



# Association between radiation-induced cell death and clinically relevant radioresistance

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## Abstract

Radiotherapy (RT) is one of the major modalities for the treatment of human cancer and has been established as an excellent local treatment for malignant tumors. However, the existence of radioresistant cells remains one of the most critical obstacles in RT. To know the characteristics of radioresistant cells, clinically relevant radioresistant (CRR) cell lines were established. CRR cells can continue to proliferate *in vitro* and *in vivo* after exposure to 2 Gy/day of X-rays for more than 30 days. Daily microscopic observation of the irradiated CRR cells has indicated that the increase in cell death is not observed within 7 days of irradiation with 10 Gy of X-rays, suggesting that cell death is involved in cellular radioresistance. Radiation-induced regulated cell death (RCD) can be classified into three categories: apoptosis, autophagy-dependent cell death and necrosis (necroptosis). This review focuses on an aspect of radiation-induced RCD that has often been neglected: the manner in which the cells are destroyed. In many studies, apoptosis is considered the primary mode of RCD in irradiated cancer cells; however, it is necessary to consider necrosis or necroptosis as one of the modes of radiation-induced RCD.

**Keywords** Apoptosis · Autophagy-dependent cell death · Necroptosis · Necrosis · Radioresistant cell · Regulated cell death

## Introduction

Radiotherapy (RT) is one of the major modalities for the treatment of human cancer and has been established as an excellent local treatment for malignant tumors. The objective of RT is to achieve local control of cancer to prevent invasion, organ dysfunction, and metastases. The efficacy of RT depends on the presence of radioresistant cells, which

remains one of the most critical obstacles in RT and radio-chemotherapy. Cellular radiosensitivity has long been a focus area in the field of radiation biology and oncology because it clearly influences the outcome of therapy. To develop more effective RT, it is necessary to understand the characteristics of radioresistant cells. Many studies have attempted to identify the molecules involved in cancer cell radioresistance (Guo et al. 2005; Chang et al. 2007; Ishigami et al. 2007; Ogawa et al. 2007). However, these studies did not necessarily aim to improve conventional fractionated RT. Moreover, it is noteworthy that cellular radioresistance has primarily been studied in cells with different genetic backgrounds and different origins. Therefore, the concordant mechanisms underlying cellular radioresistance have not been clarified yet. Therefore, it is necessary to develop a system to compare radioresistant and radiosensitive cells with isogenic background. On the other hand, published studies about the genes responsible for cellular radioresistance have analyzed surviving cells after exposure to single or several rounds of fractionated radiation. Surviving cells include many senescent cells that lack the ability to undergo mitosis, which suggests that the group of cells surviving irradiation is highly heterogeneous.

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Conventional fractionated RT consists of 2 Gy of X-rays, administered once a day, 5 days a week, for 5–7 weeks, to constitute a total of 60 Gy. The rationale underlying this schedule is to allow easier recovery of normal cells, compared to cancer cells, from the sub-lethal radiation damage occurring between the fractions. The concept of fractionated RT has evolved from the empirical evidence obtained through clinical trials. Recently, the authors have established radioresistant human cancer cells that can continue to proliferate *in vitro* after exposure to 2 Gy/day of X-rays for more than 30 days (Table 1). These cells have been termed as “clinically relevant radioresistant (CRR)” cells (Kuwahara et al. 2009, 2017). An *in vivo* experiment using a xenograft tumor model of CRR SAS-R cells showed that the tumors of CRR cells are also radioresistant to 2 Gy/day of X-rays (Fig. 1). Our previous study revealed that CRR cells could escape death caused by X-ray irradiation both *in vivo* and *in vitro* (Kuwahara et al. 2011). Therefore, to overcome radioresistance in cancer cells, it is necessary to study the mechanisms underlying radiation-induced cell death. Mammalian cells exposed to unrecoverable perturbations of the intracellular or extracellular microenvironment can activate one of many signal transduction cascades ultimately leading to their demise. Each of such regulated cell death (RCD) modes is initiated and propagated by molecular mechanisms that exhibit a considerable degree of interconnectivity (Galluzzi et al. 2018). Although the underlining molecular mechanisms exhibit considerable overlap, RCD is involved in two diametrical scenarios. On one hand, RCD can occur in the absence of any exogenous environmental perturbation. These completely physiological forms of RCD are generally referred to as programmed cell death (PCD). On the other hand, RCD can originate from perturbations of the intracellular or extracellular microenvironment, when

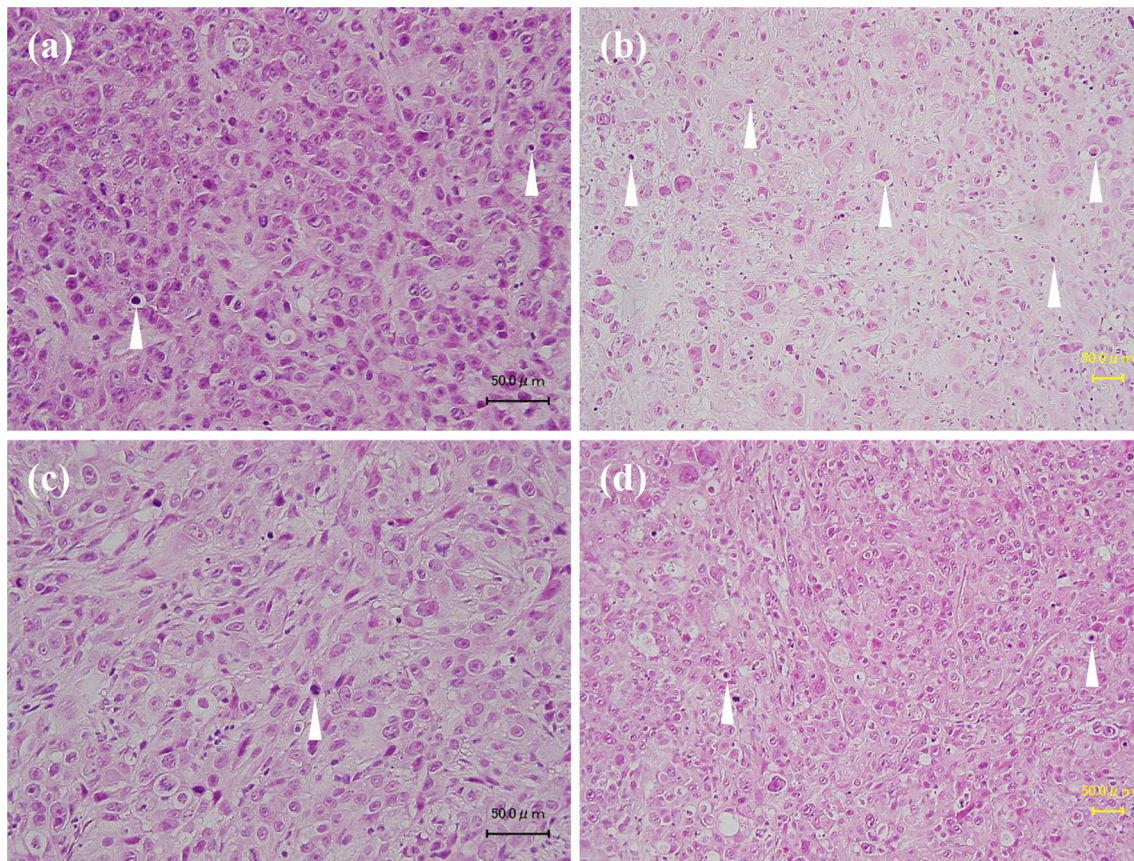
such perturbations are too intense or prolonged for adaptive responses to cope with stress and restore cellular homeostasis (Galluzzi et al. 2016).

Elucidation of the molecular mechanisms underlying radiation-induced RCD remains one of the hot topics in the field of RT (Lomax et al. 2013; Kim et al. 2015). It is generally accepted that ionizing radiation kills tumor cells primarily by causing double-strand breaks (dsbs) in the DNA directly and indirectly through the formation of intermediate radicals. Survival of cancer cells mainly depends on their capacity for oxidative defense as well as the efficacy of DNA repair. The level of reactive oxygen species (ROS)-mediated oxidative stress is also linked to cellular radiosensitivity (Dayal et al. 2014). Formerly, the mechanisms responsible for radiation-induced cell death are divided into two distinct forms: interphase death and reproductive death (Shinohara and Nakano 1993). Since they are defined based on the classical radiobiological concepts using the clonogenic cell survival assay, the biochemical and molecular mechanisms involved in the induction of radiation-induced cell death are not fully understood in relation to the modes of cell death. In 1972, Kerr and colleagues classified cell death under two headings (Kerr et al. 1972): apoptosis and necrosis. The former is PCD, which is genetically influenced. On the other hand, the latter is a form of accidental cell death, and not PCD. In 1990, the four types of PCD are reviewed based on the morphological features of dying cells, as observed during the development of both vertebrates and invertebrates (Clarke 1990). Type I PCD corresponds to apoptosis, which proceeds as cell shrinkage, chromatin condensation, nucleosomal DNA degradation, and fragmentation of the cell into apoptotic bodies. Activation of the caspase family gives rise to apoptosis. Type II PCD

**Table 1** clinically relevant radioresistant cell lines

Parental cell line	Origine	Clinically relevant radioresistant cell line
A549	Human alveolar adenocarcinoma	A549-R
H1299	Human non-small cell lung carcinoma	H1299-R
HeLa	Human cervix adenocarcinoma	HeLa-R
HepG2	Human hepatocellular carcinoma	HepG2-8960-R
HSC2	Human oral squamous cell carcinoma	HSC2-R
HSC3	Human oral squamous cell carcinoma	HSC3-R
HSC4	Human oral squamous cell carcinoma	HSC4-R
KB	HeLa contaminant	KB-R
LS174T	Human colon adenocarcinoma	LS174T-R
PC3	Human prostate adenocarcinoma	PC3-R
SAS	Human tongue carcinoma	SAS-R
U2OS	Human bone osteosarcoma	U2OS-R

Based on Kuwahara et al. (2017) with some modifications



**Fig. 1** Xenograft tumor model of parental SAS and clinically relevant radioresistant SAS-R cells. SAS-R cells show *in vivo* resistance to 2 Gy/day X-rays. **a** SAS tumor not exposed to X-rays. **b** SAS tumor exposed to a total of 60 Gy of X-rays (30 fractions). The irradiated SAS tumor showed an increase in connective tissue. Many pyknotic

cells are also detected in these tumors. **c** SAS-R tumor not exposed to X-rays. **d** SAS-R tumor exposed to a total of 60 Gy of X-rays (30 fractions). Unlike SAS tumor, SAS-R tumor do not show an increase in connective tissue. Arrow heads, pyknotic cells

corresponds to autophagy-dependent cell death, which is characterized by the appearance of double- or multiple-membrane cytoplasmic vesicles engulfing bulk cytoplasm and/or cytoplasmic organelles. Autophagic vesicles and their contents are destroyed by the lysosomal system. Type III PCD is defined as non-lysosomal degradation on the vesicular membrane. This mode of cell death corresponds to necrosis or necroptosis, which can be further subdivided into type IIIA (non-lysosomal degradation) and IIIB (cytoplasmic type of degeneration).

Apoptosis, is considered the main mode of radiation-induced RCD, and autophagy-dependent cell death and necrosis are ignored until recent. On the other hand, advances in molecular biology revealed many modes of RCD (Kroemer et al. 2009; Galluzzi et al. 2018). Recently, several modes of radiation-induced RCD, such as apoptosis (Dewey et al. 1995; Mirzaie-Joniani et al. 2002a, b; Balcer-Kubiczek 2012), autophagy-dependent cell death (Gozuacik and Kimchi 2004; Tsuboi et al. 2009; Jo et al. 2015; Chen et al. 2016), mitotic catastrophe (Roninson et al. 2001),

necrosis (Rainaldi et al. 2003), and senescence-like cell death (Galluzzi et al. 2012) have been reported.

Although apoptosis is a critical mode of cell death observed during the normal process (Meier et al. 2000), apoptotic cells are also observed in tumor tissues (Brown and Attardi 2005; Ouyang et al. 2012). The main objective of RT for cancer cells is to eliminate their reproductive potential. One approach to achieve this is by inducing tumor cell apoptosis (Kerr et al. 1994; Dewey et al. 1995) or a state of irreversible growth arrest such as senescence (Sabin and Anderson 2011; Liao et al. 2014). Kerr et al. originally coined the term “apoptosis” to differentiate naturally occurring developmental cell death from necrosis caused by acute tissue injury (1972). They adopted the Greek word for the process of leaves falling from trees or petals falling from flowers (Duque-Parra 2005). In many studies, apoptosis is considered the primary mode of cell death in irradiated cancer cells, and recent advances in tumor biology showed that apoptosis is not the main mechanism underlying the death of cancer cells in response to

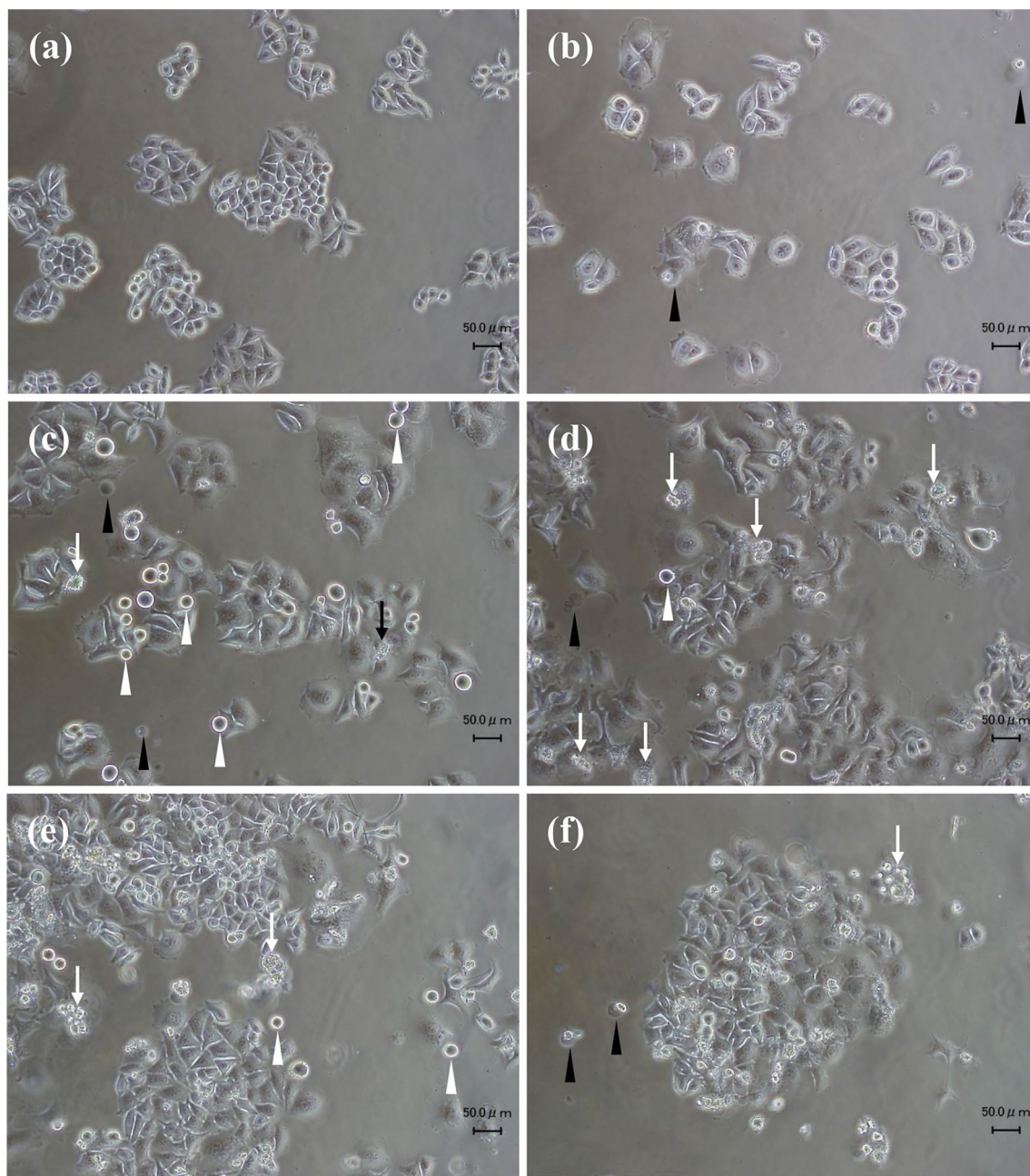
common treatment regimens, especially RT. Except for the caspase-dependent mechanism, several lines of evidences demonstrated that the caspase-independent mechanism is directly involved in radiation-induced cell death (Chipuk and Green 2005; Sun et al. 2016). Accumulating evidences also suggest that the induction of apoptosis alone is insufficient to account for the therapeutic effect of RT (Abend 2003; Eriksson and Stigbrand 2010). It has become obvious in the last few years that the inhibition of the proliferative capacity of malignant cells following IR, especially in case of solid tumors, can occur via alternative cell death modalities or permanent cell cycle arrest, i.e., mitotic catastrophe or senescence. Previously, the authors reported that apoptosis is not significantly induced in HepG2 cells after X-ray irradiation (Kuwahara et al. 2011). This review focuses on an aspect of radiation-induced RCD that has often been neglected: the manner in which the cells are destroyed. The dying cells, after exposure to IR, might adopt one of the three different morphological types, namely, apoptosis, necrosis and autophagy-dependent cell death. In this review, radiation-induced senescence and mitotic catastrophe, considered as one of the modes of cell death in tumor cells, are not mentioned because the metabolic processes of those cells continued to remain active. These cells finally perish by apoptosis, autophagy-dependent cell death, or necrosis (Fukumoto 2014).

### Effect of X-ray exposure on cancer cells

Many *in vitro* studies using cancer cell lines showed that radiation-induced cell death usually occurs within 48 h of irradiation. However, daily microscopic observation of the irradiated HepG2 cells has indicated that the increase in cell death is not observed within 48 h of irradiation with 10 Gy of X-rays (Kuwahara et al. 2011). Cell death is not induced in HeLa cells within 48 h of exposure to 10 Gy of X-rays (Fig. 2c), but begins 3 days after the exposure (Fig. 2d). Cells in mitotic catastrophe are also frequently observed around the same time. Dead cells with apoptotic bodies are seldom observed in irradiated cells. At day 5 after the exposure, a significant increase is observed in the number of dying cells (Fig. 2e). At day 7, most of the dying cells are without features of apoptosis or necrosis, suggesting that autophagy-dependent cell death is induced at this time point. During these 7 days, the induction of apoptosis is  $\leq$  not higher than 20% in HepG2, SAS, and HeLa cell lines, as determined by Annexin-V staining. Autophagy-dependent cell death is the predominant mode of cell death in HepG2 (Kuwahara et al. 2011); on the other hand, necroptosis or necrosis also seemed to be the predominant mode of cell death in irradiated cancer cells.

### Radiation-induced apoptosis

Apoptosis is generally characterized by dynamic morphological changes and energy-dependent biochemical mechanisms (Elmore 2007). Apoptosis is a vital component of various processes, including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development, and chemical-induced cell death (Elmore 2007). Apoptosis is considered the primary mode of cell death following exposure to radiation. Normal tissues such as the thymus, intestine, and testis show significant induction of apoptosis by IR (Ohyama et al. 1985; Hasegawa et al. 1997; Potten and Grant 1998). In the lymphoid and myeloid lineages, apoptosis is the primary death mechanism induced by irradiation (Radford and Murphy 1994). However, radiation-induced apoptosis is not so frequently observed in the cells of epithelial origin compared with cells of the hematological origin. Radiation-induced apoptosis has been characterized and known to include pyknosis, cell shrinkage, and internucleosomal breakage of chromatin, all of which are hallmarks of apoptotic cell death (Shinomiya 2001). Many studies discuss radiation-induced apoptosis in cancer. X-ray irradiation alone is shown to efficiently induce apoptotic bodies in wild-type p53 SAS (Ohnishi et al. 2004). Another study also showed that 4 Gy of X-rays induce apoptosis in about 20% of H23 human non-small cell lung cancer cells (Huang et al. 2010). On the other hand, 10 Gy of X-rays induce apoptosis in about 20% of HepG2 cells about 48 h after exposure to 10 Gy of X-rays (Shimura et al. 2010). Pinar et al. also showed that radiation-induced apoptosis increases with increasing dose of radiation, and that the data fit to a semi-logarithmic mathematical model; in fact, a positive correlation is noted among radiation-induced apoptosis values at different doses of radiation (2010). One of the well-known hallmarks of apoptosis is the induction of apoptotic bodies (Figs. 3b, 5a, b). To determine whether apoptosis is the main mode of radiation-induced cell death or not, morphological changes occurring in irradiated cancer cells are observed daily using phase contrast microscopy. As shown in Fig. 2, dying cells with apoptotic bodies are, at times, observed after irradiation; however, during the 7 days post-irradiation, no drastic induction of apoptosis is observed in HeLa and HepG2 cells (Kuwahara et al. 2011). In three other tumor cell lines, that is, SAS, HeLa, and U2OS-RFP-LC3, X-ray exposure induced significant rate of cell death; however, cells with apoptotic features are seldom encountered. These morphological observations strongly suggest that although X-ray exposure induces apoptosis in cancer cells, apoptosis is not the main mode



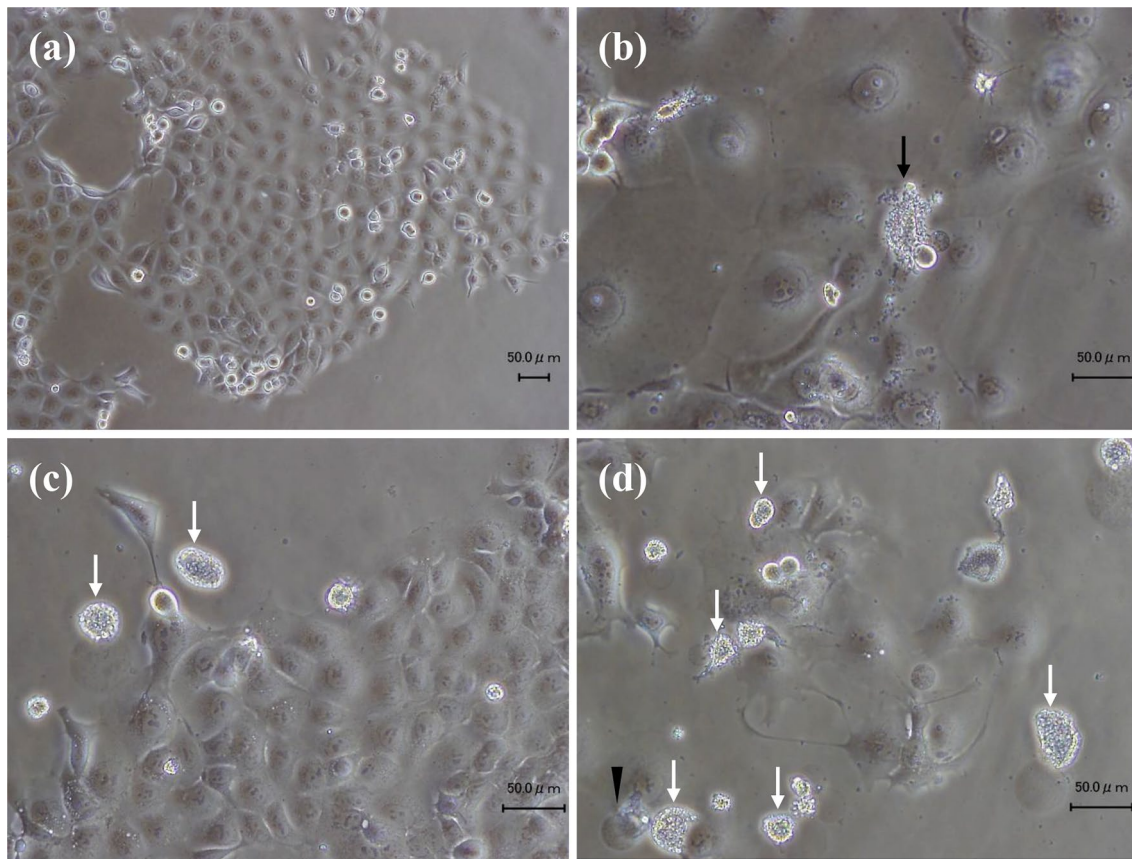
**Fig. 2** Morphological changes in HeLa cells after exposure to 10 Gy of X-rays. **a** HeLa cells not exposed to X-rays. **b** HeLa cells at day 1 after exposure to 10 Gy of X-rays. Cell death is rare. **c** HeLa cells at day 2 after exposure to 10 Gy of X-rays. Mitotic cells increase in number (white arrow heads). **d** HeLa cells at day 3 after exposure to

10 Gy of X-rays. **e** HeLa cells at day 5 after exposure to 10 Gy of X-rays. **f** HeLa cells at day 7 after exposure to 10 Gy of X-rays. Black arrow heads, necrotic cells; white arrows, dying cells without features of apoptosis or necrosis; black arrows, apoptotic death with apoptotic bodies

of cell death induced by X-ray. Time-lapse analysis of irradiated cancer cells also revealed that the extent of induction of apoptosis is very low. In CRR cells, apoptotic cells are seldom observed after irradiation with 10 Gy of X-rays (Kuwahara et al. 2011).

### Radiation-induced autophagy-dependent cell death

A relatively new discovery among the different modes of RCD is the autophagy-dependent cell death. This type of

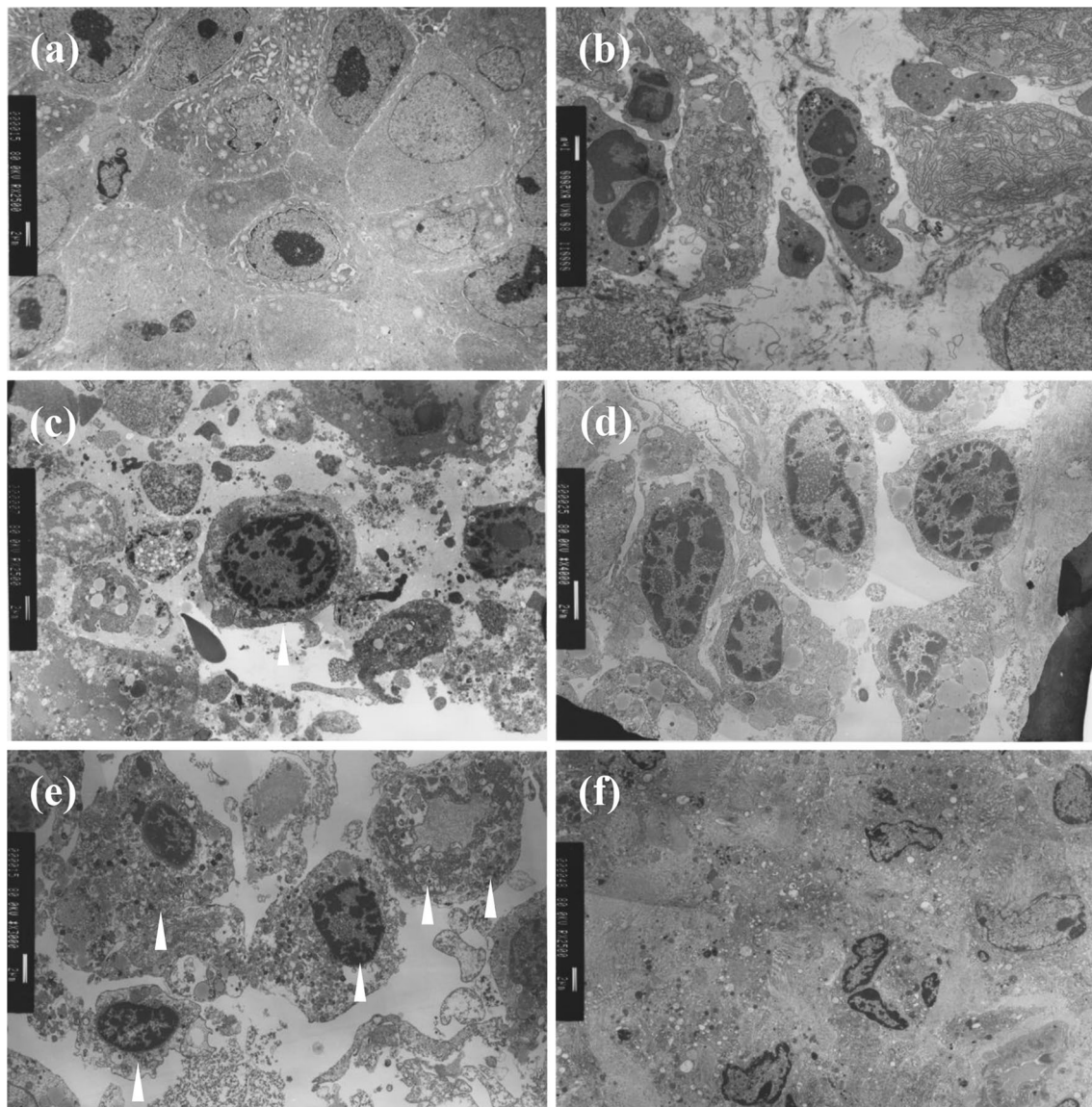


**Fig. 3** SAS cells exposed to 10 Gy of X-rays. **a** SAS cells not exposed to X-rays. **b** Apoptotic cells with apoptotic bodies (black arrow) at day 3 after exposure to 10 Gy of X-rays. **c** Dying cells without features of apoptosis or necrosis (white arrows) at day 5 after

exposure to 10 Gy of X-rays. **d** SAS cells at day 7 after exposure to 10 Gy of X-rays. Dying cells without features of apoptosis or necrosis are found to increase in number

cell death is characterized by an ultrastructurally intact nucleus and an increase in the number of autophagosomes in the cytoplasm (Tsujiyama and Shimizu 2005; Motyl et al. 2007). Autophagy is a dynamic process of protein degradation, which is typically observed during a state of nutrient deprivation (Sato et al. 2007). Cells undergoing autophagy-dependent cell death utilize the autophagic/lysosomal compartment to auto-digest proteins and damaged organelles as well as to recycle amino and fatty acids. Autophagy is characterized by the sequestration of targeted cytoplasmic components and organelles from the rest of the cell within a double-membrane vesicle called the autophagosome (Mizushima 2007). The autophagy pathway is negatively regulated by the PI3K/Akt/mTOR pathway (Chang et al. 2015). Hyperactivation of this pathway contributes to cell death, whereas controlled activation has a pro-survival effect. Recently, the interest in autophagy has been renewed among oncologists, because different types of cancer cells undergo autophagy-dependent cell death after various anticancer therapies (Moretti et al. 2007; Janku et al. 2011). This

type of non-apoptotic cell death has been documented primarily by observing morphological changes such as numerous autophagic vacuoles in the cytoplasm of dying cells. Thus, autophagy-dependent cell death is considered one of RCD processes. Autophagy-dependent cell death is suggested to be one of the modes of radiation-induced cell death (Moretti et al. 2007; Kuwahara et al. 2011; Jo et al. 2015). Autophagy-dependent cell death is involved in *in vitro* radioresistance because the extent of induction of autophagy-dependent cell death is lower in radioresistant HepG2-8960-R cells compared to parental HepG2 cells after exposure to fractionated radiation of X-rays. These observations are also confirmed in SAS and SAS-R tumors (Fig. 4). After exposure to a total of about 30 Gy of X-rays (15 fractions), an increase in the number of cytoplasmic autophagosomes is observed in the SAS tumor, but not in the SAS-R tumor. After exposure to 60 Gy of X-rays (30 fractions), autophagosome-filled SAS cells are observed, suggesting that autophagy-dependent cell death is induced in the SAS tumor. Induction of autophagy in radioresistant cells by rapamycin treatment is an effective *in vitro*



**Fig. 4** Ultrastructural analysis of parental SAS and clinically relevant radioresistant SAS-R tumors unexposed or exposed to 2 Gy/days of X-rays. **a** SAS tumor not exposed to X-rays. **b** SAS-R tumor not exposed to X-rays. **c** SAS tumor exposed to a total of 30 Gy (15 fractions). **d** SAS-R tumor exposed to a total of 30 Gy (15 fractions).

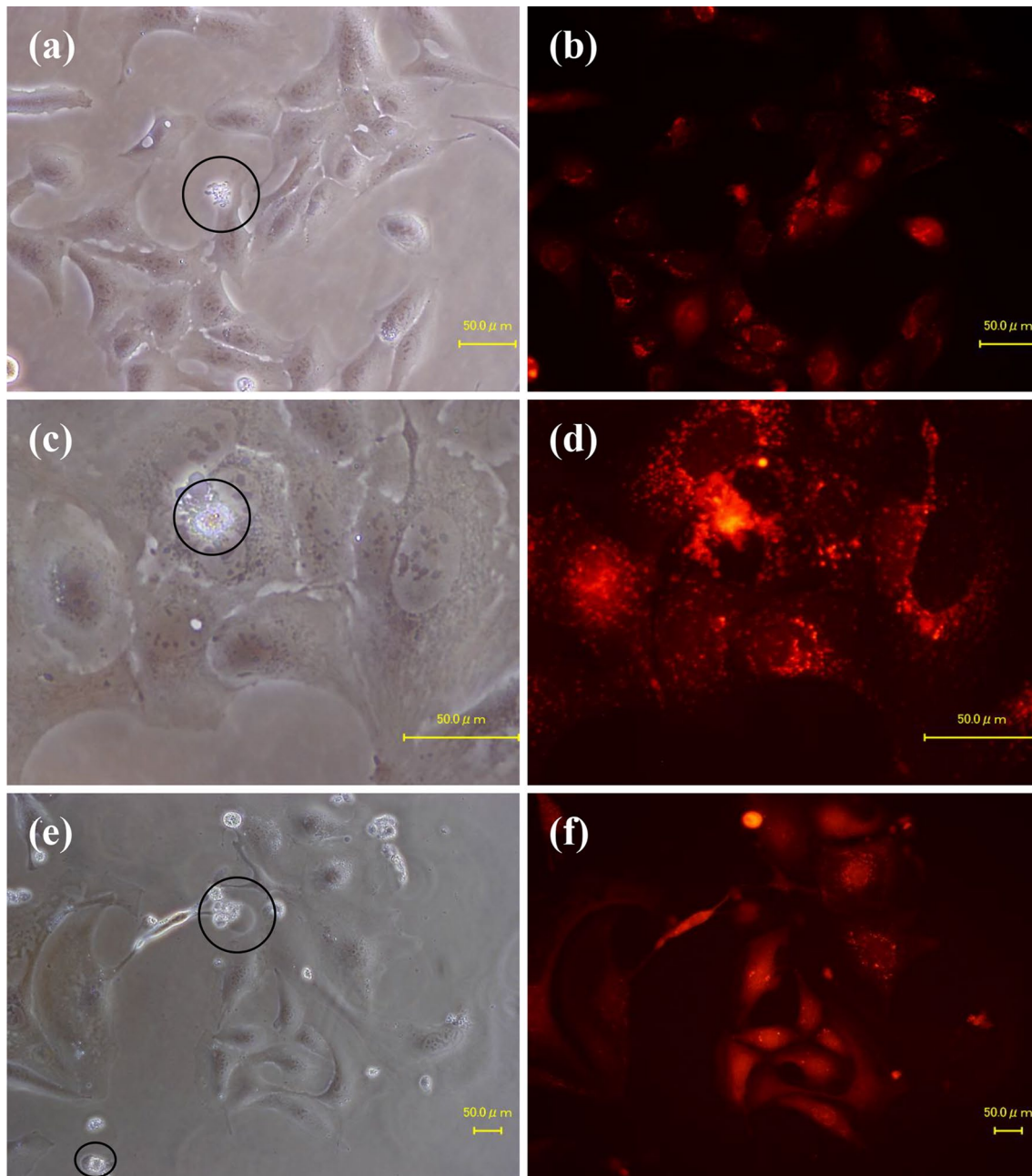
**e** SAS tumor exposed to a total of 60 Gy (30 fractions). The typical features of autophagy-dependent cell death, including an increase in the number of autophagosomes in the cytoplasm and intact nucleus (white arrow heads). **f** SAS-R tumor exposed to a total of 60 Gy (30 fractions)

approach to overcome radioresistance in tumors. The morphological changes during autophagy-dependent cell death are characterized by non-apoptotic bodies and increase in the number of cytoplasmic autophagosomes (Fig. 5). Daily microscopic observation of cancer cells revealed that this type of cell death is observed after 5 days of exposure to 10 Gy of X-rays (Kuwahara et al. 2011). Dying cells without any apoptotic bodies are filled with autophagosomes positive for an autophagosome marker, LC3 (Fig. 5c, d), suggesting that X-ray exposure induces autophagy-dependent cell death. On the other hand, autophagosomes are

seldom observed in dying cells containing apoptotic bodies (Fig. 5a, b). It should be emphasized that the frequency of autophagy-dependent cell death is higher than that of apoptotic death after irradiation.

### Radiation-induced necroptosis

Necrosis plays an important role in multiple physiological and pathological processes (Vakkila and Lotze 2004). Generally, necrosis is considered to be a form of



**Fig. 5** U2OS cells expressing RFP-LC3. **a, b** X-ray-induced apoptotic cells. Autophagosomes are seldom observed in apoptotic bodies. **c, d** X-ray-induced dying cells without morphological features of apoptosis or necrosis. A drastic increase is observed in the num-

ber of autophagosomes, suggesting that this type of dying cells are autophagy-dependent cell death. **e, f** Necrotic cell death induced by X-rays. No increase is observed in the number of autophagosomes in the cytoplasm

un-controlled cell death, observed after exposure to relatively higher doses of radiation. Recently, a new form of necrosis has been characterized as “necroptosis” (Wu et al. 2012). Necroptosis is considered as one of the modes of RCD (Jiang et al. 2011; Wu et al. 2012; Fulda 2013, 2014; Giampietri et al. 2014; Galluzzi et al. 2018). Morphologically, necroptosis exhibits the feature of necrosis; however, necroptosis includes a unique signaling pathway

involving the receptor interaction protein kinases 1 and 3 (RIP1 and RIP3) and can be specifically inhibited by necrostatins (Degterev et al. 2008; Vandenabeele et al. 2010; Silke et al. 2015). Necroptosis has been found to contribute to the regulation of the immune system, cancer development, as well as cellular response to different types of stress (Fulda 2013). Recent advances in radiation biology have shown that necroptosis plays an important role



in radiation-induced cell death in cancer cells, including endocrine cancer (Nehs et al. 2011; Su et al. 2016). Traditionally, necrosis and apoptosis after mitotic catastrophe are the principal mechanisms of cellular death caused by RT (Mansilla et al. 2006). Improperly repaired double-strand breaks in DNA can cause chromosomal abnormalities that interfere with normal segregation during mitosis, which culminates in programmed or spontaneous cell death (Castedo et al. 2004; Eriksson and Stigbrand 2010). Necrosis was previously thought to be a passive process of cellular death involving morphological changes to cells, including oncosis (increased cell volume), chromatin condensation, and swelling of organelles (Vandenabeele et al. 2010). Apoptosis, however, is a coordinated process involving the activation of specific proteases called caspases in response to the binding of specific ligands to the so-called “death receptors” on the plasma membrane. The morphological changes occurring during apoptosis, in contrast to necrosis, include pyknosis (decreased cellular volume), nuclear fragmentation (karyorrhexis), and blebbing of the plasma membrane (Elmore 2007). For years, these two processes were thought to represent categorically different cellular death mechanisms. However, more recent evidence has revealed coordinated caspase-independent programmed cellular death, which has been termed as necroptosis (Galluzzi and Kroemer 2008; Tait and Green 2008; Declercq et al. 2009).

A pan-caspase inhibitor, namely, Z-VAD-FMK, does not reduce the number of dying cells after exposure to X-rays (Kuwahara et al. 2011). Inhibition of caspase does not change the radiosensitivity of HepG2 cells. The inhibition of apoptosis has little or no effect on clonogenic survival after treatment with drugs or radiation in several tumor cell lines (Roninson et al. 2001). These observations suggest that the contribution of apoptosis to radiation-induced cell death is lower than that assumed previously. Daily microscopic observation of X-ray-irradiated cancer cells show the existence of swelling cells without apoptotic bodies (Fig. 2b). The number of swelling cells increases from day 3 after irradiation (Fig. 2c), in a manner dependent on the dose of X-rays. This can be considered as necroptosis. Necroptosis is a recently described mechanism of RCD (Galluzzi et al. 2018), and is a RIP-kinase-dependent mode of cell death (Walsh 2014). This mode is commonly observed in HepG2, SAS, HeLa, and U2OS cells (Figs. 2, 3, 5). Notably, after exposure to 10 Gy of X-rays, the frequency of induction of necroptosis is different among the four cell lines, suggesting that the dying cells with swelling cytoplasm are induced by genetic, not physical, factors. HepG2 cells are the most radiosensitive, however, the extent of induction of necroptosis is the highest among HepG2, SAS, and HeLa cell lines. These indicate that necroptosis is one of the modes of RCD induced by X-rays.

## Conclusion

Radiation-induced cell death can be classified into three categories: apoptosis, autophagy-dependent cell death and necroptosis. Many studies have shown that apoptosis is one of the primary modes of radiation-induced cell death; however, microscopic analysis of irradiated cancer cells do not show significant induction of apoptosis. Autophagy-dependent cell death is one of the main modes of X-ray-induced RCD; however, daily microscopic observation of irradiated cells in the author’s laboratory have suggested that it is necessary to consider necroptosis as one of the modes of radiation-induced RCD. To establish more efficient cancer radiotherapy, experiments are underway to clarify how and which pathway cancer cells select after radiation exposure.

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