Review Article

Importance of Proapoptotic Protein PUMA in Cell Radioresistance

(ionizing radiation / p53 / PUMA / radioprotection)

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Abstract. Protein p53 plays an essential role in the induction of apoptosis by ionizing radiation in haemopoietic cells, the damage of which is the main reason for the development of bone marrow post-irradiation syndrome. p53 activation leads to an increase in the Bcl-2 family pro-apoptotic protein PUMA level. PUMA inhibits all the five anti-apoptotic proteins (Mcl-1, Bcl-2, Bcl-X$_L$, Bcl-W and A1) and directly triggers apoptosis mediated by pro-apoptotic proteins Bax/Bak. In proliferating cells, knockout of p53 inhibits apoptosis on the one hand, but on the other disables the cellular division arrest moderated by p21$^{Cip1/Waf1}$. The radioprotective effect of p53 inhibitor pifithrin was obvious at radiation doses causing the bone marrow syndrome. Knockout of PUMA also exerts its radioprotective effect through blocking the apoptosis induction, but the arrest of cells in the cell cycle through p21 induction is not abolished. PUMA -/- mice are radioresistant in terms of the development of post-irradiation syndrome after all ra- diation doses. Small molecules are being searched for that could prevent binding of PUMA with Bcl-2 family anti-apoptotic proteins. This would result in apoptosis inhibition and radioprotective or mitigating effects of these inhibitors.

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Abbreviations: ATM – ataxia-telangiectasia mutated kinase, ATR – ATM and Rad3-related kinase, Ex-Rad – 4-carboxystyryl-4-chlorobenzylsulphone sodium salt, lin$^-$/Sca-1$^+$/CD117$^+$ – stem cells with long-term and short-term capability of haemopoiesis recovery, Mcl-1, Bcl-2, Bcl-X$_L$, Bcl-W, A1 – Bcl-2 family anti-apoptotic proteins, p16^{INK4a} – cyclin-dependent kinase inhibitor 2A, p21$^{WAF1/Cip1}$ – cyclin-dependent kinase inhibitor 1A, p53 – tumour suppressor protein 53, PIKKs – phosphoinositide 3-kinase-related protein kinases, PUMA, NOXA, Bid – Bcl-2 family pro-apoptotic proteins.

Introduction

Ionizing radiation is the major therapeutic strategy for 50 % types of tumours. The tumour destruction is, however, frequently associated with damaging healthy tissues, which is a limiting factor to the radiation dose employed. The primary target for cell damage from ionizing radiation is the molecule of DNA. During the defence against the DNA damage induced by ionizing radiation the cells activate protecting mechanisms associated with cell cycle arrest and damage repair and – in the case of too extensive damage – even induction of cell death. Central mediators of the cellular response are ATM (ataxia-telangiectasia mutated kinase) and ATR kinases (ataxia-telangiectasia and Rad3-related protein kinase) from the family of phosphoinositide 3-kinase-related protein kinases (Bakkenist and Kastan, 2003; Bartek and Lukas, 2003; Wagner and Kaufmann, 2010). Considering management of overall radiosensitivity of the cell, the most promising pathway seems to be apoptosis induction via ATM/ATR-modulated increase in tumour suppressor protein p53 and its transcriptional target – pro-apoptotic protein PUMA.
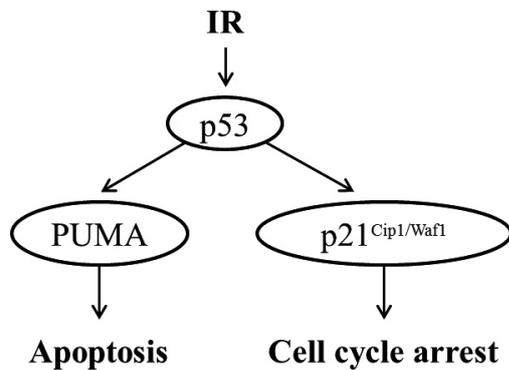
Radiosensitivity and apoptosis induction

Protein p53 acts as a transcriptional factor and regulates transactivation of multiple proteins related to cell cycle arrest and DNA repair, to permanent cell cycle arrest or to apoptosis. Protein p53 is referred to as a "Guardian of the Genome", and it is important in the tumour development prevention. Under normal conditions, the amount of p53 in the nucleus is kept at a low level. In our own experiments (Vilasová et al., 2008), protein p53 was undetectable by Western blot and/or

IR

↓

p53

↙ ↘

PUMA p21$^{Cip1/Waf1}$

↓ ↓

Apoptosis **Cell cycle arrest**

Fig. 1. Two functions of p53 after its activation by ionizing radiation. Apoptosis induction is dependent on the increase in pro-apoptotic protein PUMA. Elevation of inhibitor of cyclin-dependent kinases protein p21 leads to cell cycle arrest and repair of DNA damage.

ELISA methods in lymphocytes isolated from human peripheral blood. Its amount in the cell nucleus increased after stimulation of the lymphocytes with phytohaemagglutinin. Through the activation of phosphoinositide 3-kinase-related protein kinases (PIKKs), ionizing radiation causes post-translational phosphorylation and acetylation of p53, which leads to its accumulation and stabilization in the nucleus, where it enhances transcription of p53 responsive genes.

Thus, as an answer to the DNA damage by ionizing radiation, p53 causes cell cycle arrest or apoptosis in proliferating cells (Fig. 1) depending on particular conditions, namely the cell type and character and the intensity of the stressor. The p53-mediated cell cycle arrest is executed via increase in the protein p21$^{Cip1/Waf1}$ (inhibitor of cyclin-dependent kinases) level and results in repair of the radiation damage before the cells enter mitosis. When p21 is removed from the cells, the cells enter the cycle with unrepaired DNA, and subsequently a larger portion of them die by apoptosis (Gudkov and Komarova, 2003).

Induction of p53-mediated apoptosis is executed through induction of pro-apoptotic proteins from the Bcl-2 family. Pro-apoptotic proteins from the Bcl-2 family are divided into two structurally and functionally different groups – Bax-like factors and BH3-onlies. Bax-like death factors (e.g. Bax, Bak, Bok) are directly involved in formation of mitochondrial pores, which leads to the release of cytochrome c and other pro-apoptotic substances from the mitochondrion and onset of the intrinsic, mitochondrial pathway of apoptosis induction. This permeabilization of mitochondrial membrane is prevented by anti-apoptotic Bcl-2-like factors (Adams and Cory, 2007; Tichý, 2006). BH3-onlies (e.g. PUMA, Bid, Bim, Bad, NOXA) are not found in active form in normal, undamaged cells, but their increase and activation are observed in response to various damaging stimuli. In response to DNA damage caused by ionizing radiation, the major p53 transactivated executor of apoptosis initiation is the BH3-only factor PUMA (Erlacher et al., 2005). BH3-onlies are supposed to bind to

Bcl-2-like anti-apoptotic factors, which allows Bax-like pro-apoptotic factors to form pores in the mitochondrial membrane (Bogner et al., 2010). PUMA is capable of inhibiting all the five major anti-apoptotic Bcl-2-like proteins (Mcl-1, Bcl-2, Bcl-XL, Bcl-W and A1) (Yu and Zhang, 2008).

The question arises how the cell decides in favour of cell cycle arrest or apoptosis induction and whether these two mechanisms can affect each other. Fei et al. (2002) systematically studied tissue-specific reactions of protein p21 and further p53 targets associated with apoptosis – pro-apoptotic proteins of the Bcl-2 family (PUMA, NOXA and Bid) – to irradiation. Whereas in the white spleen pulp of mice the amount of PUMA is increased after irradiation, in the red pulp, NOXA and Bid are elevated. All the apoptotic targets of p53 (Bid, PUMA, NOXA) were also induced in the jejunum and ileum after irradiation. On the other hand, in the liver, particularly p21 was increased (Fei et al., 2002). In certain cellular types, e.g. in fibroblasts, the increase in p53 after irradiation results in an elevation of p21 level and permanent arrest of the cell cycle and condition referred to as senescence, where the cells do not divide any more, but neither die. Senescence is usually associated with increase in a further inhibitor of the cell division – p16^{INK4a} (Vávrová and Rezáčová, 2011).

Radioresistance of p53-null mice is limited to bone marrow post-irradiation syndrome

The p53-null mice were shown to be resistant to apoptosis induction after irradiation and to be (in contrast with wild-type p53 mice) able to survive relatively high radiation doses after which mice die of the bone marrow post-irradiation syndrome (Komarov et al., 1999). It is thus obvious that the limitation of apoptosis induction after irradiation has radioprotective effects in terms of the bone marrow post-irradiation syndrome (Komarova et al., 2000).

The role of p53 in the gastrointestinal tract radiosensitivity is different from the role of p53 in the haemopoietic system. Komarova et al. (2004) compared the survival of whole-body irradiated mice with and without p53. After a dose of 10 Gy, 40 % of mice with wild-type p53 survived for a period of 20 days. The animals particularly died of damage to the haemopoiesis. The p53-null mice were radioresistant and essentially all of them survived. On the other hand, the p53 deficiency resulted in reduced survival of mice exposed to a whole-body dose of 15 Gy. The dose of 15 Gy caused death in mice due to the gastrointestinal syndrome (the survival period of wild-type p53 mice was 7.4 days on average). The p53 deficiency resulted in sensitization of mice to high ionizing radiation doses (p53-null mice survived for only 4.5 days after a dose of 15 Gy). In epithelial cells of the small intestine of p53-null mice, there was no arrest of the cellular division and repair of the damage after irradiation. These cells entered the cell cycle with unrepaired DNA, which resulted in massive cellu-

lar death in intestinal crypts and accelerated dying of mice.

Radioresistance of PUMA knockout mice

The main mediator of the p53-dependent apoptosis induced by ionizing radiation is the pro-apoptotic protein PUMA. It seems that the increase in pro-apoptotic protein PUMA and subsequent apoptosis induction occur in haemopoietic system cells solely as responses to pathological stress, for example ionizing radiation. When the PUMA protein was removed from the cells, the cells were resistant to radiation-induced apoptosis (Yu and Zhang, 2005). By experiments in PUMA knockout mice, Jeffers et al. (2003) demonstrated that PUMA-null thymocytes, myeloid progenitors and pro-B lymphocytes were resistant to radiation-induced apoptosis induction. In the PUMA knockout mice, normal development of haemopoiesis can be observed. Yu et al. (2010) demonstrated PUMA +/+ mice to die more rapidly after irradiation at a dose of 10 Gy; the median survival time being 10.5 days. PUMA -/- mice survived for much longer periods with a median survival time of 215 days. The loss of one allele (PUMA +/-) also considerably prolonged the survival of irradiated mice. It was also shown that ionizing radiation at a dose of 4 Gy resulted in apoptosis induction 24 h after irradiation in 50 % of lin⁻/Sca-1⁺/CD117⁺ stem cells isolated from the bone marrow of PUMA +/+ mice, whereas most of these stem cells from PUMA -/- mice were still alive (Yu et al., 2010) and were in G0 phase of the cell cycle.

Leibowitz et al. (2011) studied the survival of mice without p53, p21 or PUMA after a dose of 15 Gy and found PUMA knockout mice to survive for the longest periods (10.5 days). Whereas p53 and p21 deficiency results in rapid apoptotic as well as non-apoptotic death of intestinal crypt cells, the PUMA deficiency blocks apoptosis and does not affect the proliferation process, thus prolonging the survival of mice. The main response of particular cell types to irradiation is shown in Fig. 2.

Radioprotective effects of substances reducing the apoptosis induction

There is the question whether p53 inhibition could be used in radioprotection. Komarov et al. (1999) demonstrated enhanced survival of whole-body irradiated mice receiving doses of about LD50/30 (lethal dose after which 50 % of animals survive up to the 30th day after irradiation) after administration of p53 inhibitor pifithrin 5 min before the whole-body irradiation. Ghosh et al. (2009) described radioprotective effects of 4-carboxystyryl-4-chlorobenzylsulfone sodium salt (Ex-Rad) in mice irradiated at a dose of 8 Gy (LD 80/30). The Ex-Rad administration resulted in reduction of p53 protein induction in cells four hours after irradiation at a dose of 10 Gy. On the other hand, mice having no p53 protein are extremely sensitive to the development of tumours, particularly lymphomas and sarcomas (Donehower et

al., 1992), and die relatively early due to tumour diseases compared to p53 wild-type mice. The increased frequency of tumours could also mean a potential risk in the use of p53 inhibitors. However, Leonova et al. (2010) demonstrated that pifithrin-β affects neither the frequency nor the time of development of tumours in p53 +/- mice after its single administration to mice irradiated by a sublethal dose of 4 Gy. These results suggested that p53 inhibitors may be used for the haemopoiesis protection/stimulation.

In terms of possible radioprotective effects, the action of substances inhibiting pro-apoptotic protein PUMA seems to be very interesting, as the increase in PUMA appears to be associated only with DNA damage by exogenic stimuli such as ionizing radiation (Yu and Zhang, 2008). In contrast to p53 -/- mice, the PUMA -/- animals survived lethal doses of radiation causing both bone marrow and gastrointestinal syndrome (Yu et al., 2010; Leibowitz et al., 2011). Thirty-two mice (PUMA -/-, -/+) exerted long-term survival after a dose of 10 Gy (100% lethal dose for wild-type mice) and development of lymphomas was demonstrated in two mice only (Yu et al., 2010). The transcriptional PUMA induction is observed
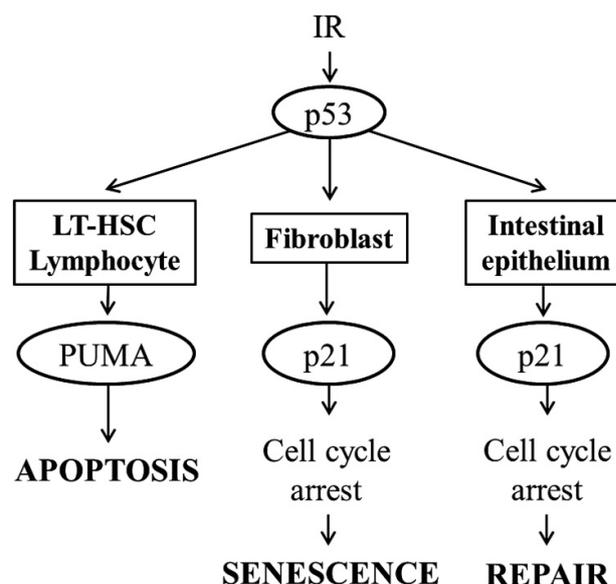
Fig. 2. The main mechanisms occurring in response of particular cell types to irradiation. Through activation of protein p53, cells of the haemopoietic system die due to relatively rapid p53-dependent apoptosis after irradiation. The absence of p53 results in reduced development of apoptosis in these cells and exerts radioprotective effects. On the other hand, the lack of p53, and particularly p53-dependent increase in p21, means that the cells of epithelial lining of intestinal crypts enter the cell cycle with unrepaired DNA and relatively rapidly die of non-apoptotic death, which results in reducing the survival period of these mice. The absence of PUMA reduces development of apoptosis only, which results in a radioprotective effect. In fibroblasts, senescent cells are developed through induction of protein p21; these cells are permanently arrested in cell cycle without further division.

four hours after irradiation (Qiu et al., 2008). The inhibition of PUMA expression with antisense oligonucleotides resulted in significant radioprotection of the gastrointestinal tract (Qiu et al., 2008). Recently, Mustata et al. (2011) synthesized 13 structurally different compounds inhibiting PUMA interactions with anti-apoptotic proteins of the Bcl-2 family, which should block radiation-induced apoptosis. The substance inhibiting interactions of PUMA and Bcl-x_L appears to be a promising radioprotectant. An important characteristic of PUMA inhibitors is also the possibility of their use as mitigating agents, i.e. they also exert protective effects when administered early after irradiation.

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