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Radiation Induced Bystander Effect: From *in Vitro* Studies to Clinical Application

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Abstract

In the past 20 years, the classic paradigm in radiobiology recognizing DNA as the main target for the action of radiation has changed. The new paradigm assumes that both targeted and non-targeted effects of radiation determine the final outcome of irradiation. Radiotherapy is one of the main modality treatments of neoplastic diseases with intent to cure, or sometimes to palliate only, thus radiation-induced non-targeted effect, commonly referred to as the radiation-induced bystander effect (RIBE) may have a share in cancer treatment. RIBE is mediated by molecular signaling from radiation targeted cells to their non-irradiated neighbors, and comprises such phenomena as bystander effect, genomic instability, adaptive response and abscopal effect. Whereas first three phenomena may appear both *in vitro* and *in vivo*, an abscopal effect is closely related to partial body irradiation and is a systemic effect mediated by immunologic system which synergizes with radiotherapy. From the clinical point of view abscopal effect is particularly interesting due to both its possible valuable contribution to the treatment of metastases, and the potential harmful effects as induction of genetic instability and carcinogenesis. This review summarized the main results of investigations of non-targeted effects coming from *in vitro* monolayer cultures, 3-dimentional models of tissues, preclinical studies on rodents and clinically observed beneficial abscopal effects with particular emphasis on participation of immunotherapy in the creation of abscopal effects.

Keywords

Radiation-Induced Bystander Effect, *In Vitro* Studies, Preclinical Investigation, Radiotherapy, Immunotherapy, Beneficial Abscopal Effect, Carcinogenic Potential, Secondary Cancers

1. Introduction

Radiation-induced bystander effect (RIBE) is a non-targeted effect commonly defined as the induction of bio-

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logical changes in cells being not directly exposed to ionizing radiation, but only subjected to signals emitted by their irradiated neighbors. For over 20 years it attracts considerable attention due to possible implications for radiotherapy ([1]-[4], and references therein) but the biological significance of bystander effect remains still open to discussion. RIBE appears in non-targeted cells as a variety of stress induced responses resembling that observed in directly hit cells. Furthermore, molecular signals secreted by hit cells can be carried far apart, possibly affecting distant targets. The molecular signals may be transmitted through intercellular gap junctions or through medium transfer mechanism. Signaling molecules in bystander effect are diverse. In addition to the short living oxygen and nitrogen free radicals, the long-living radicals, interleukin 8, transforming growth factor β (TGF-β) and other can be involved (reviewed in [1]-[4]). Furthermore, recent studies show that when irradiated cells are incubated in the vicinity of the non-irradiated cells the two populations of cells interplay. Thus, the signals are sent not only by irradiated cells leading to changes in non-radiation ones, but the non-hit cells answer the directly irradiated cells [5]-[8]. It is possible that the impact of bystander effect on responses of cancer and healthy tissues to radiation is more relevant than is believed at present. The bystander effect may be a potentially harmful (damaging of neighboring normal cells *in vivo*), or even useful event in radiotherapy (the elevation of damage to tumor cells not directly hit by radiation), both leading to modulation of the therapeutic ratio. In this paper I try to answer some questions related to bystander effect which are important from the clinical point of view, namely: Does the bystander effect occur *in vivo*? May the bystander effect have clinical implications? Does the bystander effect take place in the course of dose fractionation? Can it alter the radiation induced tumor and/or normal tissue reactions? Can the bystander effect pose a risk of secondary oncogenesis? The answers to these questions can be drown from *in vitro* experiments and preclinical data which carry some clinically useful information regarding the radiation induced non-targeted effects and their possible implications for cancer treatment. Furthermore, many clinical reports demonstrate the therapeutic benefits of non-targeted out of field/abscopal effects and even indicate a potential ability to modulate them in the appropriate direction for cancer cure. The more interesting observations from experiments *in vitro*, from preclinical studies on animals and some important clinical reports of beneficial bystander/abscopal effects are reported in the article. Preclinical investigations and clinical reports indicate that abscopal effects are immune mediated and are mainly induced in concomitant treatment by immunotherapy and radiotherapy, which operate synergistically. This immunologic aspect, and on the other hand, a possible role of abscopal effect in carcinogenesis via induction of genetic instability is pointed out.

2. Radiation Induced Bystander Effect: What Have We Learned from *in Vitro* Studies?

2.1. The Different Types of Bystander Effect

The bystander effects in non-irradiated cells often resemble the responses found in directly exposed cells. These responses are observed in cells that are in the vicinity of the irradiated cells (horizontal transmission of bystander signals via intercellular gap junction or by medium), or in subsequent generations of irradiated cells (vertical transmission of bystander signals) [9]. Classic bystander effect caused by molecular signals released by irradiated cells typically refers to the damaging effects such as: reduced clonogenic survival [10], increased sister chromatid exchange [11] [12], formation of micronuclei and apoptosis [13] [14]. In addition to the classic bystander effect (type I), other types of bystander effects were disclosed in the *in vitro* experiments [15]; the type II which elicits as increased survival of non-targeted cells when the targeted cells received a high dose of radiation, and type III, encompassing an increase in the survival of cells targeted by a high radiation dose when neighboring cells received a low radiation dose. Numerous studies *in vitro* have shown that bystander effect is dependent on the type of cells, radiation quality (LET) and dose, genetic background, and experimental condition. The reader is referred to review article [2], which discusses all these issues very accurately including putative and confirmed mechanisms responsible for the bystander effect. In the current review only some important items are bulleted.

2.2. Cell Type-Specific Response to Bystander Signaling

The various cells can demonstrate both, the differences in the ability to generate bystander signals, and a different perception of these signals [10]. The study of Gómez-Millán *et al*. [16] showed that melanoma skin-cancer cells were sensitive to radiation-conditioned medium whereas umbilical-cord stromal stem cells were not when

clonogenic cell survival or apoptosis were used as endpoints. Fortunately, it seems to be almost the rule that normal stem cells are resistant to bystander signals. Sokolov *et al*. [17] found no evidence for RIBE neither in human bone-marrow mesenchymal stem cells nor in embryonic stem cells by the criteria of induction of DNA damage and apoptotic cell death (in the range of 0.2 - 10 Gy) compared to non-irradiated cells ($p > 0.05$). Such features may be promising for a possible regenerative therapy based on human stem cells, and probably can help in repopulation of normal tissues damaged by radiation. However, cancer stem-like cells of human HT1080 fibrosarcoma cell line were also found to be less active than their counterpart non stem-like cancer cells in respect to both, the generation and the response to bystander signals [18]. The normal primary fibroblasts were also resistant to bystander signaling after either low LET or high LET radiation based on clonogenic survival and DNA double strand breaks (γH2AX foci) over doses ranging from 10 - 100 cGy [19]. This is in contrast to our results showing a high apoptosis response in the normal human dermal fibroblast cell line [8]. The reason, besides the difference in doses, probably lies in the different experimental systems. Media transfer used by [19] does not allow the continuous contact of non-irradiated cells with mediators of bystander effect which can be secreted for a long time after irradiation. On the contrary, the transwell co-incubation system used by us can freely adjust the contact time of non-irradiated with irradiated cells due to shearing a common medium but being separated by a semipermeable membrane. Such experimental system at least in part simulates an *in vivo* system. Our recent study indicated that different response to bystander signals may depend on genetic status of cells including *TP*53, the gene controlling cell fate in response to radiation but which is often mutated in cancer [20]. The viability of exposed to X-rays, and of bystander cells of colorectal carcinoma cell lines HCT116 with wild type *TP*53 and knockout gene showed a roughly comparable decline with increasing dose (0 - 8 Gy). However, both lines highly differed in apoptosis induction. Whereas cells with knockout gene were susceptible to apoptosis, wild type cells were not, but were much more vulnerable to radiation-induced premature senescence which was associated with NFκB pathway activation [20]. Cellular senescence is defined as the irreversible mitotic arrest which is normally triggered by the exhaustion of proliferating potential. Initially, cellular senescence was believed to be a side effect of culturing cells *in vitro*, but recently senescent cells have also been found *in vivo* in a variety of tissues and organs in response to different stress, among them ionizing radiation [21]. Recent studies have shown that cells undergoing senescence acquire characteristic biological features called senescence associated secretory phenotype (SASP) characterized, inter alia, by ability to release of many signaling factors which exert harmful effects on the tissue microenvironment [22] [23] and operate as secondary bystander signaling.

2.3. Radioprotective Bystander Effect

It is reasoned to expect that signals secreted by irradiated cells can cause changes in adjacent non irradiated cells and vice versa. We observed a mutual signaling between bystander normal fibroblasts co-incubated with irradiated cancer cells, which led to diminution of micronucleus and apoptosis frequency in irradiated cells and this was true for rodent (mice fibroblasts NIH3T3 vs Lewis lung carcinoma LLC) [7], and human (normal human dermal fibroblasts NHDF vs malignant melanoma Me45) cells [8]. This radioprotective effect was accompanied by reduction of cellular ROS in cancer cells. Similar protective ("rescue") effect via intercellular feedback signaling of human fibroblasts towards irradiated HeLa cells was also presented as significant diminution of micronucleus yield, apoptosis and DNA double strand breaks [6]. Additionally, other group reported that irradiation of human lung fibroblasts with the low dose of ionizing radiation (1 cGy, γ-rays) enhanced proliferation of bystander fibroblasts when they were treated with medium harvested from irradiated cells and subsequently irradiated with 2 and 4 Gy [5]. This radioprotective/radioadaptive bystander effect was preceded by the decrease in cellular level of p53 and cyclin-dependent kinase inhibitor 1 (CDKN1A protein), increase in intracellular reactive oxygen species (ROS), and increase in the DNA base excision repair protein AP-endonuclease (APE). Another example of radioprotective bystander effect was demonstrated in *ex vivo* study [24] for high dose-rate (HDR) brachytherapy patients. Blood serum, urine, and esophagus explants from esophageal carcinoma patients were used to assess patients' responses to radiation treatments based on *in vitro* keratinocyte colony-forming assay. Blood sera taken after the third fraction of brachytherapy caused a significant increase in cloning efficiency of human keratinocytes compared to baseline samples indicating a radioprotective ability of secreted factors produced by irradiated tumors. Earlier study of the same authors aimed to search a biochemical nature of these factors suggested that serotonin (5-Hydroxytryptamine) may play an active role as a signaling molecule in HDR-brachytherapy bystander effect [25].

2.4. Radiation Induced Genomic Instability

There appears to be a close link between RIBE and radiation-induced genomic instability (RIGI) [26]-[28]. Genomic instability is defined as delayed effect due to vertical conveyance of signals from the irradiated cells to their progenies observed in the form of lethal mutations, unstable chromosomal aberrations and delayed reproductive death [29]-[31]. RIGI can persist for a very long time when induced *in vivo* as shown in *ex vivo* studies [32]-[34]. It was indicated [33] that cells exposed to serum samples from Chernobyl liquidators and from workers in Gomel area induced significantly elevated level of micronuclei in recipient keratinocytes *in vitro*, whereas viability of cells treated with those sera was correspondingly reduced. This study has been recently repeated [34]. Almost thirty years after the accident there is still evidence of the presence of clastogenic and cytotoxic bystander factors in the serum of populations exposed to radiation from the reactor. Even though the authors call the observed phenomenon as bystander effect it is in fact an example of genomic instability. By the way, it is interesting whether clastogenic and cytotoxic agents exist in the serum for such a long time, or are they permanently produced by the offspring of originally damaged cells. It was postulated that among clastogenic factors are the lipid peroxidation end-products and cytokines which are mediated by superoxide radicals and other reactive oxygen species (ROS) [32]. Genomic instability may be the first step in carcinogenesis, and may pose a potential risk to human health.

2.5. Bystander Effect Induced by Fractionated Irradiation

Albeit the bystander effect is generally attributed to a low dose (less than 1 Gy) or low LET radiation [35] [36], a variety of *in vitro* studies, including our own [8] [20] [37] show that it occurs after exposure to doses used in conventional therapy, or even at higher doses. And though the results of *in vitro* studies cannot be transferred directly to the *in vivo* situation, they suggest that bystander effect possibly occurs during fractionated radiotherapy. Our study applying co-incubation system, aimed to compare bystander effect in malignant melanoma (Me45) cells after single dose and after division of the dose into 3 fractions (administered every 24 h) showed that fractionation at low doses (3 × 0.5 Gy) induced higher level of micronuclei in hit and bystander cells than single dose of 1.5 Gy. This was less evident when we used conventional dose fractionation (3 × 2 Gy) vs single dose (6 Gy). However, both fractionation schemes were much more effective in inducing apoptosis, especially in bystander cells, than single dose irradiation [38]. The results are in accordance with those presented by others [39], who studied the effects of dose fractionation on RIBE in a keratinocyte cell line and found that the fractionated dose was more toxic than the single dose and was comparable for 2.5 Gy and 1.5 Gy fraction doses. Thus bystander effect, if it appears *in vivo* during fractionated radiotherapy may reduce the expected sparing effect of fractionation to adjacent tissues and even increase normal tissue damage. On the other hand, no differences were observed in micronuclei induction in normal human lung fibroblasts (MRC5) treated with conditioned medium harvested from cultures of the same line or human lung tumor cell line (QU-DB) previously exposed to 1, 2, and 4 Gy of single acute or fractionated irradiation by equal fractions with a gap of 6 h [40]. It seems that human normal fibroblasts are relatively weak recipients of bystander signaling, especially when conditioned medium is harvested shortly after irradiation.

Summarizing, the bystander effects *in vitro* have been demonstrated using a wide range of experimental approaches like different types of radiation and doses, different types of cells including human and animal fibroblasts, endothelial cells and tumor cells and evaluating variable endpoints. Short characteristics of *in vitro* studies are presented in **Table 1**.

3. Translation of *in Vitro* Studies to *in Vivo* Situation

Some transition from *in vitro* studies of bystander effect to *in vivo* situation represents a 3D model of tissue. Using the model of artificial skin comprising of both layers, keratinocytes and fibroblasts, Belyakov *et al*. [41] demonstrated that irradiation with microbeam of alpha particles smaller than the diameter of the cell (<5 μm), induced a bystander effect in the cells spaced about 1 mm from the irradiated line. This effect was observed as significant increase of apoptosis and micronucleus frequencies. Similarly, Sedelnikova group [42] using 3D artificial tissues of skin or respiratory epithelium and microbeam irradiation found complex damage in bystander cells, comprising the DNA double strand breaks (γH2AX foci), increase of micronucleation, apoptosis, senescence and epigenetic changes in DNA methylation.

Table 1. Summary of experimental investigations characterizing radiation induced bystander effect *in vitro*.

Cell type	Radiation type and dose	Observed bystander response	Reference
Epithelial (IR), fibroblasts (By)	γ-rays, 1 - 100 cGy	clonogenic survival decreased	[10]
Chinese hamster fibroblasts	α-particles, 0.31 & 0.49 mGy	SCE increased	[11]
Human fibroblasts	α-particles, 1.5 - 8.4 cGy	SCE increased	[12]
Human fibroblasts	α-particles, X-rays (0.1 Gy)	micronuclei and apoptosis increased	[13]
Human malignant melanoma	γ-rays, 5 Gy	micronuclei, apoptosis, DNA ssb, SOD, MDA all increased	[14]
Human dermal fibroblasts vs human malignant melanoma	X-rays (6 MV), 2 & 4 Gy	micronuclei and apoptosis decreased in irradiated cells	[8]
Human malignant melanoma	X-rays (6MV), 3 - 20 Gy, spatially modulated field	clonogenic survival decreased in low dose regions, increased in high dose regions	[15]
Human embryonic stem cells and bone marrow stem cells	X-rays, 0.2, 2.0, 10 Gy	DNA damage and apoptosis unchanged	[17]
Human fibrosarcoma stem-like cells and non-stem-like cells	photons (3.7 MeV), 2.2 Gy	DNA dsb increased more in non-stem-like cells	[18]
Human normal fibroblasts, human colon carcinoma	X-rays, γ-rays, energetic electrons, 10 - 100 cGy	clonogenic survival, DNA dsb and micronuclei, all unchanged	[19]
Human colorectal carcinoma	X-rays, 2 - 8 Gy	survival decreased, apoptosis micronuclei, and senescence increased	[20]
Murine hemopoietic stem cells	α-particles, 0.25 - 1 Gy	clonogenic survival decreased, chromosomal aberrations increased	[26]
Human keratinocytes	γ-rays, 0.1 - 0.5 Gy	clonogenic survival decreased	[35]
Human colorectal carcinoma	X-rays (6 MV), 2 - 8 Gy	clonogenic survival decreased, senescence increase	[37]
Human lung fibroblasts and lung adenocarcinoma	22 MeV electrons, 6 MV photons, 5 Gy	micronuclei and apoptosis increased	[44]
Human malignant melanoma	X-rays (6 MV), 3 × 0.5 Gy vs 1.5 Gy; 3 × 2 Gy vs 6 Gy	higher increase of micronuclei and apoptosis in fractionated RT	[38]
Human keratinocytes	5 mGy-5 Gy, γ-rays given as single or split doses	clonogenic survival declined deeper after split dose	[39]
Human lung fibroblasts and lung carcinoma	1, 2, 4 Gy given as single or split doses	micronuclei and apoptosis inreased comparably in both schemes	[40]
Normal human lung fibroblasts	γ-rays, radioadaptive 1cGy, challange 2 - 4 Gy	clonogenic survival increased	[5]
Murine fibroblasts vs murine lung carcinoma	X-rays (6 MV), 2 & 4 Gy)	apoptosis and micronuclei decreased	[7]
Human primary fibroblasts vs human cervical carcinoma	α-particles, 20 & 40 cGy	apoptosis and micronuclei decreased	[6]
Human normal fibroblasts vs human malignant melanoma	X-rays (6 MV), 2 & 4 Gy	apoptosis and micronuclei decreased	[20]
Umbilical stromal cells	γ-rays, 2 - 8 Gy	clonogenic survival and apoptosis unchaneged	[16]
Human malignant melanoma	γ-rays, 2 - 8 Gy	clonogenic survival decreased, apoptosis increased	[16]

A primary explant culture of human and porcine urothelium was also applied to study bystander effect in a multicellular model that reconstructed the *in vivo* microarchitecture of normal tissue [43]. The outgrowth of urothelium composed of proliferating and differentiated cells was irradiated with helium ions ($^3He^{2+}$) possessing characteristics similar to α-particles. Using a microbeam facility the total of 10 individual cell nuclei were irra-

diated either on the periphery, where proliferating cells were located, or at the center of the explant outgrowth, which consisted of terminally differentiated cells. Bystander effect was observed as increased frequency of micronuclei and apoptosis presenting cells, however, in proliferating areas only. These data indicate that bystander-induced damage depends on the proliferation activity of the cells and thus actively proliferating tissues will be probably more responsive to bystander signaling than differentiated tissues.

Interesting from the clinical point of view are the results of Konopacka *et al*. [44]. To study the bystander effect *in vitro* they constructed a system mimicking situation occurring in local radiotherapy with external beam. The cultures of human lung carcinoma cells (A549), normal bronchial epithelial cells (BEAS-2b) and normal dermal fibroblasts (NHDF) were directly irradiated (5 Gy) or exposed to scattered radiation 4 cm outside the field (dose of 0.2 Gy) using either electron (22 MeV) or photon (6 MV) radiation generated by therapeutic linear accelerator. Results showed that in all tested cell lines exposure to radiation induced apoptosis and formation of micronuclei. Lung cancer and epithelial cells placed outside the radiation field and exposed to scattered radiation developed micronuclei and induced apoptosis at the levels comparable with those estimated in bystander cells treated with irradiation conditioned medium collected from another pool of irradiated cells. These indicate that genetic damage in cells exposed to scattered radiation is caused by factors released by irradiated cells into the medium rather than by DNA damage induced directly by radiation. It seems that bystander effects may have important clinical implications for health risk after low level radiation exposure of cells lying outside the radiation field during clinical treatment. Interestingly, normal human fibroblasts were resistant to scattered irradiation and bystander signaling [44] showing that bystander effect is cell type specific event, and confirming a lack of bystander response in fibroblasts when medium transfer system is applied.

Currently used radiotherapies comprise different types of high precision radiation techniques aimed to deliver highly conformal doses to the target volume and to reduce the doses in normal tissues [45]. Due to spatio-temporal dose modulation in a close vicinity of tumor cells which obtained high dose there are normal cells obtaining lower dose and very low dose. It is not easy to simulate such situation in experiments *in vitro*. In an attempt to spatially modulate radiation dose *in vitro* Mackonis *et al*. [15] performed experiments recalling a little the situation *in vivo* using different doses of 6MV photons to irradiate different part of culture flask with malignant melanoma. The whole field was uniformly exposed to 3, 6, 10 and 20 Gy, or the quarter field at one end of flask or stripped quarter were exposed to high 20 - 30 Gy doses, whereas the rest of shielded fields obtained ~1 Gy. The bystander effect evaluated as clonogenic survival was dependent on dose, and decreased at lower doses, but increased at high or lethal dose indicating a mutual signaling between radiation exposed and bystander cells. The cell culture models are limited to two dimensions, lacking cellular architecture and physiological features, thus the subject of future researches should be focused on the use of tissue equivalents, combined with the spatial and temporal regimens irradiation. It also seems that creation of mathematical models which can take into account the spatial tissue structure and size, type of radiation, interplay of cells based on existing experimental data would be helpful in predicting the actual bystander effects *in vivo*.

4. Non-Targeted Effect *in Vivo*

4.1. Classification of Non-Targeted Effect *in Vivo*

It is important to examine the bystander effects and other radiation induced phenomena under conditions relevant to human radiation exposures. The non-targeted radiation effects *in vivo* may greatly differ from those *in vitro*. The human body is a biological system that seeks to maintain a state of homeostasis mainly due to cooperation of the nervous, endocrine and immune systems. Therefore, classification of non-targeted effects valuable *in vitro* is useless to describe them in the *in vivo* situation. Blyth and Sykes [46] proposed the classification of non-targeted effects appearing *in vivo* into three events: bystander effect, abscopal/out-of field effect and cohort effect. According to this classification, bystander effect is rather restricted to very low doses of radiations like daily natural background, inhalation of volatile products of radon decay, X-ray screening tests, and exposure to cosmic radiation during high altitude flying. This limitation of bystander effect *in vivo* is not compatible with the *in vitro* studies showing that it occurs in a wide range of doses from cGy [34] to several Gy, e.g. [8] [20] [37]. Thus actually bystander effect may also take place in the tissues adjacent to the radiation field during local radiotherapy of cancer. From the clinical point of view the most important is, recently discussed extensively, an abscopal effect ([47], and references therein). According to Blyth and Sykes qualification abscopal effect comprises effects joint with radiotherapy to localized tumors, physical contact with small radioactive source and ra-

dionuclide intake in diagnosis and treatment, e.g. radioiodine (the radionuclide induced bystander effect will not be discussed in this issue due to limitation of space). The third type, a cohort effect, is defined as interaction between irradiated cells within an irradiated volume. The authors include here radiotherapy of tumors, CT scanning and whole body exposure due to accidents/incidents. Although this is the most logical to expect a mutual signaling between irradiated cells, the cohort effect is difficult to be prove experimentally in a quantitative way. It appears that the types II and III described by Mackonis can be reflected here. In addition, the radioprotective effect that may be exerted by cancer-associated fibroblasts towards tumor cells, as observed *in vitro* [6] [8] can also occur *in vivo* within irradiated volume.

4.2. Preclinical Data-Bystander Effect in Rodents

Many studies on non-targeted/bystander effects *in vivo* including rodents, fish and plant were published (reviewed in [48]). Most of them actually disclose the abscopal effect of a damaging character and show that abscopal effect is not tumor-specific. Khan *et al*. [49], found that partial irradiation of the rat's lung led to formation of micronuclei in other non-irradiated areas of the lung. Pretreatment of animals with Cu/Zn superoxide dismutase (SOD) or L-NAME (N^{ω}-Nitro-L-arginine methyl ester hydrochloride), the inhibitor of nitric oxide synthase (NOS), reduced the DNA damage in the shielded area, indicating the role of ROS and NO as mediators of abscopal effect. Furthermore, DNA damage in the lung fibroblasts lying in the irradiation field, and to a lesser degree outside the field was accompanied by changes in inflammatory cytokine expression (IL-1, IL-6, TNF-a, TGF-b) and activation of macrophages [50] suggesting that inflammatory agents induced by reactive oxygen species in the cells of immune system are mediators of DNA damage.

The study of Koturbash *et al*. [51] showed a variety of biological changes developed in non-irradiated mouse spleen after cranial irradiation (1 Gy X-rays) at protecting the rest of the body. Immunohistochemical study of DNA damage (γH2AX), cell proliferation (Ki67), apoptosis, and p53 protein in the spleen showed an increase of these indicators relative to non-irradiated control mice. Other data of the same group show that X-ray exposure to one side of the animal body induces DNA strand breaks and causes an increase in the level of repair protein Rad51 in unexposed bystander tissues. These bystander effects were not the results of insufficient shielding or radiation scattering [52]. Camphausen *et al*. [53] observed that fractionated irradiation of non-tumor-bearing legs of mice slowed down the growth of tumors implanted into the midline dorsum in a dose dependent manner. This abscopal effect was p53-dependent since p53-null animals did not show such effect and additionally, pifithrin-α; the blocker of p53 abrogated this abscopal effect.

Abscopal effect *in vivo* was also evidenced in the form of epigenetic changes. A considerable reduction in DNA methylation in splenic cells was found after irradiation of the head of rat with 20 Gy (X-rays, 90 kV, 5 mA) given in two fractions [54]. The epigenetic changes in mouse spleen and dermal tissue after cranial exposure to acute or fractionated irradiation was also demonstrated [55]. The DNA hypomethylation in skin tissue was short lasting effect (6 h post exposure) whereas the change in spleen was seen even two weeks following exposure to 0.5 Gy indicating tissue specific responses. A significant decrease of global DNA methylation and changes in DNA methyltransferase levels were observed even in the progeny of irradiated mice [56]. Epigenetic changes in DNA methylation and histone modification are fairly permanent changes that can be passed on to the daughter cells. Methylation disorders may be induced by ionizing radiation and other DNA damaging agents. They can lead to genetic instability, rearrangement of the genome and the development of cancer. Observed at such distant time as 7 months the bystander effect is likely to have clinical consequences like carcinogenesis. Some data indicate that bystander effects *in vivo* have carcinogenic potential. Mancuso *et al*. [57] using the neonatal cerebellum of radiosensitive Patched-1 (Ptch1) heterozygous mice as a suitable in vivo model to study bystander responses of brain demonstrated the DNA double-strand breaks and apoptotic cell death in non-irradiated cerebellum when the rest of body was exposed to X-rays. In consequence, these genetic events had led to tumor induction.

Several animal experiments revealed abscopal effect associated with irradiation of primary tumors [58]-[60]. It was observed that irradiation (2 or 6 Gy) of mammary carcinoma positioned on one flank of the mice as primary tumor resulted in inhibition of growth of secondary tumor of the same line implanted a few days later on the other flank. This out of field effect was tumor type specific since when lymphoma was passaged as secondary tumor, the abscopal effect was absent. This suggests that immune cells were activated by antigens released from the primary tumor subjected to irradiation and therefore were not able to recognize antigens of other type

of tumor cells transplanted some time later. Furthermore, abscopal effect appeared when immune system was stimulated by Fms-like tyrosine kinase receptor 3 ligand (Flt3-L), a growth factor that stimulates generation of antigen-presenting dendritic cells (DCs) [58]. In similar experimental model where primary and secondary tumors of the poorly immunogenic mammary carcinoma or colon carcinoma were implanted on two opposite legs the fractionated but no single dose radiotherapy induced an abscopal effect when used in combination with anti-CTLA-4 antibody (targeted the cytotoxic T-cell lymphocyte antigen-4) [60]. Frequency of CD8+ T lymphocytes showing tumor-specific IFNγ production was proportional to the inhibition of the secondary tumor. Another research group [59] applied ECI301 chemokine, an active variant of macrophage inflammatory protein-1 (MIP-1) to enhance the direct radiation effect and induce abscopal effect in mice bearing adenocarcinoma, fibrosarcoma or Lewis lung carcinoma. This agent is able to activate different type of lymphocytes, macrophages and DCs. Repeated i.v. administration of ECI301 after local exposure to 6 Gy not only eradicated irradiated tumors but also inhibited growth of non-irradiated tumors transplanted on second flanks. Abscopal effect was associated with increase of CD4+, CD8+ and NK cells in tumor tissues, and depletion of these cells by monoclonal antibodies reversed abscopal effect indicating that abscopal effect is immune mediated and dependent on T lymphocytes. Further example of abscopal effect being a result of combining radiotherapy with immune modulator comes from the study of Deng *et al*. [61]. They evaluated the role of potential synergistic effect of concomitant use of radiotherapy and immunotherapy with anti-PD-L1 (αPD-L1). PD-L1 (programmed death-ligand 1) binds to PD-1 receptor expressed on T-cells playing an important role in inhibiting a proficient T cell response [62]. Combination of anti-PD-L1 and single high dose (12 - 20 Gy) significantly enhanced the inhibition of primary TUBO tumor growth in BALB/c mice and MC38 tumor in C57/BL-6 mice. Systemic effect of combined treatment also greatly reduced the growth of secondary tumors transplanted on opposite flanks thirty days after primary tumor eradication. Thus administration of anti-PD-L1 enhanced the efficacy of irradiation through a cytotoxic T cell-dependent mechanism. These all indicate an important role of immunologic system activation in abscopal effect and suggest that immune therapy synergizes with radiotherapy to enhance systemic effects after irradiation.

5. Clinical Data on the Abscopal Effects

5.1. Abscopal Effect with Respect to Tumor Response: An Immune-Mediated Phenomenon

Clinical cases of abscopal effects comprise both abscopal tumor regression and normal tissue response. The history of spontaneous regression of cancer and abscopal effects are presented in review articles by Siva *et al*. [47] and Reynders *et al*. [63]. Although spontaneous regressions of metastases are documented [64], the clinical abscopal effect is connected with local radiotherapy (RT) of primary tumor or metastases. So far over a dozen clinical cases confirming the existence of beneficent abscopal effect have been published. As summarized in [47] abscopal effect was observed after conventional radiotherapy (dose range from 12 Gy to almost 70 Gy) of different types of non-hematological tumors like renal cell carcinoma, hepatocellular carcinoma, cervical, lung, esophagus and skin carcinoma and melanoma. Some of them are illustrated herein a little wider. One of the early cases of abscopal phenomenon documented in the literature is an abscopal regression of lung and mediastinal node metastases in renal cell carcinoma patient treated with 20 Gy of palliative irradiation to large, painful primary tumor presented by Mc Manus *et al*. [65]. The regression was accompanied by an elevation of serum level of interleukin-2 receptor indicating an involvement of interleukin-2 in abscopal signaling. Ohba *et al*. [66] presented the case of an old patient with hepatocellular carcinoma that regressed after palliative radiotherapy for thoracic vertebral bone metastasis. An abscopal regression was associated with a decrease in tumor marker alpha fetoprotein (AFP), and elevation of serum level of TNF-α after RT. Increase of this cytokine suggests that observed abscopal effect might be mediated by immune system activation. Some case reports present beneficial abscopal effect after stereotactic ablative radiation therapy (SABR) the aim of which is to deliver very large dose per fraction in short courses, typically 1 - 5 fractions of doses above 6 Gy to small target volume [47]. Ablative radiotherapy certainly results in a more significant damage than low-dose radiotherapy to the stroma of tumor, and causes serious vascular injury, increase of apoptosis and necrosis of irradiated cells which release tumor-associated antigens and in turn stimulate the immune response and mediate the distant effect [62] [67]. SABR treated renal cell carcinoma caused regression of untreated metastases in three of four cases presented by [68]. The abscopal regression of pulmonary and lymph node metastases in renal cell carcinoma patient was also

noticed after SABR treatment of bone and spine lesions [69]. Unfortunately, the brain metastases relapsed during treatment. The authors postulated that mechanism of abscopal effect may be organ specific and blood-brain barrier does not allow the mediators to pass it.

Abscopal effects were also documented in non-small-cell lung cancer after conventional RT and SABR, and after RT combined with ipilimumab. Ipilimumab is the humanized monoclonal antibody that targets cytotoxic T-cell lymphocyte antigen-4 (CTLA-4), the inhibitory immune checkpoint regulator [70]. CTLA-4 blockade leads to decreased activity of regulatory T cells which normally act immunosuppressive, and are more radioresistant than other T cells [71]. Along with posttreatment tumor regression, an increase in tumor-infiltrating cytotoxic lymphocytes and normalization of tumor markers was observed in Golden *et al*. study [70].

Promising are the cases of abscopal effect in the treatment of malignant melanoma. Melanoma is one of the most malignant tumors with still increasing incidence worldwide and poor prognosis [71]. Whereas early stage of melanoma is curable by surgery, in more advanced stage the treatment option is a combined treatment with chemotherapy and different type of immunotherapy ([72], and references therein). Melanoma is generally considered as radioresistant but the recent trend in the use of high-dose per fraction therapy (including SABR) has demonstrated that radioresistance can be overpass. Furthermore, the treatment by ipilimumab combined with radiotherapy, especially stereotactic radiosurgery (SRS) reduced the risk of death in patients with melanoma brain metastases as demonstrated by Silk *et al*. [73] based on analysis of existing studies. Recent few case reports showed abscopal regression of metastatic melanoma treated by combined immunotherapy with radiotherapy [74]-[76]. Postow *et al*. [74] reported a case of the abscopal effect in a patient with pulmonary metastatic melanoma treated with ipilimumab and RT. They also measured changes in NY-ESO-1 antibodies (NY-ESO-1 is an antigen which is expressed in 30 to 40% of patients with advanced melanoma) and found increase of their titers after RT when abscopal effect was observed. Grimaldi *et al*. [75] performed retrospective study aimed to evaluate the effect of ipilimumab-radiotherapy sequence. Twenty one patients with advanced melanoma who experienced disease progression after ipilimumab were treated by RT. Radiotherapy was given to the brain or extracranial metastases mainly as fractionated radiotherapy. An abscopal response was observed in 11 patients (52%) and appeared only when patients exhibited local response. Time from RT to an abscopal response was 1 - 4 month. The treatment also increased the median overall survival (OS). The median OS for patients with abscopal responses was extended to 22.4 months (range 2.5 - 50.3) vs. 8.3 months (range 7.6 - 9.0) without. These abscopal responses to RT after ipilimumab suggest that local responses to RT may be predictive of abscopal responses.

Abscopal effect was also presented in patient with large metastases of malignant melanoma into the brain, abdomen and kidney [76]. Fractionated radiotherapy to the brain metastasis combined with temozolomide followed by ipilimumab resulted in regression of the remaining metastases. Although the incidence of abscopal effect revealing as regression of advanced malignant melanoma are largely the result of combination of radiation therapy with immunotherapy, a spectacular long-term cure of advanced melanoma after RT alone has been reported [77]. The authors describe a case of advanced melanoma (IIIC) located on the scalp of patient with multiple satellite metastases which regressed after palliative RT of primary melanoma. The regression was associated with increase of autoantibodies against melanoma antigen A3 (MAGEA3) indicating a mobilization of immune system by RT. However, after 36 months patient developed nodal and brain metastases. The use of intracranial SRS with ipilimumab resulted in further increase of MAGEA3 and complete tumor regression lasting at least seven years. These all data highlights the possible clinical benefit of the radiation induced abscopal effect in the treatment of malignant melanoma. Radiotherapy may not only control the localized tumor but may induce immune activation and development of T-lymphocytes population, that recognize tumor cell antigens and eliminate tumor cells even in organs distant from irradiated tumor. However, the scarcity of documented reports on the clinical abscopal effect and the preclinical researches suggest that RT alone is unlikely to have major clinical impact on recovery of metastases. Despite the benefits of abscopal effect that may occur during RT of advanced melanoma the stereotactic radiotherapy of brain metastases combined with drugs activating T-lymphocytes like ipilimumab is significantly more effective than either of these methods used alone [73]. The main goal of combining immunotherapy with RT is to reduce existing immunosuppression or tolerance of tumor by the microenvironment of the body, and induce the immune response to cancer [78] [79]. The synergism of action of immunotherapy with RT may result from the cellular mechanisms induced by radiation-caused damage in such structures like double-stranded DNA, RNA, chromatin, or high-mobility group protein1 (HMGB-1) generally described as DAMPs which are recognized by Toll-like receptors (TLR) on immune competent cells e.g.

macrophages or DCs [62] [63].

The combination of immunotherapy with RT is now becoming a new field in cancer therapy. Preclinical results and clinical cases of beneficial abscopal effect have stimulated the initiation of clinical testing of different combinations of immunotherapy with radiotherapy in the treatment of various cancers. The currently opened clinical trials of immunotherapy combined with radiotherapy are summarized by Crittenden *et al*. [79]. The different immune stimulators are under investigation for efficacy and safety of combined treatment and possible generation of abscopal effect after localized radiotherapy. Granulocyte-macrophage colony stimulated factor (GM-CSF), TGF-β, toll-like receptor (TLR) agonist (imiquimod), anti-CTLA-4 monoclonal antibody are applied in the New York University study in metastatic breast cancer, metastatic melanoma and non-small lung cancer. Other trials combining radiotherapy and immunotherapy are ongoing at Chiles Research Institute. These include stereotactic body radiation therapy (SBRT) and high-dose interleukin 2 (IL-2) in metastatic melanoma; anti-OX40 antibody, cyclophosphamide (CTX) and radiation in patients with progressive metastatic prostate cancer; SBRT and monoclonal antibody to OX40 in breast cancer patients with metastatic lesions; combination of gemcitabine, tadalafil, telomerase vaccine and GM-CSF and standard fractionated radiation in locally advanced and borderline resectable pancreatic cancer; and combination of gemcitabine, tadalafil with hypofractionated radiation in locally advanced and borderline resectable pancreatic cancer. OX40 is a receptor expressed on activated T cells whereas activated B cells express OX40 ligand (OXO40L), a member of the tumor necrosis factor/nerve growth factor family of cytokines. OX40-OX40L interaction *in vivo* is necessary for the differentiation of activated B cells into highly immunoglobulin-producing cells [80]. Preclinical studies which became the rationale for clinical trials demonstrated synergistic action of OX40 agonist antibody in combination with CTX [81] and with radiation [82]. Clinical trials currently open at Stanford Medical Center include, among others, the anti-CTLA-4 immunotherapy with local radiotherapy in oligo-metastatic melanoma, non-Hodgkin's lymphoma, and colorectal cancer. The aim of clinical trial of immunotherapy and radiation therapy currently open at Thomas Jefferson University is to determine the maximum tolerated dose (MTD) of ipilimumab when combined with whole-brain radiation therapy (WBRT) or stereotactic radiosurgery (SRS) in metastatic melanoma to the brain. A pilot study of drug AMP-224, a PD-1 inhibitor, in combination with SBRT in patients with metastatic colorectal cancer is an open clinical trial at the National Cancer Institute [79]. It can be expected that the results of these studies will broaden our knowledge on how to manipulate the immune- and radiation therapy to improve the therapeutic effect in different types of malignant tumors.

5.2. Bystander/Abscopal Effect in Normal Tissues and Secondary Carcinogenesis

Abscopal effect may be helpful for tumor cure, but also detrimental with regard to normal tissues. Radiation induced bystander/abscopal effects in normal tissues have been repeatedly observed in animal studies as highlighted in previous section. These effects are most of all of damaging nature which can lead to cell death, cytogenetic damage and epigenetic changes being potentially carcinogenic. The question whether radiation-induced bystander effect may have an impact on acute and late damage in human normal tissue surrounded the radiation field is essential. Although direct extrapolation of results from *in vitro* experiments to the situation *in vivo* where we are dealing with complex biological system is a simplification, they suggest that RIBE and RIGI have their share in the risk of radiation-induced complications in normal tissues including inflammation, induction of mutations and secondary cancer. The various tissues have different radiosensitivity and can have different susceptibility to bystander signals. Complex systemic tissue responses like weakness, fatigue, nausea, anorexia or vomiting are usually noticeable in patients treated with radiation especially to a large volume. These responses are mediated by molecular signals induced in and secreted by irradiated cells comprising reactive oxygen and nitrogen species, long lived hydrogen peroxide and lipid peroxidation end-products, many growth factors and inflammatory cytokines [2] [4]. Sprung *et al*. [83] proposed that genotoxic stress like ionizing radiation, ultraviolet radiation, cytotoxic drugs and even the presence of a tumor lead to generation of mentioned signaling factors which cause DNA damage (and in turn genomic instability), apoptosis, senescence, changes in metabolism and activation of immune cells such as macrophages and neutrophils. The activated immune cells and long-lived reactive molecules accelerate the production of inflammatory factors which induce local response, and transmitted via blood circulation induce in distant tissues multiple responses as DNA damage, apoptosis, micronuclei and senescence [83]. It has been shown even in *in vitro* study that macrophages can transfer secondary bystander signals and play a key role in the secondary bystander effect of photon irradiation [84].

As proposed by Hendry J.H. [85] radiation-induced genomic instability in patients who underwent radiotherapy takes the form of delayed reproductive death (DRD). DRD can be involved in creation of normal tissues side effects due to increased cell loss, longer renewal and consequently increase in damage [84]. Increased levels of chromosomal aberrations and micronuclei detected after a year in lymphocytes of patients undergoing radiotherapy for cancer of the head and neck [86] or seven years after radiotherapy of seminoma [87] may confirm this supposition. Since the frequency of chromosomal damage a long time after radiotherapy mostly did not return to the level estimated before radiotherapy, this can be an important risk factor for the second-line cancer. The typical bystander effect in normal tissue was presented by Sheridan *et al*. [88]. The large *ex vivo* study carried out on the postoperative material in patients with colorectal cancer (80 patients) treated by neoadjuvant RT + FU (fluorouracil) showed an increased level of double-stranded DNA breaks in the cells of intestinal mucosa not adjacent to irradiated field compared to the mucosa in patients not treated by adjuvant RT.

Patients irradiated for cancer show, compared with healthy individuals, increase in morbidity of primary cancer of second-line [89]-[91] though toxicity of the bystander effect should not be the only reason for this growth since genetic predisposition, environmental factors and life style may have influence. Brenner *et al*. [90] by comparing the incidence of second-line cancer in patients with prostate cancer treated with surgery alone (over 50 thousand) and patients undergoing radiotherapy (over 70 thousand) found small, though statistically significant increased risk of second-line tumors in the latter group (6%, $p = 0.02$). This risk was associated with dose and latency time and grew with the increase in survival time and was 15% for patients surviving more than 5 years and 34% for patients surviving more than ten years. The emerging tumors were of solid type and were located in the bladder, large intestine and lung. The risk of secondary neoplasm after radiotherapy for cervical cancer is similar to prostate cancer. Research of Kleinerman *et al*. [91] in which they compared the risk of secondary cancers in patients irradiated due to invasive cervical carcinoma with a group of patients non-irradiated, surviving more than 30 years has shown a 12% increase in the incidence of secondary tumors, wherein after 10 years this increase was 15% and after 20 years of 26%. Neoplasms such as cancer of the colon and rectum, bladder, vagina and ovary were within a field which was subject to high dose of radiation, but there were also a few cases of leukemia. However, half of the secondary tumors accounted for the lung cancer [90]. The cancer appearance in the lung, organ relatively distant from the irradiated field of primary tumor, wherein the radiation dose was estimated at ~0.6 Gy seems to be related to the bystander effect induced by signaling molecules generated by irradiated cells, potentially mutagenic and carcinogenic. Diallo *et al*. [92] studied the spatial distribution of second malignant neoplasm (SMN) in relation to previous irradiated volumes in 115 patients (among 4581) who were exposed to radiotherapy in childhood. They found that 12% of solid tumors were located in the central area, 66% in the beam-bordering region and 22% in the regions distant > 5 cm from the irradiated volume. A peak SMN frequency of about 31% was identified in areas that received less than 2.5 Gy and 10% - 15% of these tumors arose in tissues receiving less than 0.5 Gy. These dose and spatial relation with secondary tumors suggest that in the case of modern radiotherapy techniques, such as intensity-modulated radiation therapy (IMRT), where large areas adjacent to the high-dose target are commonly exposed to a low-dose [4], non-targeted effect have potentially an important impact on radiotherapy outcome.

6. Conclusion

Bystander effect may have likely both useful and negative influence on the results of radiotherapy. The useful influence will appear if irradiated tumor cells damage neighboring tumor cells in the margin or within irradiated volume (bystander effect, cohort effect) or if an abscopal effect inhibits the growth of metastases. Negative influences may disclose as induction of cytogenetic damage, genetic instability in normal cells and tissues and in consequence secondary malignancies, an increase in the severity of radiation-induced reactions in normal tissues (especially in modern irradiation techniques of 3D IMRT aimed to spare the normal tissue due to diminution of dose). However, we cannot predict which of these effects, beneficial or detrimental, will prevail. There is a great lack of knowledge concerning the existence and role of the bystander effect in fractionated radiotherapy, IMRT as well as conventional radiotherapy, which is still an important element in cancer treatment. Animal studies using fractionated irradiations of tumors would enable to evaluate the responses of healthy tissues adjacent to radiation field. In addition, such type of experiments would allow for testing of molecular bystander pathways and for undertaking an attempt to inhibit the damaging bystander effect, or protect of normal tissues, e.g. by the use of antioxidant vitamins, which can reduce cell damage even given after irradiation [93]. The abscopal effect ob-

served *in vivo* is a beneficial immune-mediated phenomenon. Preclinical studies and clinical cases of abscopal effects published so far suggest that radiotherapy acts synergistically with targeted immune treatment and this seems to be a field for clinical manipulation. However several issues concerning the dosage of RT and immune stimulators and sequence of modalities, toxicity and selection of patients need to be solved before the abscopal phenomenon can be manipulated to enhance therapeutic benefits in radiotherapy. Therefore, future researches should focus on one hand on the determination of optimal protocols for radiation therapy, which not only kills tumor cells, but induces their immunogenicity making them recognizable immunologically. On the other hand studies should strive for optimal targeted immunotherapy, specific for given tumor type, and possibly with factors stimulating DCs population. In this way it will be possible to get the highest synergy that may result in beneficial abscopal effect. It is expected that the recently started clinical trials testing combination of immunotherapy with radiation in treatment of different malignances will bring a significant contribution to clarifying these aspects. The integrated researches of radiotherapists, radiobiologists and physicists concerning this problem would be desirable in order to develop appropriate recommendations and protocols, which could even change the existing concept of radiotherapy.

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