Cell Death After High-LET Irradiation in Orthotopic Human Hepatocellular Carcinoma In Vivo

ANAÏS ALTMEYER1, MIHAELA IGNAT2, JEAN-MARC DENIS3, NADIA MESSADDEQ4, JOHN GUEULETTE3, DIDIER MUTTER2 and PIERRE BISCHOFF1

1EA-3430, Université de Strasbourg, Centre Régional de Lutte contre le Cancer Paul Strauss, 67065 Strasbourg, France;
2Institut de Recherche Contre les Cancers de l’Appareil Digestif (IRCAD), Hôpitaux Universitaires, 67091 Strasbourg, France;
3Laboratoire d’Imagerie Moléculaire et de Radiothérapie Expérimentale, UCL-IMRE 5469, Université Catholique de Louvain, Faculté de Médecine, B-1200 Brussels, Belgium;
4Plateforme de Microscopie Electronique, Institut de Génétique et de Biologie Cellulaire et Moléculaire (IGBMC), 67400 Illkirch Graffenstaden, France

Abstract. Hepatocellular carcinoma (HCC) represents the sixth most common cancer worldwide and a major health problem since the choice of treatment is limited due to chemo- and radio-resistance. It was previously reported that high linear energy transfer (LET) radiation induced massive autophagic cell death in the human HCC SK-Hep1 cell line in vitro. This study analyzed the effects of high-LET radiation on the same HCC tumor model, orthotopically transplanted into nude mice. For this purpose, after surgical xenograft in the liver, animals were irradiated with fast neutrons and cell death occurring in the tumors was assessed with various techniques, including electron microscopy and probe-based confocal laser endomicroscopy. Results indicate that considerable autophagy and only limited apoptosis took place in the tumor xenografts after high-LET irradiation. These data confirm the previous in vitro results, suggesting that autophagy may act as a predominant mode of cell death in the efficacy of high-LET radiation.

Hepatocellular carcinoma (HCC) is a major health problem, with its incidence in the United States, Europe and Asia constantly increasing, representing the sixth most common cancer worldwide (1-2). Its principal etiologic factors are viral hepatitis and chronic alcoholism. Despite improved diagnosis and treatment strategies in recent years (3), the overall survival of HCC patients is still poor. The highly resistant nature of HCC to conventional therapies has been partially attributed to its insensitivity to cytotoxic agent-induced cell death (4). Moreover, external conventional HCC radiotherapy is limited by radiation-induced liver disease, a clinical syndrome that can lead to liver failure and even death in severe cases (5). However, new radiation therapy techniques have recently been tested successfully for the treatment of HCC, in particular high linear energy transfer (LET) radiation such as heavy ions (6). High LET radiation is known to be more effective in inducing biological damage than low-LET X-rays and γ-rays used in ‘conventional’ radiotherapy (7). A previous study investigated the different cell death modalities occurring in a radioresistant human HCC cell line, SK-Hep1, after irradiation with fast neutrons (8). It was observed that autophagy, also termed type II programmed cell death (9), was predominant with respect to all other forms of cell death. This cytotoxic effect was reinforced by combining high-LET irradiation with oxaliplatin, a third-generation platinum-based cancer chemotherapeutic drug recently evaluated in clinical trials against HCC (10-11).

Autophagy is a concept that has recently come under intense scrutiny in the cancer therapy field (12-14). This evolutionarily conserved process mainly consists in the degradation within lysosomes of cytoplasm and organelles that occurs when cells undergo a metabolic stress induced by starvation, radiation, hypoxia or when cells are exposed to chemotherapeutic drugs (15, 16). Considered as an adaptive and survival system by certain authors, autophagy is also, and paradoxically considered as an antitumor cell death mechanism (17) and previously published results (18) concur with the latter assumption. Indeed, using a human

Correspondence to: Pierre Bischoff, EA-3430, Université de Strasbourg, Centre Régional de Lutte contre le Cancer Paul Strauss, 3 rue de la porte de l’Hôpital, 67065 Strasbourg, France. Tel: +33 388258542, e-mail: pbischoff@strasbourg.fnclcc.fr

Key Words: Orthotopic xenograft, hepatocellular carcinoma, autophagy, apoptosis, high linear energy transfer irradiation.

0258-851X/2011 $2.00+.40
glioblastoma cell line (U-87), it has been shown that through irradiation with fast neutrons, high-LET radiation induced autophagic cell death \textit{in vitro} and decreased tumor progression in nude mice that had been subcutaneously grafted with U-87 cells. However, this tumor model is rather artificial, since malignant cells proliferate in a remote site from their organ of origin. Thus, the purpose of the present study was to develop an orthotopic xenograft model of the HCC SK-Hep1 cell, in order to confirm that high-LET radiation is also able to induce autophagy in these cells when growing in their natural environment.

**Materials and Methods**

**Cell line and culture condition.** The HCC cell line SK-Hep1 was obtained from the American Type Culture Collection (ATCC; USA). Cells were grown in Dulbecco’s Modified Eagle’s Medium (Sigma, Saint-Quentin Fallavier, France) and maintained in a humidified atmosphere with 5% CO2 in air. Medium was 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). Cells were plated in 75 cm² flasks (Dominique Dutscher, Brumath, France) at an initial density of 10⁵ cells/cm² and disaggregation was carried out by 5 min incubation at 37°C with a solution of trypsin-EDTA (Sigma).

**Animals and orthotopic HCC model.** Four-week-old male athymic Swiss nude mice were obtained from Charles River Laboratories (Les Asresles, France) and housed under conventional conditions (temperature 22°C±2°C, relative humidity, 55%±10%), under a 12 h light/dark cycle. Sterile food and water were available \textit{ad libitum}. National laws on the care and use of laboratory animals were strictly followed, and all animal protocols and experiments were approved by the Animal Committee of IRCAD. Figure 1 demonstrates schematically the procedures used to perform HCC orthotopic xenografts in nude mice. For this purpose, \textit{in vitro} SK-Hep1 cells were grown in 25 cm² flasks, trypsinized, washed in PBS and transferred to a 1 ml syringe for subcutaneous injection into the right limb of a nude mouse (20×10⁶ cells/150 μl). After being allowed to grow for 2 months, the tumors were removed and fragmented into 1 mm³ sections. Another series of nude mice was then laparotomized under general isoflurane anesthesia in order to access the right lobe of the liver and the tumor fragments were then implanted in the right hepatic lobe (one fragment per mouse).

**Fast neutron irradiation.** Mice were exposed at room temperature to p(65)+Be neutrons, produced at the Cyclotron Research Center (Louvain-la-Neuve, Belgium). They were positioned on a Plexiglas plate allowing the simultaneous irradiation of four animals. The irradiation field was focused on the upper part of abdomen, using a multileaf collimator, and the delivered dose was 4 Gy.

**Histological examination.** To perform histological analysis, samples were fixed in buffered formalin for at least 24 h, dehydrated and paraffin embedded. Standard 5 μm slides were stained with hematoxylin-eosin and observed under a light microscope. The terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) assay was performed using the \textit{In Situ} Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer’s protocol. TUNEL-positive cells were observed under a fluorescence microscope.

**Apoptosis follow-up by probe-based confocal laser endomicroscopy (pCLE).** Fluorescent agent: In order to monitor apoptotic cells \textit{in vivo} and real time by pCLE, tumor bearing nude mice were intravenously injected with FLIVO (Immunochemistry Technologies, Bloomington, USA), a cell-permeable poly caspase-binding inhibitor probe allowing detection of apoptosis through labeling active caspases. Then 200 μl of injection buffer, 10× diluted and filtered, were added to the reagent dissolved in DMSO. A volume of 40 μl of this solution was injected intravenously into the tail vein of each mouse. Fluorescence detection by pCLE was performed 30 min after injection for optimal staining.

*Figure 1. SK-Hep1 cells were subcutaneously injected in the right limb of a nude mouse [1]. After being allowed to grow for 2 months [2], the tumor was removed and fragmented into 1 mm³ sections [3]. Another series of nude mice was then laparotomized under general isoflurane anesthesia, in order to access the liver and the tumor fragments were then implanted in the right hepatic lobe (one fragment per mouse) [4].*
Fas-mediated apoptosis: development and testing of a positive control for apoptosis. In order to induce apoptosis in a healthy liver, anti-Fas (CD95/APO1) antibody (BD Biosciences, San Jose, USA) was injected in the tail vein of BALB/C mice (Charles River Laboratory). With an optimal dose of 4 μg anti-Fas antibody/30 g mouse and using FLIVO in vivo marker for apoptosis, it was possible to perform a real time follow-up of apoptosis in the liver. The follow-up was performed on the same mouse, which was repeatedly laparotomized and sutured under isoflurane anesthesia, with very good tolerance.

Apoptosis detection by pCLE: Probe-based confocal laser Endomicroscopy (pCLE) analysis was performed using the Cellvizio system (Mauna Kea Technologies, Paris, France), composed of a 488 nm laser scanning unit (Mauna Kea Technologies), a flexible fiber.

Figure 2. A: Fas-mediated apoptosis observed by probe-based confocal laser endomicroscopy (pCLE), 2, 4, 6 and 8 h after anti-Fas antibody injection in the tail vein of the mice (positive control). B: Resting liver of an untreated (healthy) mouse (negative control): pCLE detection of the baseline of apoptosis (left) and hematoxylin-eosin counterstaining (right).
optic miniprobe (ProFlex, Mauna Kea Technologies) and image processing software (ImageCell, Mauna Kea Technologies). Thirty minutes after intravenous injection of FLIVO, nude mice were laparotomized under general anesthesia with isoflurane. Electron microscopy. Normal hepatic tissue and orthotopic tumor tissue was cut into 1 mm cubes, and immersion-fixed with a freshly made mixture of 2.5% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer. Postfixation was performed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at 4°C in the dark. Samples were then dehydrated through graded ethanol (50, 70, 90 and 100%) and embedded in Epon. Ultrathin sections (70 nM) were cut, contrasted with uranyl acetate and lead citrate, and then examined under a Morgagni 268D electron microscope (Philips, Eindhoven, the Netherlands).

Results

Fas-mediated apoptosis. The Fas receptor (CD95, FasR) mediates apoptotic signaling upon activation by Fas-ligand (CD95L) expressed on cell surface. Highly expressing FasR, hepatocytes are known to be very susceptible to Fas-mediated apoptosis (19-20). Anti-Fas antibody is an activating antibody, inducing apoptosis through MORT1-caspase pathway, and resulting in vivo in acute hepatitis, leading to the death of animals within a few hours (19). In order to induce massive apoptosis in mouse livers, and thus obtain a positive control for apoptosis, 4 μg anti-Fas antibody were injected into the tail vein of control (Balb/c)

Figure 3. Radiation-induced apoptosis: HCC orthotopic tumor non-irradiated (NI) or irradiated by fast neutrons (4 Gy). The two top images correspond to pCLE analysis of the tumor after injection of the fluorescent apoptosis marker; the two bottom images correspond to TUNEL staining of the same tumor areas.
mice. Ninety minutes after the antibody was injected, 8 μg FLIVO™ green were injected via the tail vein. Mice were then laparotomized under general anesthesia, and probe-based confocal laser endomicroscopy was performed at 2, 4, 6 and 8 h after antibody injection.

Apoptosis progression was demonstrated by pCLE imaging during the 8 h follow-up, with the average number of fluorescent cells/field increasing from none to almost 100% (Figure 2A). At the end of the follow-up, the liver structure was totally disorganized, with non-identifiable liver lobules and non-fluorescent areas suggestive of necrosis and hemorrhage.

As negative control, a wild-type mouse was imaged by pCLE 40 min after 8 μg FLIVO™ green intravenous injection with no anti-Fas antibody injection. The detected baseline level of apoptosis in the liver was close to zero, only 1-2 fluorescent cells being detected after thoroughly scanning the entire surface of the liver (Figure 2B). After a longer exposure, the product was excreted through the bile duct, giving a strong fluorescence in the gallbladder. This finding was subsequently used as internal control for the correct intravenous injection of the fluorescent marker in the animals with low levels of apoptosis.

These results demonstrated that detection and real-time imaging of apoptosis is feasible by probe-based confocal laser endomicroscopy. Indeed, massive apoptosis induced by anti-Fas antibody was clearly evidenced in mouse livers.

**Radiation-induced apoptosis in orthotopic tumors.** After confirmation of pCLE as an appropriate tool for in vivo detection and follow-up of apoptosis in normal and tumor hepatocytes, seven nude mice that had been orthotopically xenografted with SK-Hepl cells were submitted to 4 Gy fast neutron irradiation targeted on the upper abdomen. pCLE imaging was performed seven days after irradiation in three mice and fourteen days in four mice. Non-irradiated, but orthotopically grafted mice were used as controls.

In the non-irradiated mice, the fluorescent signal in the tumors was very low (an average of three fluorescent cells/field), and no clustering of fluorescent cells was detected by pCLE (Figure 3A). Seven days after 4 Gy irradiation of the tumor-bearing mice, some clusters of fluorescent apoptotic cells were detected, with a maximum diameter of 135 μm (Figure 3B), and an average of 13 cells/field (p=0.00006). This finding was confirmed by the TUNEL staining, which showed a stronger level of apoptosis (Figure 3D) than in the non irradiated tumors (Figure 3C). Fourteen days after irradiation, the level of apoptosis returned to baseline (an average of five cells/field, data not shown). In summary, pCLE imaging allowed detection of apoptotic cells, but only a few apoptotic events were
The cells display characteristics of necrosis: abnormal nuclei with a degraded nucleolus, and a cytoplasm which was progressively emptied of its contents. In necrosis the degraded organelles are gradually replaced by collagen fibers. This necrotic process slowly leads to cell death. No autolysosomes were detected in non-irradiated tumors.

Figure 6C shows a 4 Gy-irradiated tumor. Cells also presented the characteristics of necrosis: a dilated nuclear membrane and a cytoplasm which was emptied of its contents, to be replaced by collagen fibers. However, the particularity of the irradiated orthotopic tumor cells is the induction of the massive accumulation of large autolysosomes in the cells. These dense structures enclose various cytosolic elements in their cisternal lumen. Single-membraned autolysosomes result from the fusion of double-membraned autophagosomes with lysosomes, in order to degrade their contents. Figure 6C (top right) shows tubular membranes (see t.m.), corresponding to the first step of double-membraned autophagosome formation. As described by Gao et al. (22), the tubular membrane may roll around the substrates to form a loose bundle, which may then be compacted and delimited by the outermost layer of the autophagosome’s membrane. An accumulation of lipid droplets can also be noted, both in the cytoplasm and in the autophagolysosomes at the same time, due to the irradiation. Furthermore, organelles degenerate: the endoplasmic reticulum is strongly decreased and dilated, and mitochondria show abnormal forms.

**Discussion**

The present study assessed the early and delayed events occurring after irradiation with fast neutrons on HCC cells growing in vivo, using various methods. It was shown that only a few apoptotic events were recorded by pCLE in cancer cells, none being observed under electron microscopy, contrary to autophagic cell death. Interestingly, no autophagic cells were detected in normal liver tissue surrounding the tumor, indicating the presence of a differential effect between normal and tumor cells after irradiation. These findings confirm previously published in vitro results, which showed the induction of autophagy in SK-Hepl cells submitted to high-LET radiation. Moreover, that previously published study presented evidence that this cytotoxic effect was reinforced when oxaliplatin, a third-generation platinum drug, was added to the medium shortly before irradiation (8). HCC xenografts are frequently used for screening potentially new drugs for the treatment of this lethal disease (23-25). As evidenced here, it is also a valuable model for the evaluation of the cytotoxic effects of high-LET radiation in vivo. The sole difficulty was the extremely slow growth of the orthotopically grafted HCC, in spite of their vascularization in the liver. Interestingly, HCC cells that were transplanted subcutaneously were found to progress faster, suggesting that intrinsic factors in the liver may negatively influence tumor growth. HCCs are generally
considered as radioresistant tumors and current, non-molecular targeted chemotherapeutic agents are inefficient. The limited use of radiotherapy for the treatment of HCC also arises from the toxicity of radiation for the liver (26). Therefore, a better understanding of how normal and malignant hepatic cells respond to ionizing radiation is a prerequisite to improve the radiotherapy efficiency of these tumors. In particular, the role of autophagy should be analyzed in more depth. Several recent studies have emphasized the role of this type of programmed cell death as a new target in cancer therapy (14, 27-29). In the case of HCC, Ding et al. related the important role of autophagy on the prognosis of this tumor (30). Evidence has been given to show that the autophagy defect in HCC, associated with altered apoptotic activity, results in an aggressive cancer cell phenotype and a poor prognosis. However, other studies have demonstrated a paradoxical role of autophagy when cancer cells are submitted to ionizing radiation. In some cases, autophagy inhibitors improve the response to radiotherapy (31). This last hypothesis is in contradiction to the results of the current study which showed, using human glioblastoma U-87 and HCC SK-Hep1 cell lines, that high-LET radiation-induced autophagy contributed to the eradication of cancer cells.

In conclusion, these results encourage further investigation to elucidate the exact role of autophagy in the destruction of malignant cells by high-LET radiation, using this model of orthotopically transplanted tumor.

Acknowledgements

This work was supported by grants from the European Commission within the Sixth Framework Program through I3-EURONS (contract no. RI3-CT-2004-506065), Electricité de France (Comité de Radioprotection) and the Région Alsace. We thank Mauna Kea Technologies for the loan of the Cell-Vizio system and IRCAD for the surgical facilities, and Dr Francis J. Dumont for helpful discussion.

References


