Biochemical and Biophysical Research Communications 404 (2011) 552–558

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Lung autophagic response following exposure of mice to whole body irradiation, with and without amifostine

Christos E. Zois [a], Alexandra Giatromanolaki [b], Heikki Kainulainen [c], Sotirios Botaitis [d], Sira Torvinen [c], Constantinos Simopoulos [d], Alexandros Kortsaris [e], Efthimios Sivridis [b], Michael I. Koukourakis [a,*]

[a] Department of Radiotherapy – Oncology, Democritus University of Thrace, Alexandroupolis 68100, Greece
[b] Department of Pathology, Democritus University of Thrace, Alexandroupolis, Greece
[c] Department of Biology of Physical Activity, University of Jyväskylä, Finland
[d] Department of Experimental Surgery, Democritus University of Thrace, Alexandroupolis, Greece
[e] Department of Biochemistry, Democritus University of Thrace, Alexandroupolis, Greece

ARTICLE INFO

Article history:
Received 2 December 2010
Available online 8 December 2010

Keywords:
Autophagy
Ionizing radiation
Lung
LC3
Beclin-1
p62
bnip3
Amifostine

ABSTRACT

Purpose: The effect of ionizing irradiation on the autophagic response of normal tissues is largely unexplored. Abnormal autophagic function may interfere the protein quality control leading to cell degeneration and dysfunction. This study investigates its effect on the autophagic machinery of normal mouse lung. Methods and materials: Mice were exposed to 6 Gy of whole body γ-radiation and sacrificed at various time points. The expression of MAP1LC3A/LC3A/Atg8, beclin-1, p62/sequestosome-1 and of the Bnip3 proteins was analyzed. Results: Following irradiation, the LC3A-I and LC3A-II protein levels increased significantly at 72 h and 7 days. Strikingly, LC3A-II protein was increased (5.6-fold at 7 days; $p < 0.001$) only in the cytosolic fraction, but remained unchanged in the membrane fraction. The p62 protein, was significantly increased in both supernatant and pellet fraction ($p < 0.001$), suggesting an autophagosome turnover deregulation. These findings contrast the patterns of starvation-induced autophagy up-regulation. Beclin-1 levels remained unchanged. The Bnip3 protein was significantly increased at 8 h, but it sharply decreased at 72 h ($p < 0.05$). Administration of amifostine (200 mg/kg), 30 min before irradiation, reversed all the LC3A and p62 findings on blots, suggesting restoration of the normal autophagic function. The LC3A and Beclin1 mRNA levels significantly declined following irradiation ($p < 0.01$), whereas Bnip3 levels increased. Conclusions: It is suggested that irradiation induces dysfunction of the autophagic machinery in normal lung, characterized by decreased transcription of the LC3A/Beclin-1 mRNA and accumulation of the LC3A, and p62 proteins. Whether this is due to defective maturation or to aberrant degradation of the autophagosomes requires further investigation.

1. Introduction

Normal lung response to radiotherapy is a major factor defining the maximum tolerable dose of radiotherapy delivered to lung cancer patients. Understanding the biological mechanisms involved in acute and late lung injury will allow the recognition of critical targets for pharmacological or molecular interventions aiming to cytoprotection [1]. Ionizing radiation, at dose levels used in clinical radiotherapy, leads to cell death through induction of apoptosis. DNA strand breaks are considered the major type of radiation interaction with cells [2,3].

Macro-autophagy (for simplicity autophagy), is an important biological process responsible for the turnover and recycle of unnecessary/or dysfunctional organelles and damage long-lived proteins [4]. The degradation of damaged cellular organelles through autophagic vacuole formation and fusion with lysosomes is an important mechanism aiming to maintain the cellular functional integrity and viability. At the same time, degraded subcellular structures are processed as metabolic fuel. Excessive activation of this pathway, however, leads to autophagic cellular death, so that balanced autophagic machinery is required for the cellular homeostasis [5]. Abnormal function of autophagy may interfere the protein quality control in cells leading to a variety of degenerative disorders and cellular dysfunction [6].

In cancer cell experiments, upregulation of the autophagic process can either sensitize to or protect cancer cells from irradiation and/or chemotherapy, suggesting a rather complicated role of autophagy in the cellular response to cytotoxic agents [7]. On the other hand, the role of autophagy in normal tissue response to radiotherapy is unknown.

In the current study we investigated the early response of mice lung following exposure to a single radiotherapy fraction of 6 Gy,

* Corresponding author. Fax: +30 25510 30349.
E-mail address: targ@her.forthnet.gr (M.I. Koukourakis).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.12.024

applying techniques for the detection of key proteins participating the formation and degradation of autophagosomes, namely the MAP1LC3A/Atg8, (microtubule associated protein 1 light chain 3 or simply LC3A), the beclin-1 and the p62/SQSTM1 (sequestosome 1). The changes induced by radiation were also assessed in mice pre-treated with the cytoprotective agent amifostine, known to strongly protect normal tissues against ionizing radiation by a factor of 2.5 [8]. The BNIP3 protein, previously shown to be involved in the induction of autophagy and of mitochondrial autophagy (mitophagy) [9] was in parallel examined.

2. Materials and methods

2.1. Animal care and handling

Animal care and handling was carried out according to the guidelines set by Directive 86/609/EEC. All experimental procedures have been approved by the Veterinary Direction for Animal Research in the Department of Experimental Surgery at the Democritus University of Thrace. Male mice (Balb/c) 14–16 weeks of age (33 ± 2 gr), were under normal conditions concerning ambient temperature (21–23 °C), diet, tap water *ad libitum* and were maintained on a 12 h light:12 h dark cycle.

2.2. Experimental design and tissue procurement

The mice were divided randomly into the following groups: Control ($n = 3$) and γ-radiation ($n = 12$). The mice were exposed to a single whole body γ-radiation (WBI) at dose of 6 Gy (Cobalt 60). Following irradiation, animals were sacrificed at various time points 8, 24, 72 and 168 h ($n = 3$, per time point). Moreover, in an additional group of mice, amifostine (200 mg/kgr) was given 30 min prior to WBI and animals were sacrificed after 7 days. Lung tissue was removed and immediately placed in tubes containing RNAlater to inhibit protein and RNA degradation (Ambion) and stored at −20 °C for immunoblotting and RT-qPCR. Tissue samples were also fixed in formalin and embedded in paraffin blocks for immunohistochemistry.

2.3. Starvation experiment

The mice were divided randomly into the following groups: Control ($n = 2$) and 24 h ($n = 2$) and 48 h ($n = 2$) under nutrient starvation conditions. The mice were allowed only to have contact with tap water. After the experiment the animals were sacrificed and lung tissues was removed and immediately placed in tubes containing RNAlater to inhibit protein and RNA degradation (Ambion) and stored at −20 °C for immunoblotting. The MAP1LC3A protein level for LC3A-I and LC3A-II were validated under these conditions.

2.4. Tissue homogenization

The tissues were homogenized at 4 °C in 15 volumes (15 μL per 1 mg lung tissue) of extraction buffer [0.5 M KCl, 15 mM Tris–HCl pH 7.4, 1 mM ethylene diamine tetraacetic acid (EDTA), 5 mM dithiothreitol, 10 mM Na2P2O7, 1 mM leupeptin, 30 mM tosylamino-2-phenylethyl chloromethyl ketone (TPCK), 30 mM benzoyl-L-arginine ethyl ester hydrochloride (BAEE), 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] [10] using the Fastprep instrument (MP Biomedicals) and centrifuged at 16,000*g* for 30 min (4 °C); both the supernatant and pellet were resuspended in SDS sample buffer [11]. With this fractionation process without using any detergent such as Triton X-100 we are able to obtain a membrane fraction as a pellet which includes mitochondria and autophagy related vacuoles and nuclei. Total protein concentrations for the fractions were estimated according to the method of Lowry [12].

2.5. SDS–PAGE and immunoblotting

Both fractions were separated on a discontinuous SDS gels using 10% (for beclin-1, p62, Bnip3, b-actin), 12.5% (for LC3A-I and LC3A-II) separating and 5% stacking gels [11]. Bands on the gel were visualized with Coomassie Blue R250 and analyzed densitometrically to quantify any changes in the experimental samples relative to control. For the immunoblotting 20 μg of extracts for beclin1, p62 and bnip3, 40 μg for the LC3A-I and LC3A-II were loaded on the gels. Immunoblotting was performed according to Towbin et al. [13] utilizing PVDF membranes (Millipore Corp.). All the groups were loaded on the same gel and transferred on the same membrane. After being blocked with 5% non-fat dry milk in 150 mM NaCl, 10 mM Tris, pH 7.5 (TBS) at room temperature, the membranes were hybridized overnight at 4 °C with the rabbit polyclonal to beclin1 (1:5.000, Abcam, ab62557), mouse monoclonal antibodies to SQSTM1/p62 (1:5.000, Novus Biologicals, H00008878-M01), Bnip3 (1:5.000, Abcam, ab10433), and rabbit polyclonal antibodies to beclin1 (1:5.000, Abcam, ab62557) and LC3A-I and LC3A-II (1:30.000, Abcam, ab62720). The antibodies were tested against the recombinant full length proteins, which are commercially available from Novus Biologicals (data not shown). The immunogen peptide for the MAP1LC3A antibody is the PSDRFKQRRSFADF corresponding to amino acids 2–15 of human N-terminus MAP1LC3A. The membranes were then hybridized for 2 h at room temperature with the appropriate secondary antibody, goat polyclonal to rabbit IgG (1:100.000, Abcam, ab6721) and rabbit polyclonal to mouse IgG (1:100.000, Novus Biologicals, NB 720-H) conjugated to HRP, and developed in TMB solution (KPL Laboratories). The blots were then dried overnight, scanned densitometrically and analyzed using Scion Image (Scion Corporation). To normalize our data each of these blots was then stripped [incubated in 2% SDS (w/v), 62.5 mM Tris–HCl (pH 6.8), 100 mM β-mercaptoethanol for 30 min at 60 °C, rinsed twice for 10 min each with TBS-T], dried overnight, re-hybridized with a polyclonal antibody to β-actin (1:15.000, Novus Biologicals, NB 600-532) and processed as described (p62 and Bnip3 were probe in the same blot and they have the same β-actin loading control).

2.6. Real time qPCR

The ABI Prism 7700 Sequence Detection System was used to perform TaqMan probe-based real-time PCR reactions (Applied Biosystems). The mRNA of the MAP1LC3A (Microtubule Associate Protein 1), Beclin-1 and Bnip3, were analyzed by qPCR (Applied Biosystems). Total RNA was extracted from the tissue sample with Trizol reagent (Ambion) according to the manufacturer's protocol. Concentration and purity of RNA were determined spectrophotometrically at the wavelengths of 260 and 280 nm using the Nanodrop (Nanodrop, Thermo scientific Inc.). Agarose gels were run also to check the purity and integrity of the RNA in the samples by visualizing the 28S and 18S rRNA bands. RNA (5 ng) was reverse-transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems). Expression data were normalized to the levels of GAPDH mRNA. The GAPDH has been an appropriate candidate housekeeping gene to use as an internal control in response to ionizing radiation research [14,15]. Further as the cDNA after the reverse transcription reaction serves as the template for all subsequent PCR reactions, a measure of the total cDNA content would provide an ideal method to account for potential differences in PCR results. The cDNA content was measured using the Quant-iT™ PicoGreen® dsDNA kit (Invitrogen). Samples were analyzed in a 96-well microplate fluorescence reader, with a total reaction

volume of 200 μl in each well. Each sample was analyzed in triplicate with 3 μl cDNA sample, 97 μl TE, and 100 μl PicoGreen reagent in each well. The mean reading of each triplicate was converted to an absolute amount cDNA content using a standard curve from linear emission range = 0–100 ng DNA. Similar results have been observed between the two normalization methods GAPDH mRNA and the PicoGreen. However, since the PicoGreen method requires the assumption that the rRNA is unaffected by the experimental protocol we decide to provide the results only from the GAPDH normalization.

2.7. Immunohistochemistry

The purified rabbit polyclonal antibody MAP1LC3A (Abgent, AP1805a), raised against a synthetic peptide at the C-terminal cleavage site of the human cleaved-MAP1LC3A, was used for detecting autophagy. The immunogen sequence of the autophagy cleaved-LC3 antibody MAP1LC3A (AP1805a) at the C-terminal cleavage site of the human cleaved-LC3 (APG8a) is: DEDGFLYMVYASQETFG aa 104–120 (personal communication). The antibody is capable of detecting both the LC3A-I and LC3A-II forms, although only the

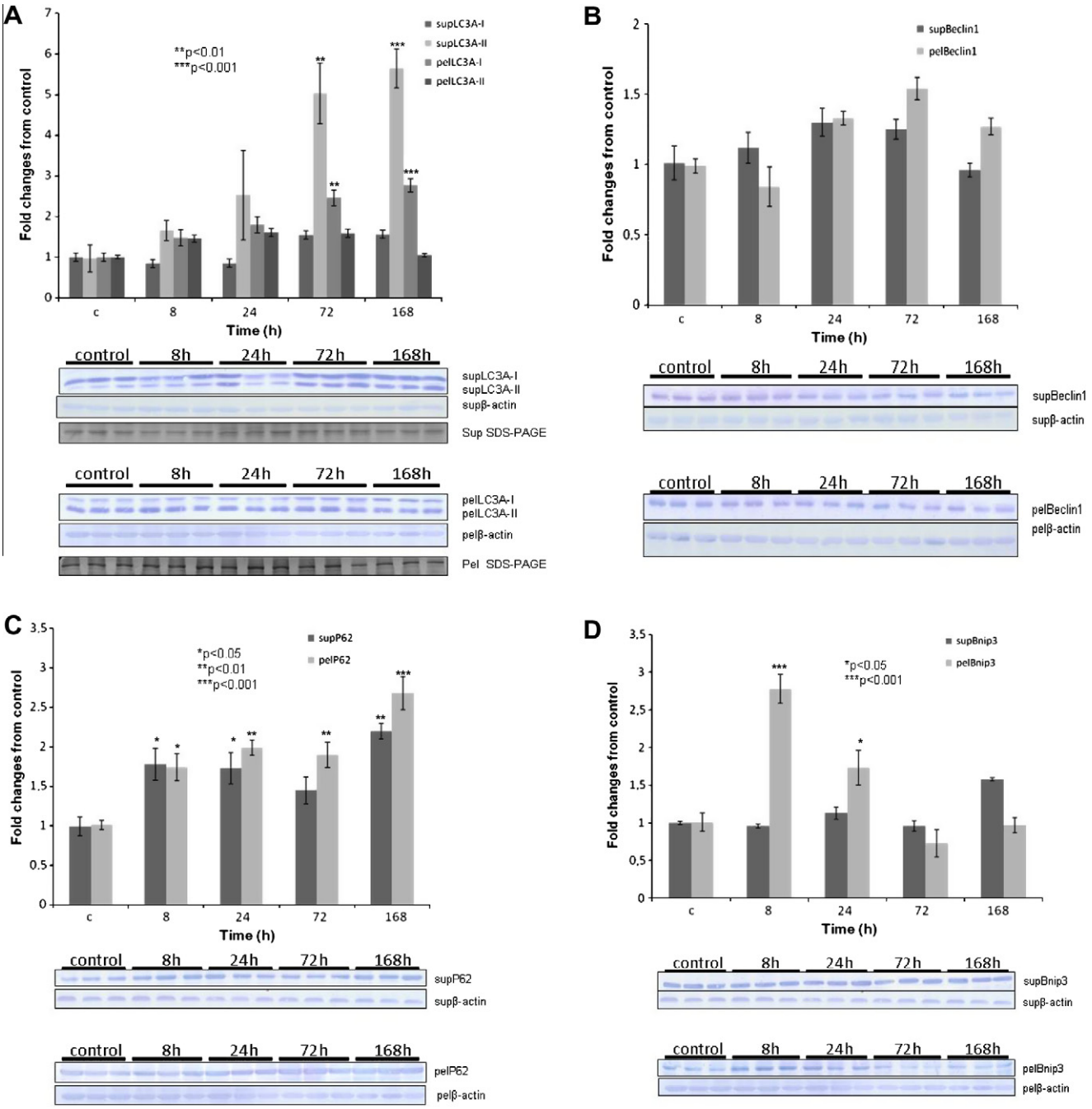

Fig. 1. Autophagic related protein expression after irradiation of lung tissue (whole body irradiation) of male Balb/c mice. Immunoblots for the LC3A-I and LC3A-II proteins in supernatant and pellet fractions and densitometrical analysis of the bands (A). Western blot of the Beclin1, p62 and Bnip3 proteins in supernatant and pellet fractions and densitometrical analysis of the bands (B, C and D). Immunoblots of the LC3A-I, LC3A-II and p62 with or without amifostine, 1 week after whole body irradiation (E). Immunoblots of the LC3A-I, LC3A-II and p62 after 24 and 48 h of starvation (F). Data show the mean ± SEM values. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with control.

LC3A-II form is membrane-bound localizing on the autophagosomal membrane. The immunohistochemical method applied has been previously established and published from our group [16]. Normal rabbit immunoglobulin-G was substituted for the primary antibody as negative control.

2.8. Statistical analysis

Data are expressed as relative changes to the control as means ± SEM, ($n = 3$). One-way ANOVA (GraphPad Prism 5.0) was performed to evaluate the significant interaction of the radiation and the protein response. Post-hoc comparisons performed with Dunnett's significant difference test with a set to $p = 0.05$.

3. Results

3.1. Radiation-induced LC3A protein

To investigate the radiation-induced LC3A protein levels in normal lung tissue we performed immunoblotting analysis to detect the LC3A-I and LC3A-II in both cytosolic and membrane fractions. Moreover, as the LC3A-II is more sensitive than LC3A-I in immunoblotting, we performed the comparison of the amount of each protein LC3A-I and LC3A-II in the various time points, instead of comparing the LC3A II/I ratio or the LC3-II/(LC3-I + LC3-II) ratio.

Immunoblot analysis showed that, following irradiation, LC3A-II protein content was increased by 5.0 ($p < 0.01$) and 5.6 ($p < 0.001$) fold at 72 and 168 h respectively in the cytosolic fraction, whereas remained unchanged in the membrane fraction of the lung tissue (Fig. 1A). Furthermore, ionizing radiation increased the LC3A-I protein content by 2.5 ($p < 0.01$) and 2.8 ($p < 0.001$) fold at 72 and 168 h respectively in the pellet fraction of lung tissue, whereas the induction in the supernatant was not significant (Fig. 1A).

The above findings were substantiated by immunohistochemical analysis of LC3A localization. Thus, immunostaining of non-irradiated lung sections with the anti-LC3A antibody showed a weak, still evident, cytoplasmic staining for both bronchial and alveolar cells, while small vacuolar structures were seen within the cytoplasm (Fig. 2A and D). After 3 and 7 days following irradiation there was a net intensification of the LC3A staining signal in the cytoplasm of both cell types (Fig. 2B, C, E and F), presumably corresponding to accumulating autophagosomes and/or accumulation of the soluble LC3A protein forms.

3.2. Radiation effect on other proteins

The Beclin-1 protein content was not significantly changed in both fractions after ionizing radiation (Fig. 1B).

The p62 protein, was significantly increased in supernatant fraction by 1.7-fold ($p < 0.05$) at 8 and 24 h, and by 2.2-fold at 168 h ($p < 0.01$) after ionizing radiation. Also the p62 was significantly increased by 2.0-fold at 24 h ($p < 0.01$), 1.9-fold at 72 h ($p < 0.01$) and 2.7-fold at 168 h ($p < 0.001$) after ionizing radiation of lung tissue (Fig. 1C).

The Bnip3 was significantly increased in the pellet fraction by 2.8-fold ($p < 0.001$) at 8 h, and 1.7-fold at 24 h ($p < 0.05$) whereas remains unchanged in supernatant fraction after ionizing radiation (Fig. 1D).

3.3. The effect of amifostine

Seven days following irradiation of mice pre-treated with amifostine, the membrane bound LC3A-II protein was increased in the pellet but not in the cytosolic fraction, in direct contrast with the findings obtained in mice irradiated without amifostine (Fig. 3A). Also, the LC3A-I protein was increased in the cytosolic fraction one week after irradiation of mice pre-treated with

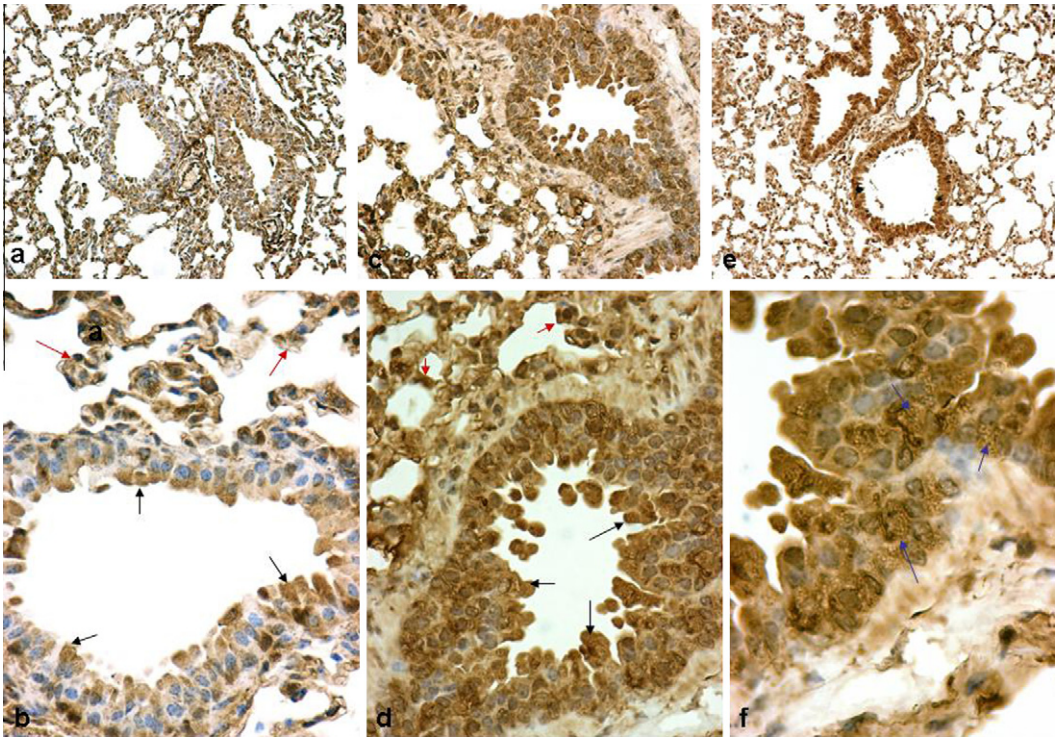

Fig. 2. Immunostaining of non-irradiated lung sections with the anti-LC3A antibody showing a weak staining of the cytoplasm of bronchial and alveolar cells (arrows; (A) magnification 100× and (B) magnification 400×). Seven days following irradiation there was a net intensification of the LC3A staining signal in the cytoplasm of bronchial and alveolar cells (arrows; (C and E) magnification 100× and (D and F) magnification 400×) and a clear and abundant presence of vacuolar structures with LC3A positive coats (arrows; (F)).

amifostine. Moreover, in mice receiving amifostine, the cytosolic p62 protein remained stable showing a normal autophagosome turn-over, as compared to mice that were irradiated without amifostine.

3.4. The effect of starvation

The LC3A-II protein was clearly increased in the pellet fraction at 24 and 48 h after starvation conditions in lung tissue of male mice (Fig. 3B). The LC3A-I and p62 remained unchanged in both fractions (Fig. 3B).

3.5. RT-PCR

Following irradiation, real time qPCR results showed that the mRNA levels of MAP1LC3A and Beclin-1 were significantly decreased compared to the control lung tissue at 8 h and remained low 7 days thereafter (Fig. 4). In contrast, the mRNA levels of the Bnip3 were significantly increased by 3-fold ($p < 0.05$) at 8 h compared to control lung tissue and returned sharply to normal at 24 h (Fig. 4).

4. Discussion

Autophagy is a fundamental process securing the cell homeostasis by degrading damaged organelles and providing energy for survival under stressful conditions. Although the main damage induced by ionizing radiation concerns the DNA, cytoplasmic organelles are also damaged by radiation [17]. The effect of ionizing radiation on the autophagy machinery is obscure [7] and there is almost complete lack of data on how ionizing radiation, at therapeutic doses, affects autophagy in normal tissues. Gorbunov et al. reported that 7 days after the exposure to 9.25 Gy γ-radiation, autophagosomes were accumulated in the crypt cells of murine small intestine [18].

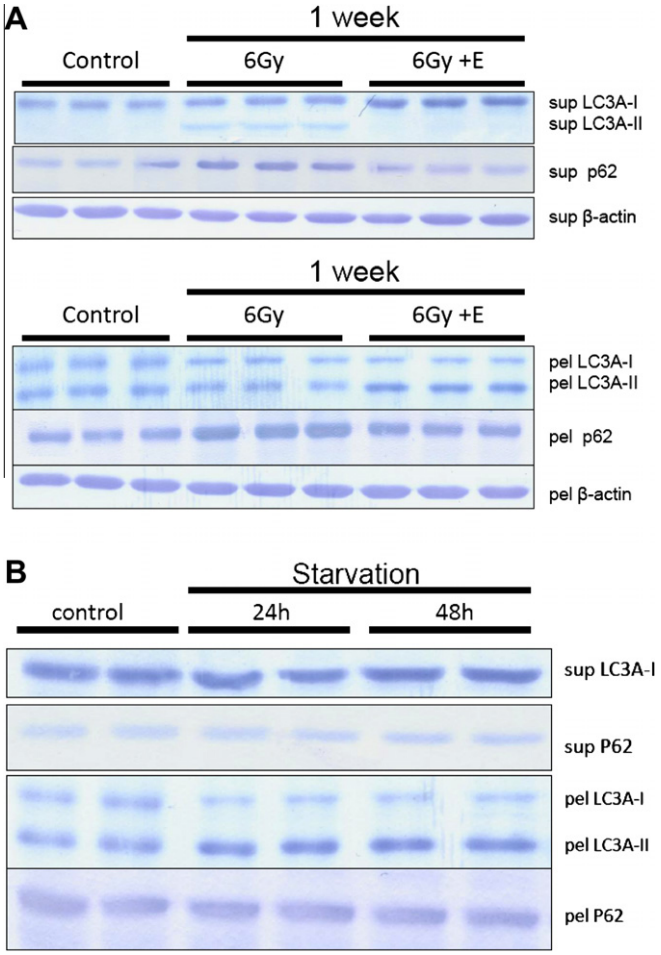

Fig. 3. Autophagic related protein (LC3A-I, LC3A-II and p62) expression after 6 Gy irradiation of lung tissue (whole body irradiation) of male Balb/c mice, (A) with and without amifostine and (B) following 24 and 48 h of starvation. Analysis has been separately performed in supernatant and pellet fractions.

In the current study we focused on the early autophagic response of normal mouse lung to ionizing radiation. Early and chronic radiation pneumonitis and fibrosis are major side-effects of radiotherapy [1]. Understanding the biological pathways involved in radiation pneumonitis would have important clinical implications.

The LC3A is a well defined autophagosomal marker with a conserved post-translation modification at C-terminal Gly-120 [19,20]. On the contrary, the post-translation modification of the MAP1LC3B isoform seems not to be conserved [19–21]. Moreover, in northern blot analysis, although the LC3A mRNA was abundant in lung rat tissue the LC3B mRNA was undetectable [19]. Thus, in the current study the LC3A isoform was chosen for experiments. LC3A derives from a proLC3 30KDa protein after cleavage by autophagin Atg4a to produce the active cytosolic form LC3A-I. Following activation by Atg7, LC3A-I is transferred to Atg3 and converted into the membrane-bound form LC3A-II. The LC3A-II resides in the inner and outer side of the membrane, where after the autophagosome formation the LC3-II located in the outer side is released to the cytosol and the LC3A-II located in the inner side is degraded by hydrolases [22]. In this latter form, LC3A localizes on the isolation membranes and the complete spherical autophagosomal and autolysosomal membranes, forming a suitable marker of autophagic activity [23,24].

Another key protein assessed was Beclin-1 that functions as a scaffold for the formation of a molecular complex to initiate autophagy [25] and is essential to initiate autophagy [25]. Moreover, we examined the p62/SQSTM1 (sequestosome 1), a multifunctional protein with an ubiquitin-associated domain that interacts with a central component of the autophagy machinery, LC3, and transport ubiquitinated proteins to degradation by the autophagosome [26]. Thus, p62 protein levels may provide a method for detecting the functionality of autophagy as normal autophagosome turn-over is linked with normal p62 levels. Finally, we assessed the expression of BNIP3 protein, which is involved in the induction of autophagy and mitophagy [9].

Western blot and immunohistochemical analysis showed that the total LC3A-I and II protein content increased at 72 h following irradiation and that was sustained up to 7 days. The most striking increase was that of the membrane bound LC3A-II protein. Differential analysis of LC3A content in cellular lysate fractions (supernatant vs. pellet), however, showed that LC3A-II was increased only in the supernatant and remained stable in the pellet. On the other hand, the decreased mRNA LC3A and Beclin-1 levels persisting throughout the 7 days of monitoring, show that ionizing radiation did not stimulate expression of these genes. Thus, the reasons of LC3A accumulation should be sought at a post-transcriptional level.

As the shift of the LC3A-II to the supernatant fraction was an unexpected finding, we further validated our technique in lung tissues of mice that were put under starvation (a stress known to induce autophagy [27]) for 24 and 48 h. The results showed that the LC3A-I remain unchanged in supernatant and pellet fraction while the LC3A-II clearly increased in pellet and remained undetectable in the supernatant fraction. These patterns of intensification of autophagy in lung tissues, sharply contrast the LC3A and p62 protein changes noted after irradiation. Since the LC3 response to

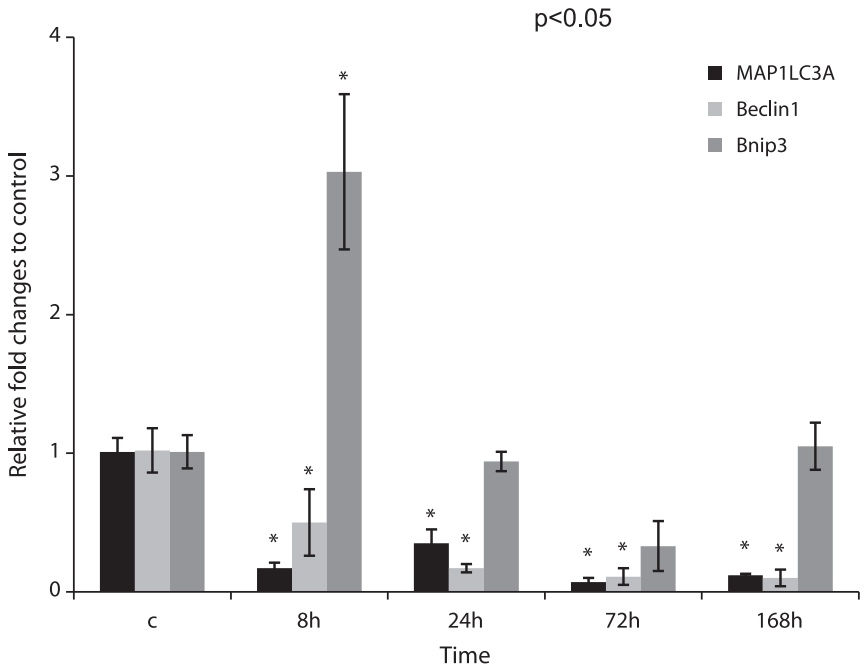

Fig. 4. Expression levels of MAP1LC3A, Beclin1 and Bnip3 mRNA in lung tissue after ionizing radiation. mRNA changes are calculated by comparing to the control group. Data are expressed as mean ± SEM values. $^{*}p < 0.05$, as compared with control.

starvation is well defined in liver tissue, we further validated our results in this tissue. In accordance to previously reported experience [27], LC3A-I and LC3A-II was clearly increased in liver tissue after 24 and 48 h starvation conditions, in the supernatant and membrane fraction, respectively (unpublished results) .

These findings are supportive of a rise in LC3A-I and II protein content in irradiated cells but are against to the expected increased presence of LC3A in membrane structures. In accordance to our study, He et al. previously reported immunoblot analysis showing that LC3A-II appears only in the pellet, whereas the LC3A-I is found in both membrane and the cytosolic fractions of unstimulated HeLa cells [28]. Nevertheless, the LC3-II found on the cytosolic side of autophagosomes is return to the cytosol and recycled [22].

We postulate that under certain stress conditions, such as irradiation, the LC3-II of the outer autophagosome membrane may accumulate in the cytosol due to the inability to be recycled, which explains the striking increase of the LC3A-II form in the soluble fraction of lung found herein. Impaired function of the proteasome machinery [29], caused by exposure to ionizing irradiation, may have disabled the recycling of LC3A-II. Of interest, the p62 protein that binds to LC3A also increased, particularly at 7 days, which supports the hypothesis of a gradual accumulation of the LC3A/p62 complex, as a result of reduced degradation ability [26]. Inhibition of autophagy leads to an increase in the size and number of p62 bodies and p62 protein levels [30]. Indeed, in a study in Atg4B mutant cells, where autophagosome maturation was blocked, increased p62 accumulation was noted, indicating that accumulation of p62 could be a good indicator of aberrant autophagy [31].

In support to this hypothesis were our findings in mice that received the potent radioprotective agent amifostine. In this case the LC3A-II protein was no longer accumulated in the cytosolic fraction but only in the membrane fraction and, furthermore, the p62 protein remained stable and did not increase. It is, therefore, suggested that the amifostine effectively contributed to the normal autophagosome turn-over and preserved the autophagic function of lung tissue.

It is postulated that following 6 Gy of γ-irradiation, normal lung cells may suffer a severe deregulation of the autophagy machinery. This deregulation is characterized by decreased transcription of the LC3A mRNA and accumulation of the LC3A, and p62 protein, either due to defective formation of autophagosomes or to an aberrant degradation processing of the formed autophagosomes. The role of this phenomenon on the radiation induced early and late lung damage is under investigation.

Acknowledgments

This work was supported by grants from Tumor and Angiogenesis Research Group. We thank Dr. Stylianos P. Scordilis (Professor at Smith College, MA) for his support during the project.

References

[1] P.G. Tsoutsou, M.I. Koukourakis, Radiation pneumonitis and fibrosis: mechanisms underlying its pathogenesis and implications for future research, Int. J. Radiat. Oncol. Biol. Phys. 66 (2006) 1281–1293.
[2] W.C. Dewey, C.C. Ling, R.E. Meyn, Radiation-induced apoptosis: relevance to radiotherapy, Int. J. Radiat. Oncol. Biol. Phys. 33 (1995) 781–796.
[3] X.P. Zhang, F. Liu, Z. Cheng, et al., Cell fate decision mediated by p53 pulses, Proc. Natl. Acad. Sci. USA 106 (2009) 12245–12250.
[4] Z. Xie, D.J. Klionsky, Autophagosome formation: core machinery and adaptations, Nat. Cell Biol. 9 (10) (2007 Oct) 1102–1109.
[5] B. Levine, J. Yuan, Autophagy in cell death: an innocent convict?, J Clin. Invest. 115 (2005) 2679–2688.
[6] B. Levine, G. Kroemer, Autophagy in the pathogenesis of disease, Cell 132 (2008) 27–42.
[7] C.E. Zois, M.I. Koukourakis, Radiation-induced autophagy in normal and cancer cells: towards novel cytoprotection and radio-sensitization policies?, Autophagy 5 (2009) 442–450
[8] C.M. Spencer, K.L. Goa, Amifostine: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic potential as a radioprotector and cytotoxic chemoprotector, Drugs 50 (1995) 1001–1031.
[9] J. Zhang, P.A. Ney, Role of BNIP3 and NIX in cell death, autophagy, and mitophagy, Cell Death Differ. 16 (2009) 939–946.
[10] H.S. Thompson, S.P. Scordilis, P.M. Clarkson, et al., A single bout of eccentric exercise increases HSP27 and HSC/HSP70 in human skeletal muscle, Acta Physiol. Scand. 171 (2001) 187–193.
[11] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[12] O.H. Lowry, N.J. Rosenberg, A.L. Farr, et al., Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[13] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.

[14] M. Banda, A. Bommineni, R.A. Thomas, et al., Evaluation and validation of housekeeping genes in response to ionizing radiation and chemical exposure for normalizing RNA expression in real-time PCR, Mutat. Res. 649 (2008) 126–134.
[15] A. Arenz, N. Stojicic, P. Lau, et al., Suitability of commonly used housekeeping genes in gene expression studies for space radiation research, Adv. Space Res. 39 (2007) 1050–1055.
[16] E. Sivridis, M.I. Koukourakis, C.E. Zois, I. Ledaki, D.J. Ferguson, A.L. Harris, K.C. Gatter, A. Giatromanolaki, LC3A-positive light microscopy detected patterns of autophagy and prognosis in operable breast carcinomas, Am. J. Pathol. 176 (5) (2010 May) 2477–2489.
[17] Z. Somosy, Radiation response of cell organelles, Micron 31 (2000) 165–181.
[18] N.V. Gorbunov, J.G. Kiang, Up-regulation of autophagy in small intestine Paneth cells in response to total-body gamma-irradiation, J. Pathol. 219 (2009) 242–252.
[19] J. Wu, Y. Dang, W. Su, C. Liu, H. Ma, Y. Shan, Y. Pei, B. Wan, J. Guo, L. Yu, Molecular cloning and characterization of rat LC3A and LC3B – two novel markers of autophagosome, Biochem. Biophys. Res. Commun. 339 (1) (2006) 437–442. Epub 2005 Nov. 14.
[20] H. He, Y. Dang, F. Dai, Z. Guo, J. Wu, X. She, Y. Pei, Y. Chen, W. Ling, C. Wu, S. Zhao, J.O. Liu, L. Yu, Post-translational modifications of three members of the human MAP1LC3 family and detection of a novel type of modification for MAP1LC3B, J. Biol. Chem. 278 (31) (2003) 29278–29287. Epub 2003 May 11.
[21] I. Tanida, T. Ueno, E. Kominami, Human light chain 3/MAP1LC3B is cleaved at its carboxyl-terminal Met121 to expose Gly120 for lipidation and targeting to autophagosomal membranes, J. Biol. Chem. 279 (46) (2004) 47704–47710. Epub 2004 Sep. 7.
[22] Y. Kabeya, N. Mizushima, T. Ueno, et al., LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing, EMBO J. 19 (2000) 5720–5728.
[23] N. Mizushima, Y. Ohsumi, T. Yoshimori, Autophagosome formation in mammalian cells, Cell Struct. Funct. 27 (2002) 421–429.
[24] Y. Cao, D.J. Klionsky, Physiological functions of Atg6/Beclin 1: a unique autophagy-related protein, Cell Res. Cell Res. 17 (2007) 839–849.
[25] X. Zeng, J.H. Overmeyer, W.A. Maltese, Functional specificity of the mammalian Beclin-Vps34 PI 3-kinase complex in macro autophagy versus endocytosis and lysosomal enzyme trafficking, J. Cell Sci. 119 (2006) 2503–2518.
[26] S. Pankiv, T.H. Clausen, T. Lamark, et al., P62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy, J. Biol. Chem. 282 (2007) 24131–24145.
[27] N. Mizushima, A. Yamamoto, M. Matsui, T. Yoshimori, Y. Ohsumi, In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker, Mol. Biol. Cell 15 (3) (2004) 1101–1111. Epub 2003 Dec. 29.
[28] H. He, Y. Dang, F. Dai, et al., Post-translational modifications of three members of the human MAP1LC3 family and detection of a novel type of modification for MAP1LC3B, J. Biol. Chem. 278 (2003) 29278–29287.
[29] W.H. McBride, K.S. Iwamoto, R. Syljuasen, et al., The role of the ubiquitin/proteasome system in cellular responses to radiation, Oncogene 22 (2003) 5755–7573.
[30] M. Komatsu, S. Waguri, M. Koike, et al., Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy deficient mice, Cell 131 (2007) 1149–11463.
[31] N. Dupont, S. Lacas-Gervais, J. Bertout, et al., Shigella phagocytic vacuolar membrane remnants participate in the cellular response to pathogen invasion and are regulated by autophagy, Cell Host Microbe 6 (2009) 137–149.