Pharmacological Enhancement of Autophagy Induced in a Hepatocellular Carcinoma Cell Line by High-LET Radiation

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Abstract. *The aim of the present study was to determine the cytotoxic consequences of high-linear energy transfer (LET) irradiation in the presence of oxaliplatin on hepatocellular carcinoma (HCC) cells in vitro. We attempted to correlate the induction of apoptosis and autophagy with the formation of DNA double-strand breaks (DSBs). SK-Hep1 cells were irradiated by 65 MeV neutrons in the presence of oxaliplatin and/or the poly(ADP-ribose) polymerase (PARP) inhibitor PJ34. DSBs were measured by the formation of γH2AX foci. Results show that in SK-Hep1 cells exposed to fast neutrons in the presence of oxaliplatin, DSBs occurred and persisted with time after irradiation. While apoptosis remained low in co-treated cells, autophagy was considerably increased after irradiation and augmented by the addition of oxaliplatin. Thus, autophagic cell death appears to play a prominent role in the cytotoxicity of the combined treatment and may be linked to the generation of heavy damage to DNA.*

Chemotherapy and radiotherapy (RT) are commonly used for the treatment of cancer. The latter offers the advantage of reaching the cancerous tissue with high accuracy, since ionizing radiation (IR) can be delivered directly inside tumor cells. This has been facilitated by new irradiation techniques that allow efficient sparing of surrounding healthy tissues. Another major advance in RT consists in the use of high linear energy transfer (LET) radiation, instead of low LET ones used in "conventional" RT (1, 2). On the other hand, significant advances have also been made over recent years in cancer chemotherapy, which involve the design of new molecularly targeted drugs as well as the development of carrier vehicles capable of delivering them more selectively to cancer sites (3). Despite such ameliorations, however, many tumors still resist treatment. In the case of RT, this unresponsiveness reflects in part the intrinsic inability of malignant cell types to undergo a cell death process in response to radiation. One approach to overcome such a resistance is to combine RT with a radiosensitizing chemotherapeutic agent so as to restore or at least augment the capacity of tumor cells to be killed by IR (4-6). Cisplatin was among the first drugs to be used in chemoradiotherapy protocols and is still associated with IR in a number of indications, including head and neck cancer (7). More recent platinum analogs have been evaluated since then, including oxaliplatin, a third-generation member of the family (8, 9). How radiation and platinum analogs interplay at the cellular and molecular level to enhance tumor cell killing has been frequently investigated, mostly with cisplatin. Results cannot, however, be extrapolated to other members of the family, since marked differences of behavior exist between them, due to their chemical structure, which in turn could affect their affinity for a particular intracellular target and also their pharmacological properties. Thus, oxaliplatin, compared with cisplatin, possesses an increased capacity to bind DNA (8, 9) and to induce apoptosis at similar concentrations.

Previously, we reported that oxaliplatin, in association with high-LET irradiation by fast neutrons, appreciably decreased the viability of human U-87 glioblastoma cells *in vitro* (10). We also showed that autophagy, another form of programmed cell death, accounted for this effect (11). Since it would be important to be able to validate our *in vitro* results on tumors growing in their original microenvironment

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in living animals, we sought to extend our analysis to another model of tumor that can be grown as xenograft in mice, the human hepatocellular carcinoma SK-Hep1 cell line. Like glioblastomas, hepatocellular carcinomas (HCC) are also indications for hadrontherapy (2), but their incidence is much higher and is increasing in developing countries faster than the former (12). Autophagy, defined as a "self-cannibalism" process, is attracting today an increasing interest in cancer therapy (13). Indeed, when the cell capacities to undergo an apoptotic response are compromised by, among other causes, an irreversible amount of lesions to DNA and possibly to other macromolecules, autophagy appears as an alternative programmed death. The objective of this study was therefore to assess its induction in SK-Hep1 cells upon a combined treatment with oxaliplatin and fast neutrons irradiation *in vitro*. Since poly(ADP-ribose) polymerase-1 (PARP-1) is one of the enzymes that facilitate DNA repair after irradiation (14), we analyzed its role in our system using a potent and specific PARP inhibitor, PJ34. We show that a co-treatment with oxaliplatin and fast neutrons results in the formation and persistence of DNA double-strand breaks (DSBs), as evidenced by the phosphorylation of the histone protein H2AX, and that this effect is enhanced when PARP is inhibited. SK-Hep1 cells respond to the co-treatment by undertaking autophagy and, to a lesser extent, apoptosis. Our results could indicate that these two sorts of programmed cellular demises co-exist and, in some situation, possibly cooperate to promote cell killing.

Materials and Methods

Cell line and reagents. The human hepatocellular carcinoma cell line SK-Hep1 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma, Saint-Quentin Fallavier, France) supplemented with 10% fetal bovine serum (Invitrogen, Cergy Pontoise, France), 1 mM sodium pyruvate, 1 mM non-essential amino-acids and 50 μg gentamycin/ml (Sigma). Cultures were maintained in a humid atmosphere of 5% CO_2. They were plated in 75 cm^2 flasks at an initial density of 10^5 cells/cm^2. Disaggregation was carried out using 5 minutes incubation at 37˚C with a solution of trypsin-ethylenediaminetetracetic acid (EDTA, Sigma). Stock solutions of oxaliplatin (1 mg/ml, Eloxatine, Sanofi-Aventis France) were prepared in bidistilled water and stored at +4˚C. Acridine Orange (AO) was obtained from Sigma. PJ34 was purchased from Alexis Biochemicals (Covalab, Villeurbanne, France). It was diluted in phosphate-buffered saline (PBS) and used at a concentration of 5 μg/ml.

Irradiation and treatment schedule. Asynchronous, exponentially growing cells were exposed at room temperature to a beam of p(65)+Be neutrons produced by the cyclotron CYCLONE at the Cyclotron Research Center (CRC) at Louvain la Neuve (Belgium). Cells were contained in 25 cm^2 flasks filled with 10 ml culture medium. They were covered with 3 cm of tissue equivalent polystyrene and placed under a multileaf collimator. The dose rate was 0.2 Gy/min, and doses ranged from 1 to 8 Gy. Unirradiated control flasks were sham irradiated. Each experiment was carried out at least in triplicate. Oxaliplatin, at 3 μM, was added to the cells 1 hour before irradiation and left in the culture medium 24 hours afterwards. At different times post-irradiation, cells were trypsinized, counted and processed for apoptosis, autophagy, or γH2AX foci determination. Cell counts were performed before irradiation and at different post-irradiation interval times, using a Coulter Counter. Using SK-Hep1 cells, a total of 23 independent series of irradiation experiments were performed over a three years period.

Clonogenic survival assay. Twenty-four hours after exposure to 1, 2, 4 and 8 Gy in the presence or absence of oxaliplatin or PJ34, Cells were trypsinised and enumerated in a Coulter Counter. This delay was required by the necessity to transport cells culture from the irradiation place (at Louvain la Neuve) to our laboratory in Strasbourg. Cells were resuspended at an appropriate number in fresh medium and plated at two different dilutions into six-well plates. Three wells were used per experimental point. Fifteen days later, cells were stained with 0.5% crystal violet, and aggregates containing more than 50 cells were scored as colonies.

γH2AX foci determination. SK-Hep1 cells were grown on microscopic glass slides placed in 10 cm Petri dishes. 24 hours after irradiation, culture medium was removed, and slides were washed once with PBS. Fixation and permeabilization were carried out using 4% formaldehyde and 0.5% Triton, respectively, according to a standard procedure. Labeling was performed using a monoclonal mouse anti-γH2AX polyclonal antibody (clone JBW301, Upstate, Lake Placid, NY, USA). Coverslips were mounted in 4',6-diamidino-2-phenylindole (DAPI)-stained Vectashield (Abcys, Paris, France). The formation of γH2AX foci in nuclei were monitored by immunofluorescence microscopic imaging using an Olympus BH-2 fluorescent microscope equipped with a digital camera. Foci were counted in 40 cells in each condition.

Apoptosis. Apoptotic cells were quantified according to (15). Briefly, cells (5×10^5) were fixed in cold 70% ethanol for at least 1 hour. Then they were washed in phosphate-buffered saline pH 7.2 (PBS) and resuspended in 100 μL of PBS containing 25 μg of RNase A, 2 mM EDTA and 10 μg of propidium iodide (PI). After incubation in the dark at 37˚C for 30 min, the fluorescence of 10.000 cells was analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and Cell Quest software (Becton Dickinson). Cells with a sub-diploid DNA content were recorded as apoptotic.

Autophagy. SK-Hep1 cells growing in 6-wells plates (Falcon 3046) were stained with acridine orange (AO) at 10 μg/ml for 15 minutes. In AO-stained cells, acidic compartments exhibit bright red fluorescence, the intensity of which is proportional to the degree of the acidity and volume of acidic vesicular organelles (16). Cells were examined and photographed in a fluorescence microscope. To quantify autophagy, cells (5×10^5) were stained with AO for 15 minutes, and then detached from the plates with trypsin-EDTA, washed with PBS and collected for flow cytometric analysis, using a FACScan and the CellQuest software (Becton Dickinson). Green (510-530 nm) and red (>650 nm) fluorescence emission from cells excited at 488 nm were measured. A minimum of 10^4 events was analyzed per sample. Green fluorescence protein-conjugated light chain 3 LC3 (GFP-LC3) transfection assays were also performed,

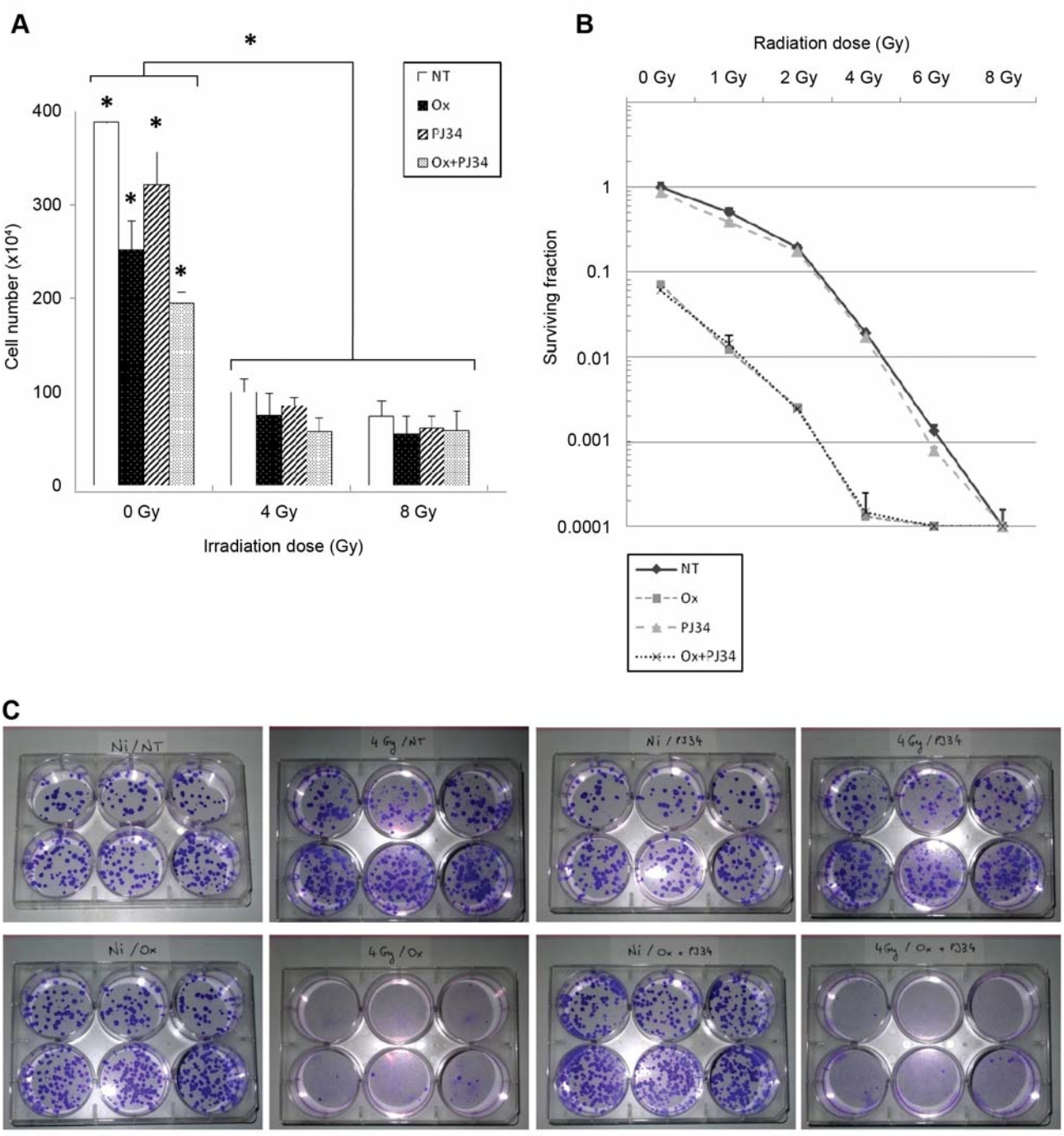

Figure 1. *A: Effect of irradiation in the presence of oxaliplatin and/or PJ34 on the growth of SK-Hep1 cells. Cells were irradiated by fast neutrons at 4 and 8Gy. At day 7 post-irradiation, they were trypsinized and their number determined using a Coulter Counter. Oxaliplatin (3 μM) and PJ34 (5 μM) were added 1 hour before the irradiation and removed 24 hours later by replacing the culture medium. Student-Newman-Keuls statistical test were performed on three independent experiments. At 0 Gy, all the groups were significantly different (*p<0.05) from the negative control (i.e. without oxaliplatin) and from each other. At 4 and 8 Gy, no significant difference can be evidenced between the different treatments. NT: Not treated; Ox: oxaliplatin-treated cells; PJ34: PJ34-treated cells; Ox-PJ34: cells treated by the combination of oxaliplatin and PJ34. B: Clonogenic survival at day 15 post-irradiation. Error bars: standard deviation (SD) of the mean of four independent determinations. p<0.05. C: Examples of colonies growing in six wells microplates. Each determination was made in triplicate. First row, from left to right: untreated, unirradiated (Ni/NT); 4Gy-irradiated, untreated (4Gy/NT); unirradiated, PJ34 treated (Ni/PJ34); 4Gy-irradiated, PJ34-treated (4Gy/PJ34). Second row, from left to right: unirradiated, oxaliplatin-treated (Ni/Ox); 4Gy-irradiated, oxaliplatin-treated (4Gy/Ox); unirradiated, oxaliplatin and PJ34-treated (Ni/Ox+PJ34); 4Gy-irradiated, oxaliplatin and PJ 34-treated (4Gy/Ox+PJ34). Three independent experiments have been performed.*

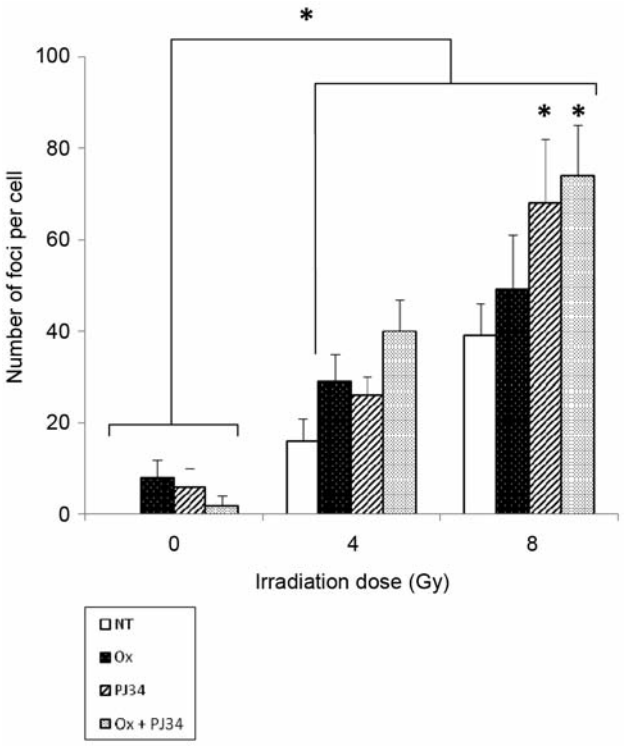

Figure 2. *Effect of irradiation in the presence of oxaliplatin and/or PJ34 on the amount of γ-H2AX foci in SK-Hep1 cells. The foci formation corresponds to the activation of the H2AX histone 24 hours after exposure to single or combined treatments. After being fixed and permeabilized, SK-Hep1 cells were labeled with an anti-γ-H2AX monoclonal antibody. Counterstaining was performed using 4, 6-diamino-2-phenylindole (DAPI), and nuclei were observed by fluorescence microscopy. NT: Untreated cells; Ox: oxaliplatin-treated cells; PJ34: PJ34-treated cells. A minimum of 40 cells were examined for each determination. In unirradiated cells, no significant differences are found between the treatments. Error bars: standard deviation (SD). At 4 and 8 Gy, the oxaliplatin + PJ34 co-treatment induces a significantly difference ($p<0.05$) from the negative control (i.e. without any treatment).*

and autophagy was quantified by the formation of GFP-LC3 tagged autophagosomes, according to (17).

Statistical analysis. Statistical analyses were performed using the MedCalc statistical software. Differences between subgroups in terms of cell count, foci number and proportion of apoptotic and autophagic cells with respect to chemical treatment and irradiation conditions were evaluated using an ANOVA one-way approach and pair wised compared with a Student-Newman-Keuls. Differences between subgroups were considered to be significant if $p<0.05$.

Results

Cell growth and survival. First, we assessed the effect of the combined treatment on SK-Hep1 cell proliferation. Control and oxaliplatin-treated cells were trypsinised at day 7 post irradiation, and their number recorded using a Coulter

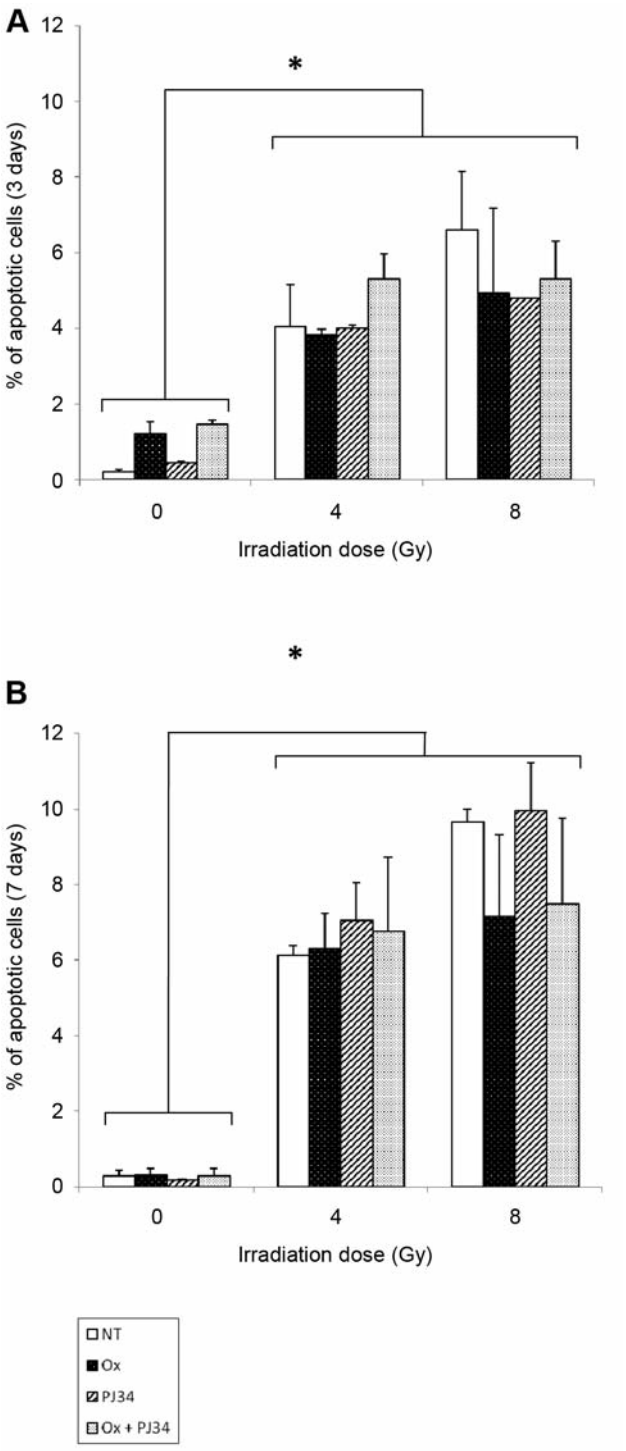

Figure 3. *Percentage of apoptosis in SK-Hep1 cells, 3 days (A) and 7 days (B) following the irradiation. Determination was done by flow cytometry, after propidium iodide staining, as described in the "Material and Methods". NT: Untreated cells; Ox: oxaliplatin-treated cells; PJ34: PJ34-treated cells. Error bars: standard deviation (SD). Student-Newman-Keuls statistical test was performed on three independent experiments. *$p<0.05$.*

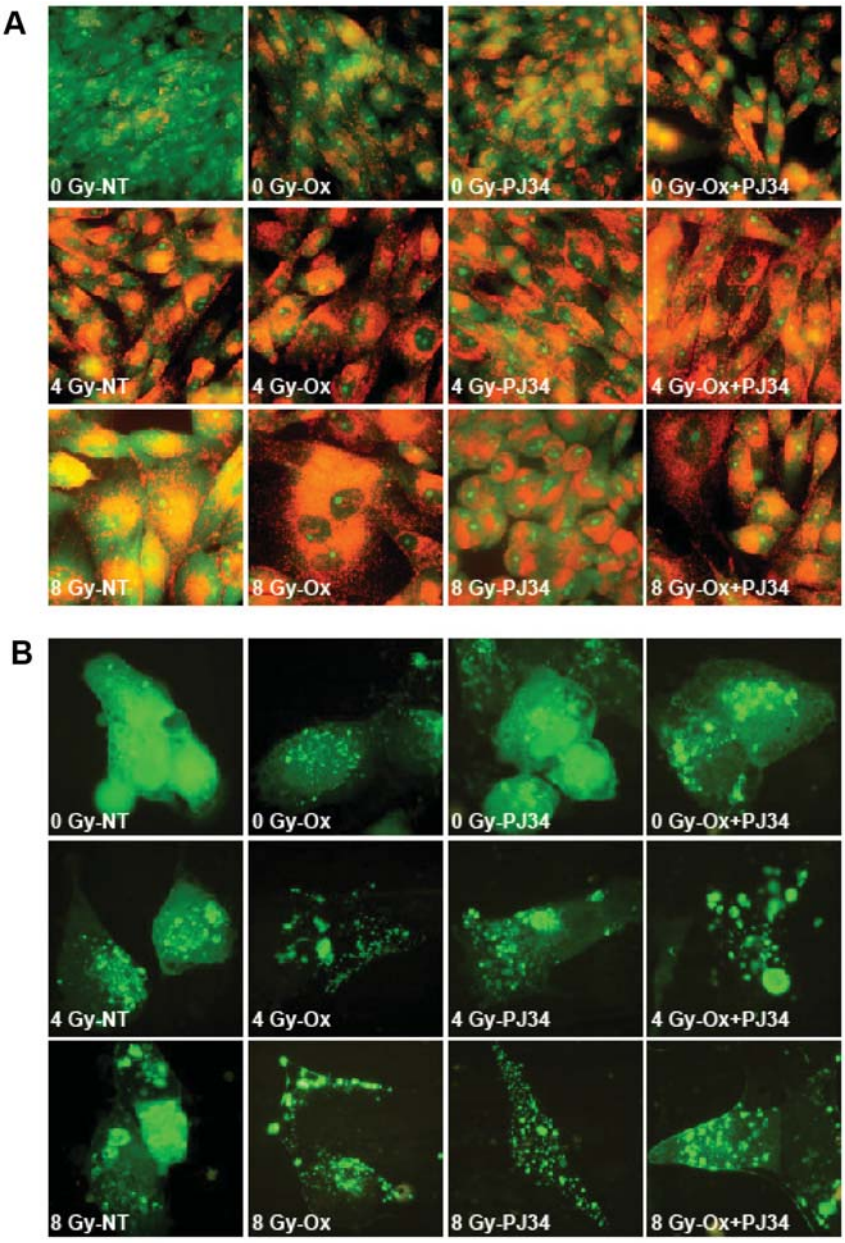

Figure 4. *Representative fluorescence microscopy images of autophagic cells in unirradiated and irradiated SK-Hep-1 cultures. A: AO staining on day 3 after irradiation (magnification of ×40). B: Expression of green fluorescence protein-conjugated light chain 3 (GFP-LC3) in transfected SK-Hep1 cells, measured 24 hours after irradiation (magnification of ×100). NT: Untreated cells; Ox: oxaliplatin-treated cells, PJ34: PJ34-treated cells.*

Counter. As shown in Figure 1A, irradiation at 4 or 8 Gy in the absence of other treatment caused a marked decrease of cell number in the cultures. At this time, treatment of cells with either oxaliplatin (3 μM) or the PARP inhibitor PJ34 (5 μM) alone significantly decreased the cell number in unirradiated groups. These concentrations were determined in preliminary experiments and shown to be devoid of short-term cytotoxicity. Co-treatment with oxaliplatin and irradiation at 4 Gy and 8 Gy resulted in a reduction of cell number comparable to that observed with irradiation alone. Figure 1A also demonstrates that addition of PJ34 to the cultures at the same time as oxaliplatin did not significantly influence the reduction of cellularity after irradiation. Using the clonogenic assay, a reduction in surviving fraction was found in oxaliplatin-treated, unirradiated cells (Figure 1B and 1C). At 4Gy, this decrease was amplified by oxaliplatin and oxaliplatin

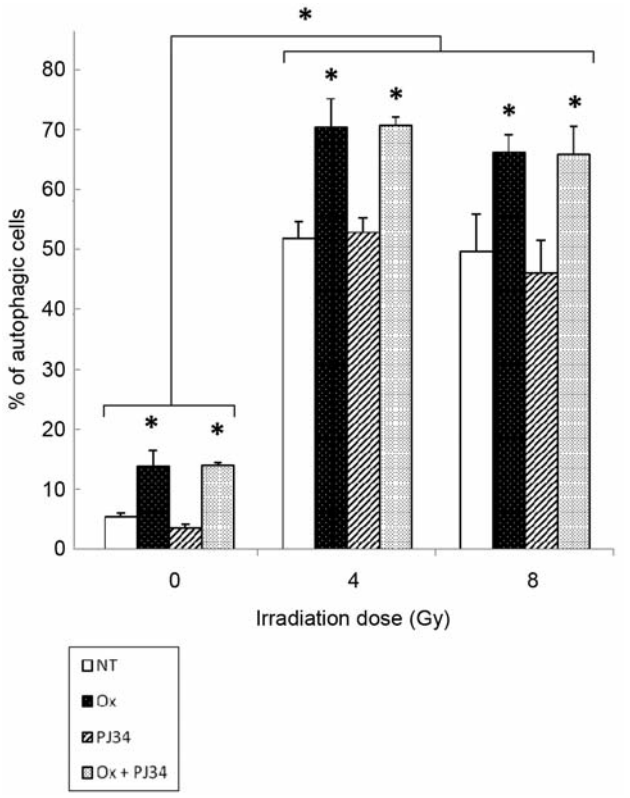

Figure 5. *Percentage of autophagy in SK-Hep1 cells 3 days post-irradiation. Determination was carried out by flow cytometry after AO staining, as described in the Material and Methods. NT: untreated cells; Ox: oxaliplatin-treated cells; PJ34: PJ34-treated cells. Error bars: SD. Student-Newman-Keuls statistical test was performed on three independent experiments. *p<0.05.*

+ PJ34 treatments. Clonogenic survival of cells irradiated in the presence of oxaliplatin and PJ34 was depressed by a 2 log factor compared to control, irradiated only. After 8 Gy, survival was completely abolished in both oxaliplatin-treated groups. In cells irradiated in the absence of oxaliplatin, survival was considerably decreased, but not totally. In comparison with U-87 submitted to the same treatment (10) these results indicate that SK-Hep1 are more radiosensitive, and that the action of oxaliplatin is less pronounced, when combined with the irradiation.

Activation of H2AX. We then monitored the occurrence of DSBs in treated cells by measuring the number of γ-H2AX-foci within the nucleus at 24 hours post irradiation. As shown in Figure 2, a very low number of foci were scored in untreated and non-irradiated cells as well as in oxaliplatin-treated cells. By contrast, numerous foci were recorded in irradiated cells. The number of foci was markedly augmented in cells co-treated with oxaliplatin and irradiation, indicating that oxaliplatin enhanced the formation of radiation-induced DSBs. We also tested the effect of PJ34 on the activation of γ-H2AX in treated cells. As seen in Figure 2, a pronounced increase of the foci number could be found in irradiated cells. Maximum values were recorded in oxaliplatin and PJ34-treated, irradiated cells.

Apoptosis. The effects of the co-treatment on apoptosis induction were then evaluated. Cells were labeled with PI and examined by flow cytometry. As shown in Figure 3, irradiation induced apoptosis in SK-Hep1 cells. Oxaliplatin, at 3 μM, was without effect on apoptosis induction. However, the percentage of apoptosis determined at day 3 post-irradiation (Figure 3A) was low. At day 7 (Figure 3B), it was slightly augmented, but maximum values did not exceed 10%. Importantly, combined treatments resulted in lower percentages of apoptotic cells, as compared to single treatments alone. This result is similar to what we previously reported with U-87 cells (11). We therefore assume that the induction of this form of cell death cannot fully account for the cytotoxic effect resulting from the co-treatment. Moreover, the inhibition of PARP was without significant effect on apoptosis induction.

Autophagy. We next examined whether autophagy could be induced by the combination of oxaliplatin and irradiation. Three days after the irradiation, SK-Hep1 were stained with AO and examined microscopically. Cells with cytoplasm expressing the characteristic red fluorescent pattern were scored as positive for autophagy. A large majority of irradiated or co-treated cells expressed this labeling, albeit to various degrees. In co-treated groups, the fluorescence intensity was dramatically reinforced, and cells were enlarged (Figure 4A).

Using another, more specific, marker of autophagy, LC3, we found that GFP-LC3-tagged autophagosomes were more numerous in cells treated by the combination of an irradiation with oxaliplatin (Figure 4B). In irradiated, oxaliplatin and PJ34-treated cells, the amount of autophagosomes was considerable, confirming results obtained with AO staining. In non-irradiated, untreated cells were largely negative for GFP-LC3 staining, indicating that autophagy is mostly induced by the irradiation. However, oxaliplatin, alone and combined with PJ34, was able to induce autophagosomes in the absence of irradiation. In order to quantify autophagy, flow cytometry analysis of AO-stained cells was performed. As shown in Figure 5, the percentage of autophagic cells was markedly increased in cultures exposed to radiation/oxaliplatin cotreatment. In unirradiated cells, the co-addition of oxaliplatin and PJ34 also augmented this percentage, confirming the data obtained by fluorescence microscopy. It is worth noting that since survival was also markedly decreased in oxaliplatin-treated, unirradiated cells (Figure 1B), autophagy might be inferred

to directly affect the clonogenicity. It should be noted that PJ34 alone did not influence the occurrence of autophagy in unirradiated as well as in irradiated cells.

Discussion

In the present study, we show that the combination of oxaliplatin and high-LET radiation markedly reduces the growth of SK-Hep1 cells and that autophagic cell death accounts predominantly for this effect. Furthermore, the overall cytotoxicity appears to be linked to the induction of DSBs and to their persistence, indicating that autophagy could be related to a blockade of DNA repair. Autophagy has emerged recently as an alternative form of programmed cell death induced by anticancer agents (18, 19), although in some situations, it could promote cell survival following DNA damage by delaying apoptosis (20). However, recently, promotion of autophagy has been proposed as a mechanism for radiosensitizing breast tumor cells (21). While damage to DNA is a major determinant in the triggering of apoptosis, its role in the promotion of autophagy by radiation remains to be more firmly established. Nevertheless, radiation-induced DNA damages have been recently reported to enhance both apoptosis and autophagy in human melanoma cells (22). Moreover, the inhibition of the DNA-dependent protein kinase catalytic subunit has been shown to sensitize malignant cells to radiation by inducing autophagy (16). Our results indicate that the combination treatment with oxaliplatin and neutrons leads to the formation of many residual DSB in SK-Hep1 cells. The induction of apoptosis and persistent γ-H2AX formation by oxaliplatin has been described in HCT116 human colorectal cancer cell line (23). On the other hand, the increased lethal effect of high-LET radiation reflects the efficiency with which they induce complex DSBs and also non-oxidative clustered DNA lesions (24, 25). Recently, oxaliplatin was reported to augment the radiosensitivity of cervical cancer cells by delaying the abrogation of gamma-H2AX and by attenuating checkpoint kinase 2 activation (26). This effect resulted in an enhancement of mitotic catastrophe, another type of cell death that we did not observe in our model. Interestingly, autophagy has also been presented as a new mechanism of synergistic cytotoxicity between doxorubicin and roscovitine in sarcoma cell lines (27). In autophagy signaling, mammalian target of rapamycin (mTOR) represents a major sensor. This kinase, one of the downstream effectors of the PI-3K/AKT pathway, is activated by a variety of stimuli, including DNA-damaging agents (20). The AKT/mTOR pathway is involved in radioresistance (28) and inhibitors of AKT have been shown to exert a radiosensitizing effect in U-87 glioblastoma cells by inducing autophagy (29). Specific inhibitors of mTOR, such as RAD-001 (everolimus) are also capable of sensitizing cancer cells to radiation through autophagy upregulation (17). Moreover, high-LET radiation has been reported to disrupt the cellular architecture, leading to autophagy induction in mouse skeletal muscles (30). Since we observed a pronounced swelling of neutron-irradiated cells, damage to plasma membranes and to cytoskeletal components might also be considered as a causative factor for the emergence of autophagy in our experimental system. Regarding the role of PARP in autophagy induction, we noted that its inhibition by PJ34 only plays a marginal role, in our experimental conditions at least. However, this inhibitor has recently been reported to enhance the suppressive effects of cisplatin in HCC cells (31). Indeed, PARP is an important molecular target for radiosensitizing tumor cells, since this enzyme plays a key role in DNA repair mechanisms (32). Thus, further experiments with higher concentrations will be necessary to verify its possible contribution in cell killing when oxaliplatin and high-LET radiation are associated.

In conclusion, the present data indicate that autophagy can be initiated by severe damage to DNA (and possibly to other macromolecules) caused by the combination of oxaliplatin with high-LET radiation. The interest in autophagic cell death induced by anticancer drugs (19) and radiation (21, 33) is now rapidly growing. Its role in the link between cytostasis and cytotoxicity remains, however, unclear (34). On the other hand, whether autophagy represents a common feature of cell death response to high-LET radiation needs to be established. For such studies, SK-Hep-1 hepatocellular carcinoma cell line could provide an efficient model.

Acknowledgements

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