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Influence of Local Temporary Ischemia on Radiotherapy Effects

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ABSTRACT

Here we summarize and discuss the body of information concerning mechanisms and regulation of local short-duration preconditioning that may accompany experimental radiation therapy. Based on the results of our previous studies in vivo we investigated possible impact of local temporary ischemia on the effectiveness of ionizing radiation-based anticancer therapy. We used total body-irradiated or abdomen only-irradiated healthy rats. Chosen blood parameters, changes in bone marrow cytological picture as well as histological picture of the small intestine were used as toxicity markers. We found a significant transient modification in lipid peroxides, triglycerides, uric acid concentration, SOD isoenzymes activity in the rat serum, increased numbers of small intestine crypts and suppression of bone marrow polychromatic erythrocytes and polychromatic erythrocytes with micronuclei. No significant differences were observed in MnSOD isoenzyme activity in serum and in small intestine homogenates after local temporary ischemia, nor after irradiation, nor combined treatment. Some differences were observed in intestinal tissue CuZnSOD activities. Nevertheless, great variations in response to ischemia, radiation or combined treatment was noted concerning this parameter. Histological picture of the small intestine from ionizing radiation-treated rats has indicated a marked protection of crypt survival by local temporary ischemic preconditioning. Some of the ionizing radiation-caused toxic effects were reduced in animals treated with local ischemic preconditioning. Together, these results provide a new insight into development of a more effective anticancer therapy combining short-duration ischemia and ionizing radiation modality.

Keywords*: *Local Temporary Ischemia*, *Ionizing Radiation*, *Lipid Peroxidation

1. Introduction

The level of oxygen in tissues influences radiotherapy effects and the physical relationship between oxygen concentration and effectiveness of photon radiation is usually expressed as oxygen enhancement ratio (OER) [1]. Hypoxic theory, being one of the fundamental problems in radiotherapy is based on the thesis that cells characterized by low concentration of oxygen are more radioresistant. Cancer tissues are recognized as heterogeneous and are characterized by specific vasculature, unique microenvironment, and altered lymphatic drainage. Inflammatory processes adjacent to cancer tissue involve some cytokines like transforming growth factor β1 (TGF β1) and tumor necrosis factor α (TNF α). The main elements in inflammatory processes are macrophages which have been shown to release different biochemical molecules including nitrogen superoxide, nitric oxide or tumor necrosis factor α. It must be pointed out that hypoxic cancer cells surviving radiotherapy show improved oxygen uptake which has a stimulatory effect on their proliferation and probably contributes to cancer recurrence and dissemination. It was generally accepted for a long time that tissue response to ionizing radiation fractionated doses results from 5 R's of radiotherapy: Repair, Reassortment, Repopulation, Reoxygenation, and Radiosensitivity [2,3]. One of these elements, Reoxygenation has been attributed to the relationship between radiotherapy effectiveness and oxygen concentration in irradiated tissue. The method underlying the oxygen enhancement was explored in clinical trials, for example by the EORTC radiotherapy group which used a mixture of oxygen with low concentrations of carbon dioxide in cancer patients undergoing radiotherapy. In one strategy, carbon dioxide-induced peripheral vasodilatation is increased by orally administered nicotinamide [4]. However, vascular status did not show carbon dioxide dependency and the treatment used. Tissue oxygen concen-

tration also depends on the status of microcirculation and thus vessels' status also influences the effectiveness of radiotherapy. It has been known for a long time that in healthy organisms at rest all tissues are correctly oxygenated. Physical exercise increases oxygen consumption, in response to which the organism must supply more oxygen, resulting in higher heart rate and higher frequency of respiration. Diseases affecting gas exchange in tissues alter these compensatory mechanisms. Similar effects have been observed under clinical conditions such as: anemia, often accompanying cancer, and characterized by lower numbers of erythrocytes which diminishes oxygen supplementation to the tissues; respiratory diseases affecting gas exchange; left heart insufficiency leading to disturbed blood flow and local peripheral ischemia and hypoxia. However, it is now clear that hypoxia is characterized by a decrease of oxygen tissue concentration below normal level whereas ischemia as a phenomenon of insufficient blood supply in a tissue which results in low oxygen concentration in such tissue. It must be underlined that cellular consequences of hypoxia as well as ischemia are different because of different mechanisms involved [5,6]. Additionally, incomplete one-electron reduction of oxygen drives the formation of reactive molecular fragments called reactive oxygen species (ROS). The OER takes into account only stable oxygen concentration in irradiated tissue, but it is clear that this is only one of all possible clinical situations related to tissue hypoxia. Along this line, oxidative stress as well as hypoxic states are both present in tumors. This helps explain why tumors, like peripheral tissues, is well oxygenated in one situation and hypoxic in another. The question is whether temporary hypoxia or ischemia can change the effects of radiotherapy?

2. Ionizing Radiation

Ionizing radiation-based treatment is one of the basic diagnostic or curative clinical procedures applied in fighting against human cancers. This widely used treatment method is characterized, besides therapeutic benefit, by toxic post-radiation side effects. The latter are the consequence of indirect formation of oxygen- and nitrogen-reactive species but they also result from direct ionization of cellular structures which leads to alteration of multiple intra- and extracellular processes. Assuming the radical-mediated mechanism of ionizing radiation toxicity, it has been suggested that probably more than half of the DNA strand breaks, as well as cell deaths, results from radiolysis of intra and extracellular water [7]. Cellular lipids and proteins are susceptible to oxidative/nitrosative modifications. Lipid peroxidation yields lipid radicals, which form bulky adducts with carbonyl derivatives (protein oxidation products), and causes aggregation of protein complexes leading to proteasome inactivation [8]. Generation of free radicals following ionizing radiation exposure, or other pathological conditions, are known to be extremely noxious to both dividing and non-dividing cells [9]. Special attention is paid at present to free electrons (e^-_{hyd}) which have lost all of their energy and can react with DNA. Water radiolysis leads to the formation of hydrated electrons which do not cause intense DNA damage but before the end of that process the electrons enter their pre-hydrated state (e^-_{pre}). This electron state allows their reaction with DNA nucleotides leading to the formation of nucleotide anions subject to both dissociation or relaxation to the ground state *via* energy release [10]. It should be mentioned that low-energy secondary electrons are recognized in cancer radiotherapy as partially responsible for intensified tumor eradication when concomitantly applied with chemotherapeutic drugs [11,12]. Ionizing radiation-mediated oxidative/nitrosative modifications of cellular macromolecules elicit biological effects through activation of signaling pathways [13]. Ionizing radiation may also induce numerous lipid peroxidation products which possess second messenger function. Examples are major fractions of aldehydes produced by lipid peroxides (LPO) such as malondialdehyde (MDA) or trans-4-hydroxy-2-nonenal (4-HNE). MDA is also a crucial signaling molecule determining the formation of Schiff bases as a result of the reaction with Lys residues; it plays a major role in low-density lipoprotein (LDL) modification attracting macrophages that later form foam cells [14]. In our study of serum lipid peroxidation in γ-ray irradiated rats (2 Gy, total-body, once a day for three consecutive days), we observed that the level of lipid peroxidation end-products was increased. This effect correlated with progressive elevation of triglycerides in rat serum. Both of these time-dependent effects appear to coincide with a minimum concentration of uric acid in the serum of animals treated with radiation [15,16]. No significant differences were noted in the lipid peroxidation level after higher radiation doses (3 × 3 Gy and 3 × 5 Gy) were applied to the treated animals. Histological examination showed that the number of crypts in the mucosa of the small intestine after irradiation gradually decreased during the first few days. The eventual damage to polychromatic erythrocytes (PCEs) was examined and the numbers of micronucleated PCEs were analysed. A late increase in the proportion of micronucleated PCEs was noted after higher doses of radiation (3 × 5 Gy) but changes of PCEs with and without micronuclei did not reach statistical significance [17]. Thus, in the next series of experiments, the animals were irradiated in the abdomen area with 2 Gy doses for ten consecutive days. The results showed that the level of lipid peroxidation prod-

ucts in serum was almost doubled, compared to control one day post-irradiation. Normalization of their concentration down to the control level was observed on the fourth day after the termination of ionizing radiation treatment. Moreover, no regular changes in the lipid peroxidation level were seen in small intestine homogenates [18]. It has been found that one of the many effects of γ-rays irradiation is iron release from cellular deposit of ferritin [19]. The released iron ions are preferably sequestered by intracellular citrate or ADP which may cause autooxidation of Fe^{+2} and acceleration of free-radical reactions and mitochondrial lipid peroxidation [20]. One of the eminent events is genetic instability induced by oxidative stress and disturbed function of mitochondria because of damage sustained by subunits of electron transport chain proteins. Mitochondria seem to be the primary target of ionizing radiation exposure. Mitochondrial dysfunction contributes to the development of persistent genetic instability observed not only *in vitro* in cell cultures for many generation but also *in vivo* in animals progeny [21]. Our experimental design was to compare enzymatic activities of mitochondrial (MnSOD-2) and cytoplasmic (CuZnSOD-1) isoenzymes of SOD (EC 1.15.1.1) which, as concluded previously [22-24], modulate the effects of ionizing radiation. Our data indicate that fractionated irradiation of rat abdomen (10 × 2 Gy) induced a small, not significant activity increase of MnSOD-2 isoenzyme from intestinal homogenate during the first few days of post-treatment. Contrary to the early increase of MnSOD-2 activity observed in the small intestine, a significant decrease in serum was noted during the first few days after conclusion of fractionated irradiation. Contrarily, we observed high variability of cytoplasmic CuZnSOD-1 isoenzyme activity in serum and also in small intestine homogenates. It should be therefore considered that various exogenous and endogenous factors affecting radical reactions during and after exposure to ionizing radiation probably determine the character of tissues response.

3. Ischemic Preconditioning

Several studies have shown that the basic endogenous factor involved in vasodilatation is nitric oxide secreted by endothelial cells and probably affecting radiotherapy effectiveness, depending on vascular endothelial activity. The growing number of findings support the importance of nitric oxide in biochemical signaling pathways initiated by the vascular endothelium activity [25-27]. It has been early recognized that blockade of endogenous nitric oxide synthesis alters pro-survival effects induced by ischemic preconditioning [26]. From the experiments using nitric oxide synthesis inhibitors it was concluded that ischemic preconditioning depends on the activity of vascular endothelium. Further, stimulation of nitric oxide increase gives rise to pro-survival effects and stimulates the accumulation of inducible nitric oxide synthase (iNOS) demonstrating a positive feedback between these two pathways. Studies on modulation of endogenous nitric oxide level by inhibitors or stimulators of its synthesis provided evidence that activities of the endothelium are a necessary element of C3H mice protection against whole-body ionizing radiation toxicity [28]. Exposure of dogs to short-term myocardial infarction induced by temporary clamping of the coronary artery in most cases induced partial protection against myocardial infarction [29]. The results clearly showed that the field of infarction was reduced close to 70% in comparison with that in groups of animals without ischemic preconditioning. It seems that experimental clamping of the right coronary artery may protect against infarction by total occlusion of the left coronary artery. Whether this mechanism is unique to defined regions of the body or applies to the whole body needs further experimental confirmation. Pro-survival effects of this phenomenon are also observed in clinical practice during coronary angioplasty when the stenocardiac pain during the first occlusion of the coronary artery was more severe than during subsequent occlusions. A likely pro-survival effects of ischemic preconditioning have also been observed in studies with skeletal muscles [30,31], liver [32], brain [33], jejunum [34], kidneys [35]. The temporary clamping of the renal artery resulted in prevention of myocardial infarction [36]. Analysis of data obtained investigating cells injured by brief periods of ischemia/reperfusion have been indicated free radicals toxicity [37,38]. It is well known that in ischemic diseases intensity and duration of hypoxia represents risk of lethal damage. Contrary to the deleterious effects of long lasting hypoxia, short periods of sub-lethal stress such as ischemia/reperfusion can be used as pre- or post-conditioning based technique to prevent their adverse effect [29]. This way of treatment was termed preconditioning with ischemia and is characterized by a bimodal protection schedule. The first (early) phase is observed between 2 to 4 hours and defined as "classic preconditioning", the second (late) phase appears between 12 to 24 hours and extends up to 4 days, named "second window of protection—SWOP" [26,39]. The suggested pathways leading to reduction of ischemia/reperfusion-induced injury are the lowering of oxidative stress [40], leukocyte adhesion [41], intracellular Na^+ accumulation [42], conversion of xanthine dehydrogenase to oxidase [43] and opening of mitochondrial ATP-dependent potassium channels [44]. Our own recent experimental results coincide with the second phase of maximum radioprotection [15-18]. Wu *et al.* investigated critical importance of the threshold

level of ischemic preconditioning status [45]. Its protective effects included amelioration of mitochondrial respiratory dysfunction, reduced ROS generation, suppressed mitochondrial GSH oxidation whereas harmful effects resulted from mitochondrial respiratory damage and increase in ROS generation, mitochondrial GSH oxidation and lipid peroxidation increase; they should be both carefully evaluated under various conditions of administration. Evidence has been presented by several authors that ROS in high concentration are toxic [46], but low levels of ROS have a stimulating effect on cellular defense mechanisms and prevent tissue injury [47,48]. In this manner, in ischemia-reperfusion injury, it leads to tissue damage due to increased concentration of cytosolic iron potentiated by a six-fold increase in intracellular citrate level and damage to mitochondrial ultrastructure; this may cause some reduction of oxidative phosphorylation processes and disturbances in the physiological level of high-energy phosphates [49]. Several authors found that the small intestine mucosal mitochondrial respiratory function was injured by ischemia/reperfusion because of ROS generation. The evidence for this stems from the results of mitochondrial dehydrogenase-dependent assay involving conversion of MTT to its formazan derivative MTT-FZ, from increased mitochondrial GSSG levels and decreased GSH and GSH/GSG ratio and from the rise of the mitochondrial lipid peroxidation level [45,50]. It has been found that moderate tissue oxidative stress induced by ionizing radiation preconditioning provokes resistance to a subsequent, much stronger stress, enforced by application of ischemia/reperfusion. The suggested mechanism of this action involves superoxide level increase which stimulates MnSOD isoenzyme activity, as well as expression of HSP27 protein. This effect could be ameliorated by administrating manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin radical scavenger which lowers superoxide formation and MnSOD-2 expression. However, our results indicate that local temporary ischemic preconditioning had no influence on MnSOD-2 activity in serum and that there were no differences in the activity of that isoenzyme in small intestine tissue homogenates. Such an effect suggests that cellular resistance against radiation induced by local temporary ischemia depends on MnSOD-2 expression and activity. It thus seems probable that the superoxide radical reacts rapidly with nitric oxide, leading to the formation of peroxynitrite. The resulting radical anion-driven nitration may cause inactivation of MnSOD-2 isoenzyme [51] which is tightly linked with ischemia and reperfusion [52]. In addition, it was striking that manipulations such as irradiation only, irradiation with preconditioning and/or preconditioning ischemia initially diminished serum activity of the cytoplasmic CuZnSOD-1 isoenzyme. Some fluctuations of this isoenzyme activity were observed in intestinal tissue, although differences were not significant. It has been suggested that lack of influence of ischemic preconditioning upon SOD activities would appear related to endothelial protection. Endothelial cells might activate a SOD-like anti-$^{\bullet-}O_2$–mechanism which attenuates the $^{\bullet-}O_2$ burst while simultaneously increasing the $^{\bullet}OH$ burst. Thus, specific activity of SOD enzyme might not have been detectable because it was limited to a small, although functionally important, enzyme fraction, like that bound to glycocalyx [53]. Along this line, superoxide radical anions formed can dismutate into oxygen and hydrogen peroxide and the later then reacts with transition metals ions to create hydroxyl radicals [54]. A significant decrease, not only in SOD but also in glutathione peroxidase (GSHPx) activities has been reported, contrary to strong elevation of catalase activity [55]. Histological picture of the small intestine indicates that in the control group of rats, compared to rats with applied ischemia, the number of crypts in mucosa showed only small fluctuations. The difference in the number of crypts in irradiated animals with and without local temporary ischemia was statistically significant [18]. Oxidative damage following irradiation, as well as ischemia, may create end-products of lipid peroxidation which may activate caspases, subsequently leading to increased DNase activation and DNA degradation leading to apoptosis [56].

4. Combination of Ischemic Preconditioning and Ionizing Radiation

A recent approach to improve the clinical value of some anticancer treatments is to prevent their adverse toxic stress effects with temporary treatment using the same or different inducer. This kind of preconditioning mode of action has been characterized in many models of protection against oxidative stress-inducing stimuli such as ionizing radiation exposure [17]. Such trials using segmental liver ischemic preconditioning, as well as liver reperfusion during intraoperative radiotherapy (IORT), were successfully undertaken against ionizing radiation toxicity in animals. Ischemic preconditioning (IP) was found to reduce histological, laboratory and flowmetry alterations caused by high single doses of ionizing radiation, especially during ischemia, which are suggested to be involved in post-radiation liver injury [57]. In the course of our study on the dose- and time-dependent events induced by a combined treatment, local ischemic preconditioning induced by clamping the rat's tail base (3 × 5 min with 1 min pause) followed by whole body ionizing radiation (2 Gy once a day for three consecutive days), we observed significant modification of peroxidizing response after total body irradiation in the pres-

ence of local temporary ischemia. This led us to the assumption that local ischemic preconditioning *in vivo* prevents the peroxidizing effects of total body irradiation. To check this possibility, a relationship between the concentration of thiobarbituric acid-reactive substances (TBA-RS), triglyceride and uric acid levels in serum of treated animals were examined. Parallel estimation of uric acid concentration indicated that the state of these time-dependent effects measured by TBA-RS and triglyceride levels appeared to coincide with low levels of serum uric acid. The relationship between TBA-RS and triglycerides revealed that their slowly progressive increase in ischemia preconditioning preceding irradiation correlated with progressive decrease of uric acid concentration. This suggests a much more intense use of this antioxidant to prevent the over-production of end products of lipid peroxidation and also oxidative transformation of low-density lipoprotein (LDL). The significance of this relation, if any, is difficult to explain at present [15]. Thus, in the following series of experiments, we studied the effect of local temporary ischemia on radiation-induced lipid peroxidation, superoxide dismutase isoenzymes' activities and intestinal crypt number. The animals were irradiated in the abdomen area with 2 Gy doses for ten consecutive days and local temporary ischemia was induced by clamping the tail base before each irradiation. Our results indicate that application of local temporary ischemia diminished the concentration of ionizing radiation-induced lipid peroxides in serum. In contrast, there was no evident effect on the level of lipid peroxides in tissue homogenates in any investigated groups of animals. Local temporary ischemia had no influence on MnSOD-2 activity in serum and there were no differences in the activity of this isoenzyme measured in tissue homogenates and in both irradiated groups the behavior of this isoenzyme was similar. Some fluctuations of CuZnSOD-1 isoenzyme activity in intestinal tissue were observed but the differences were not significant [18]. Antioxidant enzymes SOD, catalase and radical scavengers attenuating the post-ischemic lesions of the mucosa prevent reperfusion damage of the intestine. This suggests the role for therapeutic maneuvers that focus on counteracting oxidative damage under conditions involving oxidative stress. The putative underlying mechanism may involve direct molecular recombination of free radicals induced both by brief ischemia and ionizing radiation. It seems that ischemia may prevent post-irradiation damage when the irradiation starts during the reperfusion period. We have previously shown that the protective effect of ischemic preconditioning disappears with the use of higher doses of irradiation [17]. Thus, it may be concluded that the nature of ischemic preconditioning probably limits its capacity to buffer massive radicals' burst created by high doses of ionizing radiation. However, it was reported that ionizing radiation doses up to 50 Gy of intraoperative radiotherapy (IORT) can be tolerated if they are delivered during ischemia and also if they are combined with ischemic preconditioning. Ischemia was found to reduce the massive histopathological changes in IORT-treated rats which probably reflects smaller amounts of free radicals formed during ischemia [57]. Our experiments indicate that changes in local ischemic preconditioning prevent partial and even total body post-irradiation toxicity in rats [15-18]. In the case of radiotherapy, the timing of protection produced by ischemic preconditioning is probably the same as that in protection against infarction.

5. Conclusions

Based on the literature review, the results of our experiments relating local temporary ischemic preconditioning with the prevention of ionizing radiation toxic side effects to whole body or its parts can be discussed. Since the conditions of radiation treatment employed by us, i.e. relatively low dose and/or short exposure, were the same as in typical clinical practice, the observation that some radiation toxicity symptoms in non-ischemic tissues can be ameliorated by local temporary ischemia is obviously valuable. However, the introduced research model has many gaps that need to be investigated and improved. These results were proposed to stimulate collaborative studies between radiotherapists and radiobiologists to explore this field which is obviously under investigated and full of knowledge gaps. If local temporary ischemic preconditioning is found to have a significant role in decreasing ionizing radiation toxicity, and if it can prevent tissue and organ damage, it may become clinical reality, like 3D conformal radiation therapy (3DCRT) or intensity-modulated radiation therapy (IMRT).

6. Conflict of Interest Notification

Authors read and approved the final version of manuscript and declare that they have no competing or conflicting interest.

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