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REVIEW ARTICLE

DOSE-RATE AS A CRITICAL ASPECT OF CELLULAR RESPONSE TO GAMMA-RADIATION

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Summary

Ionizing radiation (IR) induces various types of damage in the cellular DNA, of which the most deleterious are double strand breaks. Double strand breaks lead to activation of signaling cascade aiming to repair the damage or to transiently or permanently arrest cell cycle, and/or induce cell death. In the case of high doses of ionizing radiation with a high dose-rate (0.5-1 Gy / min) where the cell repair capacity is insufficient, cell death often occurs in response to double-strand breaks. The response to the radiation exposure depends on many factors such as the cell type, its proliferation activity, and p53 status. In tumor cells, cell death is associated primarily with apoptosis or mitotic catastrophe. In normal fibroblasts, cells accumulate in the G1-phase of the cell cycle and so-called premature senescence occurs after irradiation.

In cells with functional p53 protein an increase in the p21 protein (cell division inhibitor) and accumulation of the cells in the G1-phase occurs. In the case of very low-dose rate (LDR), this accumulation is transient; after DNA damage repair, the cells continue to divide. Upon irradiation with higher doses at a LDR, accumulation in the G1-phase is irreversible; p16 protein is upregulated and the status of premature senescence is induced. The same dose of radiation administered at LDRs results in more senescence than after an acute exposure.

In the case of the use of IR for the eradication of tumor cells, the status of these cells is important in terms of p53 and proliferation. About fifty percent of tumor cells do not possess p53 protein or are mutant, and after irradiation they accumulate in the G2-phase and repair the IR-induced damage (e.g. HL-60 cells). In HL-60 cells ($p53^{-/-}$ human promyelocytic leukemia), G2-phase accumulation occurs during irradiation with low dose rate, and their radioresistance increases if the cells are irradiated in the G2-phase. When the dose-rate is very low, the cells enter the mitotic cycle during irradiation, and because cels in mitosis are highly radiosensitive, apoptosis is induced and thus their radiosensitivity increases as well.

Key words: cell death; apoptosis; senescence; p53; p21; ionizing radiation; dose rate

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Introduction

Radiotherapy (RT) is often used to treat malignant tumors. RT is usually performed with gamma- or X-rays with hight dose rate (0.5-1 Gy / min) in five fractions per week. Another method used for the eradication of cancer cells by IR is radioimmunotherapy (1,2). Therapeutic monoclonal antibodies have been a major breakthrough in the treatment of both indolent and aggressive B-cell non-Hodgkin's lymphoma (NHL), and have become the standard treatment for these diseases. Radiolabeled antibodies (e.g. ^{131}I, ^{90}Y) emit continuously exponentially-decreasing radiation. However, some patients may not respond to antibody therapy, or resistance may develop (2). It is therefore important to study the molecular mechanisms underlying the response of cells to IR at a low dose rate.

Molecular mechanisms of cell response to IR exposure

Cellular damage induced by IR involves particularly DNA modifications, including single-strand breaks and DSBs. While single-strand breaks in DNA are repaired rapidly under the critical participation of poly-(ADP-ribose)-polymerase (PARP), DSBs are a potentially lethal type of cellular damage and their repair is difficult. In response to DNA damage by IR, proteins from the phospatidylinositol-3-kinase related family are activated: ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) (3). After exposure, ATM dissociates to monomer and is phosphorylated at serine 1981. ATM and ATR are both extremely large protein kinases (350 and 301 kDa, respectively) which phosphorylate many down-stream substrates in order to repair DNA or to trigger apoptosis, and in some cases so-called stress-induced premature senescence (SIPS) of the cells is induced.

It seems that very early changes occur in the chromatin surrounding the DSB. At the DSB site, histone H2AX is phosphorylated by ATM kinase rapidly (within minutes) after irradiation. This phosphorylation is further propagated through the adjacent chromatin. Modified chromatin then binds other proteins such as p53-binding protein-1 (53BP1), which recruits several proteins associated with reparative and lethal processes, and creates IRIF. Proteins that co-localize with gamma-H2AX in IRIF include ATM, Mre11, Rad50, Nbs1 (the MRN repair complex), Mdc1, 53BP1 and BRCA1. All these proteins are able to surround the DSB at all phases of the cell cycle and also in the interphase. Interactions of all these proteins are important for an efficient DSB repair (4). If the damage is not repaired, cell death is induced.

Apoptosis

IR-induced apoptosis is mediated through phosphorylation of p53, and further activation of pro-apoptotic members of the Bcl-2 family follows (Bax, Bad, Bim and Bid, PUMA and NOXA). Under stress-free conditions, pro-apoptotic Bcl-2 family members are found in different compartments of the cell. Whereas the anti-apoptotic proteins of the Bcl-2 family are integral parts of the membrane structures (mitochondrial and nuclear) and the endoplasmic reticulum, the pro-apoptotic proteins are found in the cytoplasm, possibly bound to the cytoskeleton. After irradiation, Bcl-2 pro-apoptotic members (especially PUMA and NOXA) are activated, and they interact with anti-apoptotic members where they meet at the surface of the mitochondria in order to regulate the release of cytochrome c. When pro-apoptotic proteins prevail, cytochrome c is released from the mitochondria and forms a complex with Apaf-1 and procaspase-9 (the apoptosome). The apoptosome subsequently activates procaspase-3. Active caspase 3 is the most important effector caspase, and is responsible for the execution of the apoptotic process (5).

P53 is a significant tumor suppressor gene that is stimulated by cellular stresses such as IR, hypoxia, carcinogens, and oxidative stress. After activation of p53, cell cycle arrest occurs in the G1-phase. Low doses of radiation lead to DNA repair, while high doses, especially in hematopoietic cells, lead to apoptosis. When p53 protein is mutated or absent in tumor cells, the induction of apoptosis decreases and the tumor cells proliferate. In addition to p53, the tumor suppressor family consists of two other proteins p73 and p63.

P63 and p73 are structural homologues of p53 that may act similarly to this protein, but also possess other functions. Currently, more than 40 different isoforms of the p53 family members have been identified. The role of p63 and p73 in tumor formation is controversial. For some tumors, these proteins are downregulated, in others

they are overexpressed or their genes are amplified. Some isoforms have carcinogenic properties and confer resistance to chemotherapy (6). The role of individual proteins under in vivo conditions was clarified by knockout mouse experiments for $p53^{-/-}$, $p63^{-/-}$, and $p73^{-/-}$. The role of p53 in suppressing carcinogenesis has been clearly demonstrated. Mice without p53 died of cancer 100-250 days after birth, unlike p53-positive mice that lived 500 days or more (7,8). P63 plays a pivotal role in the skin development, and p73 in the development of the nervous and immune system (9). In addition, $P63^{-/-}$ mice die soon after the birth due to dehydration associated with poor skin and epithelial tissue development (10).

Among the many pathways by which p53 can induce apoptosis associated with IR-induced damage, a major activation is mediated through the pro-apoptotic protein PUMA, which triggers apoptosis by the mitochondrial pathway. The pro-apoptotic activity of p53 seems to be regulated by p73. P73 induces apoptosis in T-cells by promoting the transcription of Bim. In activated T-cells NF-κB induces Mdm2 that in turn forms a complex with p73, which inhibits p73-dependent activation of Bim and results in apoptosis (11). Additionally, Yoshida et al. (12) demonstrated that in the absence of p53, apoptosis can be induced through ATM phosphorylation, which leads to the phosphorylation of nuclear IKK-α which stabilizes p73 in the nucleus. P73 then replaces p53 as a pro-apoptotic signal transducer.

Senescence

The cell division of normal human fibroblasts in cell cultures is limited. After approximately 60 divisions, growth arrest occurs, the number of cells in the S-phase decreases and the cells are irreversibly blocked in the G1- or G2-phase of the cell cycle. This state is referred to as replicative senescence. Such cells are characterized by the presence of positive beta-galactosidase (at pH 6), elevated expression of p21 and p16, and hypophosphorylation of the Rb protein (13). Replicative senescence is caused by progressive truncation of the telomere, which is shortened with each division. Normal somatic cells have a limited lifetime; they lose telomere DNA located at the ends of the chromosomes in accordance with the number of divisions, both *in vivo* and *in vitro*. The critical length of the telomere is 5 kb, at which point p53 is activated and the cell enters the irreversible block. Replicative senescence can be avoided by increased telomerase activity (14).

In some cells (e.g. fibroblasts), IR induces damage which is referred to as stress-induced premature senescence (SIPS). Cells in SIPS possess all cellular features typical for replicative senescence, i.e. beta-galactosidase-positivity, p21 and p16 accumulation, and pRB hypophosphorylation. SIPS has been shown to be induced by a p53-dependent block in the cell cycle (15). The signaling pathway is similar to repair of radiation damage activated through ATM kinase, H2AX phosphorylation and IRIF induction, and subtelomeric DNA production (16). It seems that activation of p53 and the increase in p21 (a pathway associated with truncation of telomeres) are reversible pathways: for example, telomerase increase may lead to resumption of proliferation on the one hand, or on the other the signaling leading to p16 increase may be activated. In senescence, both pathways are significant, but only the p16 increase is responsible for irreversible cell entry into senescence (15). In terms of the impact of IR on hematopoietic cells, Wang et al. (17) showed that while hematopoietic progenitor cells died after IR via apoptosis, hematopoietic stem cells (HSC) induced senes-cence. Induction of senescence in HSC was associated with an increase in cell cycle inhibitory proteins p21, p19, and p16.

Mitotic catastrophe

While hematopoietic cells die after irradiation via apoptosis, some cancer cells in solid tumors respond to RT by induction of aberrant mitosis accompanied by formation of gigantic cells with multiple nuclei and several micronuclei (18). Mitotic catastrophe is often observed in cells with DNA damage and deficient cell cycle checkpoints. At least 50% of tumor cells harbor mutated p53, or p53 is absent. $P53^{-/-}$ cells accumulate in the G2/M block after irradiation, and mitotic catastrophe occurs with premature entry into mitosis with unrepaired DNA. These cells may survive for days, transit to senescence or delayed apoptosis or delayed necrosis, respectively. Amornwichet et al. (19) compared the ability to induce apoptosis and mitotic catastrophe in colorectal cancer cell lines HCT 116 with and without TP53 after irradiation with carbon-ion beam or X-ray. $P53^{-/-}$ cells were more resistant towards X-radiation than $p53^{+/+}$ cells. Unlike sensitivity towards X-radiation, radiosensitivity to carbon ions is comparable to $p53^{+/+}$ and $p53^{-/-}$ cells. While in $p53^{+/+}$ HCT 116 cells both X-ray and carbon-ion beam

irradiation primarily induces apoptosis, in $p53^{-/-}$ cells carbon-ion beam irradiation induces mitotic catastrophe and is associated with entry into mitosis with unrepaired DSBs. If the number of DSBs is lower than 10-20, the cell can enter mitosis, complete it and proceed to the G1-phase. Cells with a higher number of DSBs are not capable of full DNA repair, and mitotic catastrophe occurs when entering mitosis. Finally, cells with $p53^{-/-}$ have a higher tendency to extend the G2/M-phase after irradiation than the $p53^{+/+}$ cell.

The importance of IR dose-rate for cell death

As we have already mentioned, apoptosis is the major form of cell death in hematopoietic cells. In the case of immune RT, hematopoietic cells are irradiated by continuous gamma radiation at a LDR (20,21). From the perspective of LDR therapy, two crucial points are important: the presence of p53 and the length of the G2 block. In our previous work (22), we irradiated T-lymphocytic leukemia MOLT-4 cells ($p53^{+/+}$) and promyelocytic leukemia HL-60 cells ($p53^{-/-}$) with a high dose-rate (HDR) of 0.6 Gy/min, a LDR of 3.9 mGy/min, and a sub-LDR of 1.8 mGy/min. The radiosensitivity of MOLT-4 and HL-60 cells was determined by clonogenity test, and D0 (the radiation dose at which 37% of the cells survived) was calculated. Decrease in dose-rate had no effect on the radiosensitivity of MOLT-4 cells (D_0 for HDR 0.87 Gy, for LDR 0.78 Gy and for sub-LDR 0.70 Gy). In contrast, a significant increase in radioresistance after LDR irradiation was observed for p53-negative HL-60 cells (D_0 for HDR 2.20 Gy and for LDR 3.74 Gy). After an additional decrease in dose-rate to sub-LDR, the D_0 value was not significantly different from HDR irradiation (2.92 Gy). Taking into account that during the HDR the cells are irradiated in all phases of the cell cycle and during LDR the irradiation impacts the cells mainly in the G2-phase, we failed to prove that the G2-phase is the most radioresistant phase of the cell cycle of HL-60 cells. On the other hand, we showed that irradiation of cells in this phase induced DNA damage repair and increased radioresistance. When the dose-rate was lowered to sub-LDR level (approximately 1.8 mGy/min), an opposite effect was observed, i.e. D_0 value decreased from 3.74 Gy to 2.9 Gy. Taken together, we have demonstrated that during sub-LDR irradiation at first the cells accumulated in G2-phase, but later they entered mitosis, which had a radiosensitizing effect, or (if cell damage was not repaired sufficiently) the cells entered apoptosis.

Unlike hematopoietic cells, fibroblasts are driven after irradiation into senescence. Cao et al. (23) compared the colony-forming ability of non-hematopoietic tumor cells (MCF10A, HCT-116, MCF-7, U2OS, and HeLa) with that of human fibroblasts (HHDF p9, TIG-3 p27, and BJ1/hT) after acute gamma irradiation (1 Gy), and irradiation at a sub-LDR of 0.347 and 0.694 mGy/min respectively, up to a total dose of 5 Gy. After acute irradiation, the D_0 in all studied lines ranged between 1.1-1.4 Gy. In tumor cells, LDR had little effect in terms of decreasing the formation of colonies. By contrast, in fibroblasts there was a marked decrease in proliferation. In the case of irradiated fibroblasts (sub-LRD 0.694 mGy/min for 10 days), the cells accumulated in the G1-phase of the cell cycle and did not proliferate. After another 10-day incubation, the cells remained in an irreversible G1-phase (state of senescence) and did not form colonies either. The status of senescence was confirmed by beta-galactosidase test. In the case of an even lower dose rate sub-LDR (0.347 mGy/min), after 10 days of irradiation (total of 5 Gy) the cells also accumulated in the G1-phase, but the state of senescence was reversible and after an additional 10 days of incubation without irradiation the cells proliferated well and formed colonies.

The induction of p53 together with p21 was responsible for the previously mentioned transient block in the cell cycle. Thus, fibroblasts were able to repair the long-term irradiation damage induced at a dose-rate of 0.347 mGy/min. When studying IRIF, it was shown that they are formed at a dose-rate of 0.694 mGy/min. IRIF were not induced with a lower dose-rate than that. P53 regulated the cellular response to irradiation and cell cycle arrest, and in the case of higher dose-rates, the G1-arrest was irreversible and senescence was induced.

In the case of HDR-irradiated fibroblasts (1 Gy/min), only 20% displayed senescence within 6 days after a total dose of 4 Gy, whereas 90% of fibroblasts were in senescence when sub-LDR (0.694 mGy/min) was applied. Similar results were obtained in the study of pulmonary fibroblasts isolated from whole-body irradiated mice at a total dose of 2 Gy. Irradiation at a dose-rate of 0.694 mGy/min resulted in a higher number of senescent cells (23).

Tsai et al.24 reported that exposure of human mammary fibroblasts to X-rays twice a day with a dose of 50 mGy (100 mGy per day) up to a total dose of 5 Gy resulted in senescence in 70% of cells, which was in contrast to 25% of senescence when HDR X-rays were applied. Fibroblasts were able to survive in culture for up to 1 year. Senescence was demonstrated with beta-galactosidase positivity and an increase in p16.

Such senescent fibroblasts produce degradative enzymes, cytokines and growth factors, and this microenvironment paradoxically promotes tumorigenesis. Tsai et al. (25) irradiated fibroblasts with sub-LDR up to a total dose of 10 Gy, and demonstrated that senescent fibroblasts co-cultivated with breast cancer cells activated the Akt pathway that increased tumor cell survival by numerous mechanisms. There was also an increase in the resistance of these tumor cells towards IR and adriamycin. The results clearly showed the importance of microenvironment in the behavior of tumor cells.

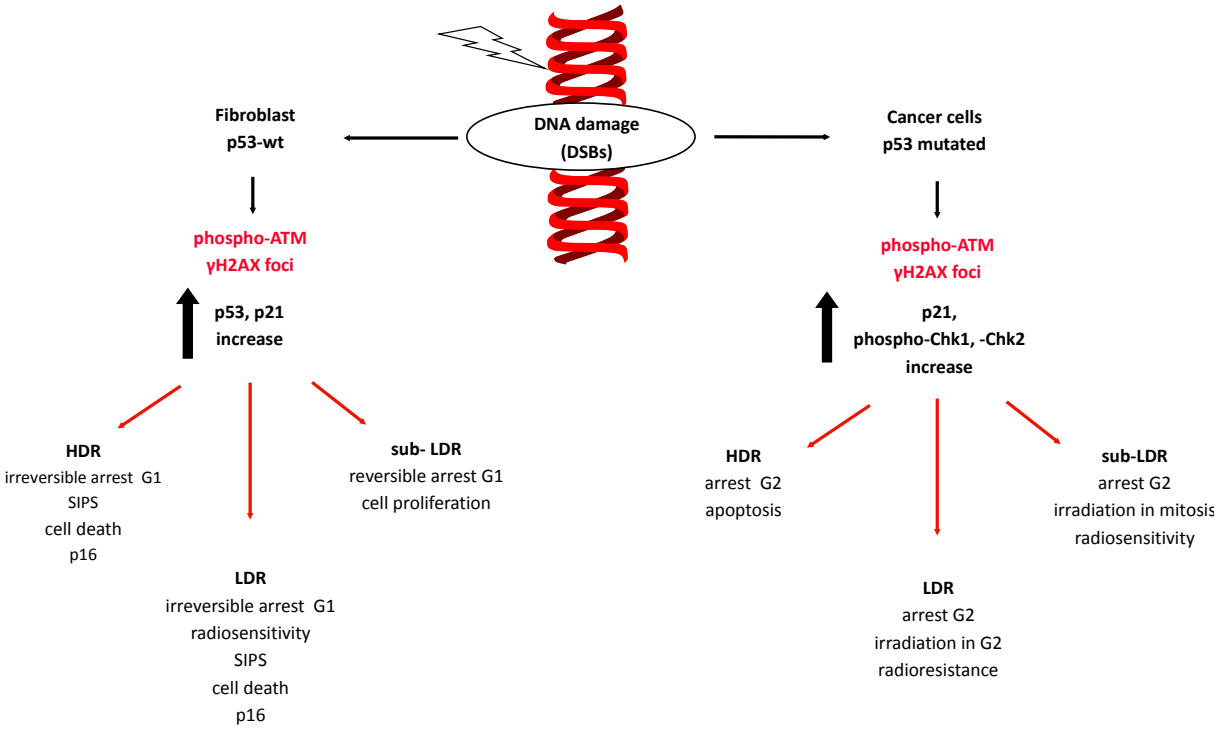

Figure 1. Impact of dose-rate on radiation-induced DNA damage in normal and cancer cells. DSBs, double strand breaks; HDR, high dose-rate; LDR, low dose-rate; phopsho-ATM, Ataxia-telangiectasia-mutated kinase phosphorylated at serine 1981; phospho-Chk-1/2; Checkpoint-kinase-1 phosphorylated at serine 345 and Checkpoint-kinase-2 phosphorylated at threonine 68; γH2AX, phosphorylated histone H2A.X at serine 139.

Conclusion

After gamma irradiation of fibroblasts at very LDR, there is a reversible increase in p21 and accumulation of cells in the G1-phase. After the end of exposure, the damage is repaired and the cell division of fibroblasts proceeds. If the dose-rate is higher, the p21 increase is irreversible as well as that of p16, and a permanent block is induced in the G1-phase. In this phase, the cells are positive for beta-galactosidase and are in a state of replicative senescence.

P53-negative cells, in contrast to fibroblasts, accumulate after irradiation in the G2-phase, where they repair the damage and thus are more resistant towards IR. Interestingly, in the case of LDR (when the irradiation happens during the G2-phase), the radio-resistance is further increased. By contrast, sub-LDR irradiation allows the cells to enter mitosis, which is the most sensitive phase during irradiation, and their radiosensitivity rises again.

To summarize, consideration of the dose rate is extremely important in planning of radiotherapy protocols. At very low dose rates, replicative senescence of fibroblasts is transient, and after some time, radiation damage could be repaired. P53 $^{-/-}$ cells can, when irradiated at a very low dose rate, slip off the G2-phase and enter mitosis, which increases their radiosensitivity.

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Conflict of interests

The authors report no conflict of interest.

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