolysis, swelling cytoplasm) were similar to those of oncosis. They included obvious swelling cell organs, expanding rough endoplasmic reticulum, swelling and cavitation of mitochondria, condensed chromatin and widening of the intermembrane space of karyotheca. These changes are consistent with the findings observed under the light microscope. Oncosis might be the main mode of cellular necrosis induced by high dose in 1 fraction.

Apoptosis is the main necrotic style in traditional radiation therapy. After irradiated by X-ray, all of the tumor cells are not immediately injured, however, they die by means of apoptosis. Inside the cell checkpoint, there is an inherent monitoring mechanism, guaranteeing fidelity of cell duplication. Because of this, DNA injury and mutation can be detected. The damaged cell is blocked and repaired at corresponding checkpoints in the cell cycle. If DNA repair fails, cellular apoptosis can occur.

Some studies [11-14] showed that the percentage of the spontaneous cellular apoptosis depended on the time and dose of the tumor irradiated in several tumor cell lines (e.g. MIA Panco-1), and the peak of spontaneous cellular apoptosis was different in different cell lines.

Zheng et al. [14] irradiated cylindromatous SACC-83 cells in vitro with different doses (2, 5, 10, 15, 20, 25, 30 Gy) of X-ray. They found cellular apoptosis at 2-15 Gy and obvious necrosis at more than 15 Gy. Fifteen Gy was a lethal dose to SACC-83 cells in vitro. Morphologically, the cells showed necrosis at 20-30 Gy, transition between necrosis and apoptosis at 10-15 Gy, simple apoptosis at 10 Gy and transition between apoptosis and original tumor cell at 2-5 Gy. At more than 15 Gy, cellular colony forming efficiency and colony proliferation all vanished. At this time, there were few living cells. It was illustrated that the irradiation effect on cylindromatous SACC-83 cells was cellular apoptosis and direct cytotoxic action. As the single dose was boosted, necrosis became more obvious [15-17].

In our study, when MIA PaCa-2 was irradiated with different doses and then continuously cultured during 12 h, the percentage of the spontaneous cellular apoptosis increased by degrees following the dose being increased and the highest percentage was in the 17 Gy group. After 12 h, the percentage of the spontaneous cellular apoptosis decreased steadily in the 17 Gy group and increased continuously in the other groups.

In the 17 Gy group, cellular apoptosis and blockage in G1/M of cell cycle appeared earlier and more obviously than those in the other groups. It was suggested that the DNA injury in the 17 Gy group was so severe as to easily activate the check point of the cell cycle. With this phenomenon, apoptosis was not the exclusive mechanism causing cell death. Cell necrosis induced by high dose in single fraction may greatly account for the cell death. When major cells directly became necrosis, the number of remnant living cells decreased, which impacted the relative value of apoptosis. On the surface, apoptosis in the high dose group was less than in the low dose group. Considering major necrotic cells, the radiological damage should be more obvious. Apoptotic
peak occurred quickly a short time after irradiated with high dose. In this period of time, cellular apoptosis and blockage were increased. At the same time, intense and quick necrosis made the major cells start to leave the cell cycle, which led the cells entering the apoptotic procedure. As the number of cell blockage decreased, apoptotic percentage was lowered.

Based on DNA injury, this article has confirmed the deduction about oncoric injury mechanism.

Klassen\(^{[18]}\) proposed that apoptosis was an active process which required gene expression, protein synthesis and energy. When irradiated at more than 10 Gy, cellular membrane integrity and genetic transcription were destroyed. Ion gradient in the microenvironment between the inner membrane and outer membrane couldn’t be kept stable, therefore, the apoptotic mechanism was not switched on. Thus oncosis is usually a passive cellular death mode after irradiated with high dose. With the same stimulus, the cellular death mode, apoptosis or oncosis, are determined by amount of ATP. If energy is sufficient, apoptosis cannot complete its normal procedure. In this way cellular oncosis occurs.

It is concluded that the quick and severe cellular death evident in the high dose group is oncoric necrosis. Because high dose radiation results in vast wide-bound cellular injury, and DNA injury induced by high dose radiation is more significant than that induced by low dose radiation. In the cellular microenvironment, ATP was not supplied adequately in a short time, therefore, passive cellular oncosis occurred. It is difficult to explain radiation injury with traditional apoptotic theory. Direct evidence is required to prove this.

It has been reported in the literature\(^{[4,14]}\) that DNA degradation of apoptotic cells shows regular and different length of DNA fragments (also called ladders) in gel electrophoresis. When cellular oncosis and DNA degradation have an atypical DNA ladder, which is irregular and has unequal length of fragments. It is due to diffuse and random DNA fragments degraded from nucleosomes\(^{[19]}\). The results from the study led by Zheng et al.\(^{[1]}\) are consistent with this rule.

It was concluded in the present study that the change of tail moment (DNA injury) had a positive correlation with dosage (0-10 Gy) and this was extenuated in the 17 Gy group. As oncosis was the main cellular death mode in the 17 Gy group, cellular necrosis was very quick. There was little DNA injury detected because of only a few remnant apoptotic cells.

Accompanying an increase of dose, lytic fragments of DNA were increased shown in gel electrophoresis. Apoptotic bands presented a typical ladder in the 10 Gy group, which was analogous with the standard DNA marker. When the radiation dose increased, the bands became vague and not obvious in irregular, lamellar shadow. This fits with theoretical deduction\(^{[11]}\) and findings reported in the literature\(^{[4]}\).

Some studies\(^{[20-23]}\) have shown that treatment through inducing oncotic necrosis of tumor cells can effectively control tumors, if apoptosis process has been inhibited. Oncosis is an important factor in the process of tumor occurrence and development.

**Conflict of interest statement**

No potential conflicts of interest were disclosed.

**References**

15. Cao RF, Cao YX, Zhang X, et al. Effect of γ ray irradiation of different doses on glioma cell lines culture in vitro and the protection of γ ray irradiation to neuro-