Activation of Caspase-3 in Radioinduced Apoptosis in Developing Brain

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ACTIVATION OF CASPASE-3 IN RADIOINDUCED APOPTOSIS IN DEVELOPING BRAIN

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ABSTRACT

ICE/ced-3 related proteases (caspases) have been implicated in programmed cell death in a wide variety of cell types. However, their roles in radiation-induced cell death in cultures of mixed, neuronal and glial precursors cells are poorly understood. In order to further elucidate the molecular mechanisms underlying radiation induced death in this system; we have examined the ability of ionizing radiation to induce cell death and the caspase-3 activity. Survival decreased in a dose-dependent manner 24 h after a single 0.1 to 4 Gy dose of ionizing radiation. Irradiation resulted in a significant induction in caspase-3 activity, as measured by increased cleavage of colorimetric caspase substrates. Specific inhibitor of caspase-3, zDEVD-fmk, protected only partially from radiation induced cell death. These results demonstrate that cell death occurred despite of caspase-3 inhibition, and suggest that radio-induced cell death may occur by other mechanisms.

Key Words: caspase-3, developing brain, ionizing radiation, apoptosis.

I. INTRODUCTION

Programmed cell death (PCD) usually called apoptosis, was described by Kerr et al. (1). It involves a network of metabolic events and may be triggered by a variety of biological and physical stimuli and has been implicated in normal and multiple pathological processes. (2).

Apoptotic death requires an active self-destruction program with activation of proteases and endonucleases, early chromatin condensation followed by internucleosomal DNA fragmentation and associated with cell shrinkage. Plasma membrane integrity is maintained until later stage controlled auto digestion.

The activation of the caspase family of proteases has been detected in numerous cell systems and appears to function as a common pathway through which apoptotic mechanisms may operate. Caspases are synthesized as precursors (procaspases) and are converted into mature enzymes by apoptotic signals.

There has been an increase in studies on caspases demonstrating presence of 14-16 members of this new super family. Many of them play multiple roles in the initiation and execution of cell death in normal and pathological process. (3,4).

In the normal central nervous system in development, approximately one-half of the original cells die by apoptosis. (5).

In neurons, the PCD occurs in neuro-degenerative disorders, such as Alzheimer’s disease and Parkinson’s disease, and after irradiation or metabolic deficiencies following ischaemic or epileptic injury.

The embryonic development of mammalian is highly susceptible to disturbance caused by ionizing radiation (IR). Radiation-induced injuries during the earlier implantation stages, often lead to
embryonic death. During organogenesis period results in malformations or deaths. During fetogenesis, the irradiation of developing brain causes growth retardation, small head, maturation disturbances and abnormal neuronal migration, which most likely results in long-term damage.

Epidemiological studies of the prenatally exposed survivors of the atomic bombing of Hiroshima and Nagasaki have demonstrated brain damage in humans. This damage was manifested as an increase in the frequency of mental retardation, diminished performance on intelligence testing. (6).

Gamma and X irradiation-increased apoptosis in fetal rat brain was observed in vivo and in vitro and the activation of different member of the family of caspase was associated with this process and is a relatively early event. (7).

In general it precedes or appears concomitantly with all the morphological and biochemical changes specifically associated with apoptosis. The cysteine protease caspase-3 is thought to be one of the most common mediators of mammalian apoptosis.

The activation of caspase-3 was observed in vivo in rat developing cerebellum but it was not essential for radiation-induced apoptosis.

Irradiation of primary cultured hippocampal neurons resulted in a significant induction in caspase activity, as measured by increased cleavage of fluorogenic caspase substrates. However, specific inhibitors of caspase activity (zVAD-fmk) failed to protect from radiation-induced cell death.

The fact that cell death occurred despite caspase inhibition suggests that radiation-induced neuronal cell death may occur in a caspase-independent manner. (8, 9).

Cell-culture provides an interesting model for studying the effects of different doses of IR on morphological and functional development.

Dissociated precursors of neuronal-glial brain cells, can be maintained as mixed cultures and have been extensively used to monitor the evolution of neuronal-glia interaction after single dose and fractionated X irradiation (10).

In this study, we irradiated rat primary mixed cell cultures of an age corresponding to the fetogenesis period in vivo (day 17 of gestation), when the neuronal and glial cells, are still dividing and are not fully differentiated.

From the neuroembryological viewpoint this period correspond to human period from the 8th through the 15th week following ovulation in which the radiosensibility of developing brain is maximal.(6).

The aim of this work was determine the relationship between level of apoptosis and activity of caspase-3 in rat cortical precursors cells for different doses of gamma irradiation.

Understanding the cellular mechanisms involved in radiation damage on this putative target cell population, will contribute to the understanding of the cellular effects of radiation in developing brain.

II. MATERIALS AND METHODS

Cell culture. Primary cortical cultures were prepared from the forebrain of rat embryos (Wistar) at gestational day 17 (E17). The mother was sedated with sodium pentobarbital (100 mg/kg, ip). The fetus was then surgically removed and the brain was dissected out under sterile conditions. The meningial tissue and any connective tissue were removed from the brain with the aid of the stereo dissecting microscope, to assure that only cerebral and sub cortical tissue cells were used for culture. A combined enzymatic and mechanical procedure prepared cell suspension, was employed. Briefly, cell suspensions of rat cerebral cortex were prepared by chopping the tissue, followed by digestion with 0,25% trypsyn (Gibco) and 10 μg/ml DNAse (Sigma) for 15 min.
After adding fetal calf serum, the cell suspension was centrifuged at 500 xG for 5 min. After centrifugation culture medium was added and cell were counted in the hemocytometer chamber. Dissociated cells were plated at a density of 3x10^5 cells/cm^2 on coated dishes with poly-L-lysine or 22 mm^2 cover slips and gamma irradiated.

Cultures were kept in 199 medium (Sigma) supplemented with 24 mM D-glucose; 19 mM KCl, 50 ug/ml gentamin, 2 mM L-glutamine, 100µg/ml transferrin, 20 mM progesterone, 30 mM selenium chloride, 60 µM putrescine and 25 ug/ml insulin. The cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2 in air.(11)

**Immunocytochemical studies.** Cells were fixed overnight on cover slips in 4% paraformaldehyde culture at 4 °C on days 1, 2 and 10, blocked with 5% normal goat serum in phosphate–buffered saline, and exposed to one of the following primary antibodies for 60 minutes at 37 C a) a rabbit polyclonal antibody against nestin b) glial fibrillary acidic protein (GFAP) (Dako Corp., Carpinteria, CA).(12)

**Irradiation of cultures.** For gamma irradiation, dissociated cells were plated and cultures were irradiated in growth medium at a dose rate of 0,3 Gy/min from ^60^Co source with dose ranging 0,1 to 4 Gy.

**Assay for cell viability and flow cytometric analysis.** Cell viability of primary cortical culture was quantified by trypan blue at 24 h post irradiation. Cultures were incubated with 0,4% trypan blue solution (Sigma) for 4 min. Only death cells with a damaged cell membrane are permeable to trypan blue. The number of trypan blue-permeable cells and viable white cells was counted in hemocytometer chamber.

Occurrence of apoptosis was analyzed by flow cytometry. Cells were fixed in 10 ml cold 80% (v/v) ethanol and 20% (v/v) fetal calf serum at 4 °C overnight. The cells were then centrifuged, washed and resuspended with 2 ml of PBS. 30 units of DNAase-free RNAase and 100 µg propidium iodide (PI) were added and the cells were incubated for 20 minutes under a dark condition.

The PI fluorescence of the cells was measured using about 1x10^6 cells in a FACS flow cytometer. The fraction of cells in apoptosis was estimated from the cellular DNA content The analysis was carried out with the Winmdi 2.8 program.

**DNA fragmentation analysis.** 5x10^6 cells from 4, 8, 12 and 24 h post irradiation were incubated in lysis buffer (1% sodium dodecyl sulfate (SDS), 0,2 mg/ml proteinase K, 100 mM NaCl, 20 mM Tris-HCl (pH 8) for 4 hs at 37 °C, DNA was extracted with phenol/chloroform and precipitated in ethanol. Ten micrograms of extracted DNA, suspended in TE buffer and then incubated with RNAase for 4 hs at 56 °C was loaded onto 1,6% agarose gel containing 0,09 M Tris-borate (pH 8,3), 2 mM EDTA and 0,2 µg/ml of ethidium bromide. Following electrophoresis, DNA bands were visualized and photographed on an ultraviolet transilluminator.

**Colorimetric caspase assays.** At specific times after irradiation, cells were collected in cool phosphate buffered saline solution, centrifuged 5000 x g 5 min. The cell were frozen and maintained at −70 °C until the time of assay. Caspase activity was determined using the Clontech ApoAlert TM CPP32 Assay Colorimetric Kit according to the manufacturer’s instructions (Clontech, Palo Alto, CA). The levels of relative absorbance were normalized against the protein concentration of the extract, which was determined using the Bio-Rad Protein Assay reagent.

**Caspase inhibition.** The specific peptide inhibitor of caspase-3 activity, z-Asp-Glu-Val-Asp-fmk, (zDEVD-fmk), were added to the cultures 1 hr before irradiation at 20 µM and was not removed until caspase-3 assays was performed.(9).
III. RESULTS

Developmental of fetal progenitor cells in culture.

On initial day, embryonic cells assume a small round shape with short processes, consistent with their identity as progenitor cells. On day 2 this was confirmed by positive staining for nestin, an intermediate filament present in the cytoplasm of pluripotent CNS progenitor cells. Starting from day 5 to 6 the number of GFAP positive cells increased and a decline in nestin expression occurred. By day 10 the number of GFAP-positive cells comprised 60 to 70% of the culture. (Data not shown). These data indicate that the cultured E17 cells were behaving as pluripotent stem cells, differentiating over time into cells demonstrating neuronal and glial properties.

Radiation-induced dose-dependent cell death. In the current study, primary cultures of developing brain from wild-type rat showed extensive, dose-dependent cell death after exposure to a single dose of ionizing radiation using a $^{60}\text{Co}$ source (Table 1).

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>% survival</th>
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<tbody>
<tr>
<td>0</td>
<td>92,0</td>
</tr>
<tr>
<td>0,1</td>
<td>92,0</td>
</tr>
<tr>
<td>0,25</td>
<td>90,3</td>
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<tr>
<td>0,5</td>
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<td>2</td>
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<td>3</td>
<td>44,7</td>
</tr>
<tr>
<td>4</td>
<td>10,6</td>
</tr>
</tbody>
</table>

Table 1. Survival of irradiated cells

Cells were dissociated and maintained in culture as described in the materials and methods. The cultures were irradiated with 0,1 to 4 Gy. Control cultures (0 Gy) were sham-irradiated. Cell survival was assayed 24 hr later by trypan blue method. Irradiation caused a dose dependent decline in cell survival.

Involvement of apoptosis in the process of cell death. The increase of the apoptotic process was determined by flow cytometry at different times after irradiation in control cultures (0 Gy) and in 2 Gy irradiated cells. (Table 2).
Table 2. Time course of the increase of apoptotic process

<table>
<thead>
<tr>
<th>Time aft Irradiation</th>
<th>Dose</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0 Gy</td>
</tr>
<tr>
<td>4 h</td>
<td>2,3</td>
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<tr>
<td>8 h</td>
<td>2,5</td>
</tr>
<tr>
<td>12 h</td>
<td>2,8</td>
</tr>
<tr>
<td>24 h</td>
<td>8,0</td>
</tr>
</tbody>
</table>

nd: not determined

Apoptosis was characterized by nuclear condensation, cell shrinkage, membrane blebbing, DNA fragmentation and formation of apoptotic bodies. (Figure 1).

Figure 1. Rat E17 cortical cells were plated in poly-L-lysine-coated cover slip. 24 h after irradiation the cells were observed by May Grunwald–Giemsa staining.

DNA preparation and electrophoresis. The DNA samples extracted 24 h after irradiation from $5 \times 10^6$ cells were subjected to agarose gel electrophoresis to investigate the presence of DNA fragmentation induced by apoptosis. Irradiation-dependent internucleosomal fragmentation was manifested as a ladder of oligonucleosomal of DNA fragments, which were multiples of 180-200 base pairs bands. (Figure 2). The extent of DNA fragmentation appeared maximal with 3-4 Gy of $\gamma$-rays.

Analysis of DNA fragmentation as a function of time after irradiation (3Gy), showed a low level of internucleosomal DNA fragmentation as early as 4-6 h after irradiation, (non shown). The ladder became gradually more pronounced over the period of 8-24 h after irradiation. This internucleosomal DNA fragmentation is a biochemical evidence for apoptosis rapidly induced by radiation.
Figure 2. Radiation-induced internucleosomal DNA fragmentation. DNA was extracted from the 12000 g supernatants at 24 h after irradiation and separated on a 1.6% agarose gel. Lane 1 sham-irradiated cells, lane 2, 2 Gy, lane 3, 3 Gy, lane 4, 4 Gy.

Induction and activation of caspase-3. Colorimetric assays showed that caspase-3 activity was increased after irradiation for the dose 0.25 Gy to 4 Gy. Peak induction was at 4-5 h post irradiation for dose ranging 2 to 4 Gy. The time of peak activity was not determined for dose below 2 Gy. (Table 3)

Table 3. Increase of caspase-3 activity

| Dose (Gy) | % of activity<sup>(1)</sup> |
|-----------|----------------|---|
| 0.1       | nd            |   |
| 0.25      | 16            |   |
| 0.5       | 49            |   |
| 1         | 86            |   |
| 2         | 111           |   |
| 3         | 130           |   |
| 4         | 130           |   |

<sup>(1)</sup> Increase of activity was determined respect to the control
nd: increase of activity was not detected by this method

Caspase-3 inhibition failed to completely inhibit radiation induced cell death. Basal level of caspase-3 in non irradiated cells cultures was not detected by this method. However a single dose of ionizing radiation above 0.25 Gy induced increase in caspase-3 activity after 4-5 h as measured by cleavage of the colorimetric caspase substrate DEVD-pNA. To confirm if the observed increase
in caspase-3 activity was responsible of radiation-induced cell death, the cells irradiated with 3 Gy were pretreated with zDEVD-fmk, an inhibitor of caspase-3, since 1 h before the irradiation until 4 or 24 h post irradiation. The preliminary results show that the caspase-3 activity decrease up to 80 % of top level 4 h post irradiation and the survival of cells treated with inhibitor increased only 5%, 24 h post irradiation. These findings indicate that although caspase-3 activation occurs it does not play a central role in radiation-induced cell death under these conditions.

IV. DISCUSSION

This study was undertaken to apply recently acquired knowledge about the molecular aspects of apoptosis, to the fetal forebrain, a tissue of potential interest and importance to the cell death field because of its exquisite radiosensitivity.

For many years it has been recognized that ionizing radiation has a harmful effect on mammalian development, even at quite low doses.

Recent experiments in vivo and in vitro focusing on the effects of ionizing radiation on neocortical development have demonstrated that low-dose ionizing radiation interferes with the migration of neurons (6, 13)

This study demonstrated caspase-3 activation in primary cultured neuronal and glial precursors after irradiation at different dose. However, caspase-3 inhibition had not a clear effect on survival of 3 Gy irradiated cells, which, suggests that this activation was not a central process of radiation-induced cell death at the dose studied. We cannot exclude the possibility that the necrotic processes at this dose was important and/or other caspases which are not inhibited by the substrate antagonists employed in the present study, may be involved in the radiation-induced cell death in this system.

Further analysis are in progress to study if the zDEVD-fmk inhibitor can increase de survival at dose below 3 Gy were the necrotic process is not important, or there are the others mechanisms caspase-3 independent.

V. REFERENCES


