Radiation-induced apoptosis of neural precursors cell cultures: early modulation of the response mediated by reactive oxygen and nitrogen species (ROS/RNS)

Pablo Gisone, Diana Dubner, Elizabeth Robello, Severino Michelin María del Rosario Perez.

Autoridad Regulatoria Nuclear (ARN), Gerencia de Apoyo Científico, Laboratorio de Radiopatología, Avenida del Libertador 8250, (C1429BNP) Buenos Aires, Argentina
TEL: 54 11 43 79 83 86        FAX: 54 11 43 79 84 60
e-mail: pgisone@cae.arn.gov.ar

Abstract. Apoptosis, the typical mode of radiation-induced cell death in developing Central Nervous System (CNS), is closely related with the oxidative status. Enhanced radiation-induced generation of ROS/RNS has been observed after exposures to low radiation doses leading to cellular amplification of signal transduction and further molecular and cellular radiation-responses. Moreover Nitric oxide (NO) and hydroxyl radical are implicated in domapinergic neurotoxicity in different paradigms. This study is an attempt to adress the participation of radiation-induced free radicals production, the contribution of endogenous NO generation, and the excitotoxic pathway, in the radiation-induced apoptosis of neural cortical precursors. Cortical cells obtained from at 17 gestational day (gd) were irradiated with doses from 0,2 Gy to 2 Gy at a dose-rate of 0,3 Gy/m. A significant decrease of Luminol-dependent Chemiluminescence was evident 30 m after irradiation reaching basal levels at 120 m follow for a tendence to increasing values Incubations with Superoxide Dismutase (SOD) decreased significantly the chemiluminiscence in irradiated samples. NO content estimated by measuring the stable products NO$^2$ and NO$^3$ released to the culture medium in the same period, has shown a time-dependent accumulation from 1 h post-irradiation. The apoptosis, determined 24 h post-irradiation by flow citometry, morphology and DNA fragmentation, revealed a dose-effect relationship with significant differences from 0,4 Gy. The samples pre-treated with 10 mM of N-acetyl cyteine (NAC) a precursor of intracellular GSH synthesis, shown a significant decrease of the apoptosis. Apoptosis was significantly increased in irradiated cells after inhibition of nitric oxide synthase (NOS) byL-NAME. We conclude that ROS/RNS play a pivotal role in the early signaling pathway leading to a radiation-induced cell death.

1. Introduccion

Even under very low doses of radiation, the developing brain is one of the most radiosensitives organs in view of the amounts of structural abnormalities which can be induced in this tissue

Developmental radiation-induced abnormalities of the cerebral cortex of fetuses are expressed in different ways, depending on the dose and on the gestation day of exposure. An increased prevalence of Severe Mental Retardation (SMR) has been epidemiological evidenced in children who were prenatally exposed to the atomic bombing of Hiroshima and Nagasaki. The incidence of microcephaly in the Hiroshima and Nagasaki cohort was higher among atomic bomb survivors who were exposed at 8-10 weeks of gestation. In order to supplement the knowledge of radiation-induced perturbations of brain development specifically in regards to potential mechanisms at the cellular levels, different animals models have been employed.

The animal studies have shown that prenatal irradiation results in microcephaly impairment in neurotransmitters contents, behavioural deficits in adult life and significant decreases in cortical thickness. The embrionic day 13 in mice and 15 to 17 days in rats corresponds to the highly radiosensitivity stage in humans. In these developmental periods, doses of 15 cGy are able to decelerate neuronal migration associated with a changing pattern expression of neural cell adhesion molecules. Rats exposed to 1 Gy of X rays on day 15 showed microcephaly and impaired postnatal development of dendritic spines. This high susceptibility of the developing brain might be related with the rate of membrane lipid peroxidation, the development and modulation of
NMDA receptor sites, the intracellular Ca\(^{2+}\) influx mechanism, the expression of apoptotic and antiapoptotic genes, and the activation of caspases.[14].

The responses of cells to radiation are most likely initiated by the radiation-induced free radicals production. In fact, enhanced cellular generation of ROS/RNS has been observed after exposures to low radiation doses production and could lead to cellular amplification of signal transduction and further molecular and cellular radiation-responses [15].

Nitric Oxide (NO) and hydroxyl radicals (OH) are implicated in dopaminergic neurotoxicity in different neurotoxic paradigms.[16].

In the last years growing evidence of NO as a neuroprotector is accumulating in the literature [17]. Since the effects of NO are modulated by both direct and indirect interactions that can be dependent of its content and of cell type specificity, pro-apoptotic and antiapoptotic effects has been described [18]. Low concentrations of NO can inhibit the apoptotic pathway through eGMP-dependent mechanisms, and caspases inhibition. In contrast, NO may have pro-apoptotic effects via mitochondria, DNA damage and inhibition of proteasome [18]. We have recently communicated that ionizing radiation induces an early increase of nNOS activity that correlates with a further augmentation in NO steady-state concentration playing an antioxidant role in irradiated developing rat brain.[19].

A number of studies have confirmed that radiation induced cell death in developing brain follows the apoptotic pathway [20,21] predominantly triggered by double strand breaks in the DNA.

In a similar model of irradiated neural precursor cells in vitro, we have shown that the radiation-induced programmed cell death is a caspase-3 dependent event [22].

The increased proteolytic activities of caspases might be responsible of a burst of ROS production as it has been proposed.[23].

This study is an attempt to address the participation of radiation-induced free radicals production and its behaviour, as well as the contribution of NO endogenous generation, in modulating the apoptotic response, in an in vitro model of neural cortical precursor cells exposes to Gamma radiation.

2. Material and methods

2.1 Cortical cell culture

Wistar female rats were housed with males overnight and pregnant dams, identified by sperm positive vaginal smears, were considered at gestational day (gd) 0.

Primary cell cultures were prepared according to the method of micromass described by Flint et al [24] with some modifications. Living fetuses were removed at the 17th gd and the brain was dissected out under sterile conditions. The meninges were removed and the cortical plate was collected in Ca\(^{2+}\)/Mg\(^{2+}\) free Hank’s balanced salt solution (HBSS) containing 0.25% trypsin and 10ug/ml DNAse. The tissue was digested for 15min and then mechanically dissociated by flushing through glass pipettes. The large clumps were allowed to descend to the bottom and the supernatant was centrifuged at 500xg for 5min. The pellet was redispersed in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 50ug/ml gentamine and then transferred into petri dishes or 24-well plates at a density of 10\(^6\) cells/ml.

Cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO\(_2\).

2.2 Irradiation and drug treatments

The irradiation were carried out with a \(^{60}\)Co teletherapy unit (Picker C4M60;Atomic Energy Commission/CAE). Doses from 0.2 to 2.0 Gy were given at a dose-rate of 0.3Gy/min. The exposure commenced 2h after plating in order to let cell attachment. Control samples were sham irradiated. The cells were then incubated and harvested at different time-points for different assays.

The various drugs were added to the culture medium and gently mixed. The cultures were incubated in the aforementioned conditions for the times indicated in the figures.
2.3 Immunocytochemical studies

Nestin was determined on days 2 post-irradiation (pi). Cells were fixed overnight at 4 °C on cover slips in 4 % paraformaldehyde buffered in 50 mM sodium borate at pH 9.5, blocked with 5 % fetal serum in PBS, and exposed for 60 minutes at 37 °C to the anti-nestin monoclonal antibody at 1/25 dilution (Chemicon International Inc. (Cat MAB353). Secondary antibody staining was performed by exposure to goat anti mouse IgG FITC at 1/10 dilution for 30 min at 37 °C, (Boehringer Mannheim Biochemical). Samples stained only with goat anti mouse IgG FITC in the same conditions were used as negative controls.

For the determination of glial fibrillary acidic protein (GFAP) the cells were fixed in precooled methanol for 10 min at -20°C. The cells were covered with the specific monoclonal antibody (clone G-A-5) (Boehringer Mannheim Biochemical) for 30 min at room temperature. Secondary antibody staining was performed by exposure to goat anti mouse IgG FITC at 1/10 dilution for 30 min at 37 °C (Boehringer Mannheim Biochemical). Samples exposed only with goat anti mouse IgG FITC in the same conditions were used as negative controls.

2-4 Morphological features

Apoptotic cells were identified using conventional morphological criterio after staining with May Grunwald-Giemsa according to standard procedure.

In order to discriminate between cells in the process of apoptosis, living and dead (either necrotic or apoptotic) cells, a mixture of three fluorescent dyes, according to Piñero et al [25] was used. Briefly, 500ul of cells suspension (3 x 10^6 cells/ml) were incubated with 10ul of the fluorescent mix prepared in PBS ph 8, containing 2ug/ml of Hoescht 33342 (H), 15ug/ml of fluorescein diacetate (FDA), and 5ug/ml of propidium iodide (PI). After 5 minute-incubation and once washed, samples were dropped onto slide and observed by a fluorescence microscopy (Carl Zeiss™ MC 80 DX).

2-5 DNA fragmentation in agarose gels

Cortical cells (5 x 10^6) were lysed in a buffer containing 1% sodium dodecyl sulfate, 100mM NaCl, 20mM Tris-HCl (ph 8) and 20mM EDTA for 30min in an ice-cold bath and centrifuged at 27000xg for 15min at 4°C. The soluble DNA recovered in the supernatant was incubated with 100ug/ml Proteinase K for 3h at 56°C and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The nucleic acids were precipitated with 2.5vol of ice-cold ethanol in the presence of 0.3M ammonium acetate (final concentration). DNA pellet was resuspended in 10ul of TE buffer and incubated with 3ul of 200ug/ml DNAsse-free RNAse at 56°C for 1h. Electrophoresis was carried out in TAE buffer (ph8.3) after loading equal concentration of DNA (10ug) onto wells of 1.8% agarosa gels added with 0.2ug/ml ethidium bromide. DNA fragments were visualized and photographed on a UV transilluminator.

2-6 Analysis of apoptosis rate by flow cytometry

The assay was performed as described by Darzynkiewicz et al [26] with minor modifications. Briefly, 1x10^6 cells were collected from dishes and fixed in 70% ethanol at 4°C for 2h. After centrifuging, cells were washed and resuspended in PBS containing 50ug/ml RNAsse and 40ug/ml propidium iodide (PI). After 30min at room temperature, the apoptosis rate was analyzed using a Becton Dickinson® FACStar Plus flow cytometer. Apoptotic cells were identified in a DNA histogram as a sub-G1 hypodiploid population.

2-7 Measurement of NO production

To ascertain the NO generation, nitrite/nitrate levels were measured in the culture supernatant. Nitrate was reduced according to Verdon et al. [27] , before assaying the resultant nitrite using the Griess reaction.
2-8 Measurement of intracellular ROS production

a) Chemiluminescence measurement
Luminol–dependent chemiluminescence was measured in a Packard 1500 Tri-CARB® Liquid Scintillation Analyzer in the single photon counting mode. Stock solution of Lum (2mM) was prepared by dissolving in 0.1M bicarbonate and was stored in the dark at 4°C. The reaction mixture included 1x 10^6 cells, 250ul of luminol stock solution and bicarbonate 0.1M (final concentration 25mM, pH 8.5) in a 5ml total volume of PBS.

b) Fluorescent assays
Two oxidation – sensitive fluorescent dyes 2’,7’ Dichlorodihydrofluorescein diacetate (H_2DCFDA) and dihydroethidium (DHE) were employed. H_2DCFDA was reported to be preferential for hydrogen peroxide and DHE was used as an indicator of superoxide anion. For the determination of hydrogen peroxide cells were washed in PBS and then H_2DCFDA was added to a final concentration of 10µM for 30 min at 37°C. The cells were analysed by FACS with excitation at 488 and emission at 530nm. For determination of superoxide anion cells were washed in PBS and stained with DHE 5µM at 37°C for 30min. The cells were examined by FACS with excitation at 488 and emission at 610nm.

2-9 Statistical
All data are represented as means +/- SEM. Statistical analysis was performed using the Student t-test.

3. Results

3-1 Immunocytochemical studies
Cell cultured from the embryonic rat forebrain showed positive staining (90%) for nestin, an intermediate filament present in the cytoplasm of pluripotential central nervous system progenitor cells, and negativity for GFAP a glial fibrillary acidic protein present in astrocytes. This confirmed that cells cultured on 17 GD were neural precursors.

3-2 Apoptosis
In this study, at first, we characterized the apoptotic response of the system. The morphological changes observed in irradiated samples were those typical of apoptotic death, and included chromatin condensation with nuclear shrinkage and fragmentation. Conventional agarose gel electrophoresis showed the characteristic DNA ladder pattern of 200 bp owing to internucleosomal fragmentation. Apoptosis quantitation was assessed by flow cytometric measurement of DNA content after fixation and PI staining and by fluorescence microscopy. In this case, and after staining cells suspensions with three fluorescent dyes, apoptotic cells exhibited a green cytoplasm and blue spotted nuclear bodies. The apoptotic response to radiation injury revealed a dose-effect relationship, showing a significant difference starting from 0.4 Gy (Fig. 1). Figure 2 shows the time-course of apoptotic death up to 20 hours after sham or irradiation procedure. Even though the spontaneous rate increased progressively in control samples during the studied period, the irradiated cells showed a significant higher rate for a 2 Gy dose since 4 hours pi.
FIG. 1. Radiation induced apoptosis. Dose-effect relationship

The effect of pretreatment with N-acetyl cysteine (NAC) a precursor of intracellular GSH synthesis on radiation induced apoptosis was evaluated. The treatment with 10mM NAC from 24h before irradiation up to 24h pi, blocked apoptosis in the irradiated samples. Inversely incubation of the cells with 0.1mM BSO from 24h pi resulted in a significant increase in the apoptosis rate. No significant changes were observed in the control cells treated with either of the two drugs (Fig 3).

When the cultured cells were incubated in the presence of No-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS, a significant increase of the apoptosis rate could be detected (Fig 4).

FIG. 3. Effect of NAC and BSO on radiation induced apoptosis
Significant differences from 2Gy: * p<0.01, **p<0.001

3-3 NO production.
We then examined NO content, estimated by measuring the stable oxidation products NO₂⁻ and NO₃⁻, released to the culture medium. Exposure of cells to gamma radiation stimulated NO production, from 1h pi, and thereafter, a time-dependent accumulation was observed through the early phase of the response studied (Fig 5).

FIG. 4. Effect of L-NAME 0.5mM and NA 0.1mM on radiation induced apoptosis. The drugs were added 2h prior to irradiation up to 24h pi
Significant differences from 2Gy: * p<0.01
3-4 ROS production
The intracellular ROS production measured by chemiluminescence showed a significant decrease in the irradiated samples up to 1 hour post-irradiation and then tended to normalize compared to control cells (Fig 6).
When precursor cortical cells were incubated in the presence of L-NAME, the initial decrease observed in the irradiated samples disappeared and no changes with respect to control values were detected up to 4 hours post-irradiation (hpi) where gradually increased to about three-fold by 6 hours post-irradiation (hpi) (Fig 6).

Examining the formation of superoxide anion by DHE revealed that the irradiated samples showed lower levels than the control ones since 2 hours post-irradiation (hpi) whereas the presence of L-NAME significantly enhanced the superoxide anion levels up to 2 hours post-irradiation (hpi). These changes suggest that the early generation of NO could be scavenging the superoxide anion resulting in peroxynitrite production. (Fig 7).
When it was examined the time dependency of hydrogen peroxide levels by DCF staining it was found that the irradiated cells had lower levels than control ones during the first two hours pi and the inhibition of NO production did not change the pattern. By 4h pi, the addition of L-NAME resulted in a small but detectable increase (Fig 8). These results suggest that in the early phase, the superoxide anion is not being converted to hydrogen peroxide by superoxide dismutase (SOD).

**FIG. 7.** Time course of DHE fluorescence intensity following irradiation. A: Control, B: 2Gy, C: 2Gy + 0.5mM L-NAME
Significant difference from control: *p<0.05
Significant difference from 2Gy: **p<0.05

**FIG. 8.** Time course of H₂DCFDA fluorescence intensity following irradiation. A: Control, B: 2Gy, C: 2Gy + 0.5mM L-NAME
Significant difference from control: *p<0.05
Significant difference from 2Gy: **p<0.05

Taken together these results indicate that NO and superoxide anion are the main species playing a role in the oxidative status of precursor neural cells in the first hours following irradiation. As it has been reported that caspase activation during the apoptotic process may lie on a pathway leading to generation of ROS [23] the effect of zDEVD-fmk a caspase inhibitor was tested. Interestingly, zDEVD-fmk prevented the increase of reactive species induced by radiation since 2h pi (Fig.6).

4.Discussion

The effects of ionising radiation on cellular response systems are mediated through the interaction of radicals and reactive oxygen and nitrogen species. In this study, our goal was to investigate the relationship between oxidative stress, and endogenous NO production in the radiation induced early signalling related with programmed cell death in a cortical precursor cell cultures model, irradiated with Gamma radiation. Within the complex network of cell signals elicited by ionising radiation the cell pathway controlling cell survival has a key role.

Our present results revealed that following 2 Gy of Gamma irradiation, the augmentation of the apoptotic fraction was already present at 4h reaching the maximum at 24h pi. This pattern could be related to the fact that as it has been reported, Gamma radiation is able to induce an early increased level of p53 protein and a down-regulation of IGF-I levels in cells cultures from developing brain as well an in vivo models which could lead to cell death by apoptosis [29,30].

Moreover, we have previously shown in an in vivo model of prenatal irradiation of developing brain an early augmentation in nNOS activity that correlates with a further increase in NO steady-state concentration with antioxidant effects [19]. NO is a free radical and, hence, has a relative short half-life due to its reactivity with other intracellular constituents [31]. Likewise, NO is able to react and scavenge highly reactive oxygen species i.e, superoxide anion (O₂⁻) and Hydroxyl radicals (OH⁻) and converts them into
non radicals such nitrites and nitrates [32]. In physiological systems such nitrate is the major NO-derived metabolite. [33].

We observed in this in vitro model a significant increase of Nitrite/Nitrate concentration that was evident from 30 min p.i. to 6 h p.i and whose time-dependent accumulation might result from their stability in vitro systems [34]. Since this finding is the indirect evidence of the augmentation of NO production and that on the other hand, the oxidative status assessed by QL showed significant decreased levels up to 60 min after irradiation, our results suggest that NO could have in this in vitro model, a neuroprotective role early after irradiation.

A transient generation of reactive ROS/RNS within few minutes after irradiation has been demonstrated in several systems [35]. While it is reasonable to assume that, these reactive species are generated from the primary ionizations events and according to several reports the hydroxil radical would be the major component [36], secondary ROS products are also generated in latest time-points as a consequence of extranuclear amplification mechanisms, particularly superoxide anion and hydrogen peroxide [37].

Incubations with L-NAME and SOD allowed to demonstrate the increased production of the $\text{O}_2^-$ performed by dihydroethidium, a fluorescent dye sensitive to superoxide anion, up to 2 h p.i. in the irradiated sample. Then, it is possible to conclude that during the early phase of the response, the radiation-induced generation of endogenous NO is responsible of the scavenger effect on the $\text{O}_2^-$. Similarly, incubation with L-NAME was able to modify the apoptotic response in the irradiated samples by increasing significantly the apoptotic levels 24 h post-irradiation.

It has been described that low at moderates levels of NO can increase $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ production by inhibiting mitochondrial respiration, whereas at high level it inhibits $\text{H}_2\text{O}_2$ production by scavenging the precursor superoxides [38]. In our experiments incubation with L-NAME seems do not modify the low level of $\text{H}_2\text{O}_2$ found at early times in the irradiated sample related with its control ones. The difference between the increase of $\text{O}_2^-$ and the decrease of $\text{H}_2\text{O}_2$ might reflect the activity of peroxidases such as glutathion peroxidase.

In a previous work we have demonstrated a dose-dependent radiation induced activation of caspase-3 detectable by 2 h p.i. and showing a peak at 4 h coinciding with the beginning of the apoptosis [22].

In this sense, the later increase of ROS is coincident with the augmentation of the Caspase-3 activity. The efficacy of caspase inhibitor zDEVD-fmk to reduce the free radicals production in our experiment suggest the possibility that this secondary burst of ROS production would be directly dependent of the caspase-3 activation. These results are consisting with those communicated by Chen et al. [39]. Likewise it has been described a two-step process for cyt c release during radiation-induced apoptosis and a link between ROS production and mitochondrial cyt c depletion. This fact could explain the coincidence observed between the start of the apoptotic process and the late ROS burst.

GSH is the most abundant thiol in cells that can protect them from oxidative stress damage by scavenging peroxides in the cytosol and mitochondria [40]. The oxidative stress could lead to GSH consumption, which is also a major oxidant signal for apoptosis. Using incubations with NAC 24 h prior irradiation we have shown a significant decreased level of apoptosis at 24 h postirradiation, suggesting again that ROS are playing a relevant role in modulating the early signaling system that lead to programmed cell death. Although DCF has a high affinity for $\text{H}_2\text{O}_2$, the exact nature of the ROS depicted by DCF in our experiment will need further confirmation. Nevertheless it is possible to assume that $\text{H}_2\text{O}_2$ is the mean component in the late ROS burst, considering the potential intracellular GSH depletion and the inhibitory effect of NO on catalase activity. [38]

In conclusion, in vitro irradiation of neural precursor cells allowed us to demonstrate that, consisting with previous results obtained in an in vivo model, there is an early radiation-induced increased generation of NO exerting neuroprotective properties. In addition we found that ROS/RNS are able to modulate the apoptotic response and, at least in part, caspase-3 is responsible to the late ROS burst.
5. References


[38] Brown, G.C., and Borutait, V., Nitric Oxide, Mitochondria, and cell death. IUBMB; 52:189-95. (2001)

