SYNTHÈSE

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Hypoxic tumor cell radiosensitization: role of the iNOS/NO pathway

Radiosensibilisation de cellules tumorales hypoxiques par production endogène de monoxyde d'azote

Mark De Ridder
Gretel Van Esch
Benedikt Engels
Valeri Verovski
Guy Storme

Oncologisch centrum UZ Brussel, Department of Radiation Oncology, Laarbeeklaan 101, B-1090 Brussels, Belgium
<Mark.Deridder@uzbrussel.be>

Résumé. L'hypoxie est une propriété typique du micromilieu tumoral et une cause principale de radiorésistance. Pendant ces dernières dizaines d'années, plusieurs stratégies ont été développées pour améliorer l'oxygénation de tumeurs solides au cours du traitement radiologique, par exemple la respiration d'oxygène sous conditions hyperbares (3 atmosphères) et l'amélioration de la perfusion tumorale par nicotinamide, qui est utilisée en combinaison avec l'administration de carbogène et un traitement radiologique hyperfractionné pour inhiber la repopulation des cellules tumorales (ARCON). D'autres stratégies sont l'utilisation de radiosensibilisateurs qui miment l'effet d'oxygène au niveau de l'ADN, comme les nitro-imidazoles, et l'utilisation de drogues bioréductives comme la mitomycine C et la tirapazamine. Une méta-analyse des études randomisées qui ont évalué une forme de radiosensibilisation hypoxique montre un net avantage au niveau local et de la survie, surtout pour les tumeurs ORL et du col utérin. Pourtant, aucune des ces stratégies est utilisée en routine clinique, pour raisons pratiques et suite à leur toxicité. Nous avons développé une stratégie alternative, qui exploite les propriétés du micromilieu tumoral pour cibler la radiosensibilisation. La plupart des tumeurs solides expriment la synthase inductible de NO (iNOS) qui produit une quantité importante de monoxide d'azote (NO). Cette enzyme est induite, entre autres, par les lipopolysaccharides bactérielles (LPS) et par le lipide A, une de ses dérivées qui est utilisée dans les vaccins. Nous avons démontré que la production intracellulaire de NO par iNOS radiosensibilise des cellules tumorales hypoxiques à des concentrations extracellulaires non toxiques. De plus, la transcription d'iNOS est soutenue par l'hypoxie chronique et par des cytokines pro-inflammatoires comme l'interféron γ. Pour cela, nous avons proposé l'infiltrat tumoral pro-inflammatoire comme nouvelle cible pour des stratégies de radiosensibilisation et avons identifié deux modes d'action : 1) les cellules de l'infiltrat tumoral pro-inflammatoire (macrophages, cellules T/NK) peuvent être une source de cytokines qui induisent iNOS dans les cellules tumorales avoisinants ; 2) des macrophages tumorales proprement stimulées peuvent devenir une source intratumorale importante de production de NO. Nos recherches actuelles sont concentrées sur l'intégration de stratégies immunitaires dans des protocoles de radiosensibilisation. ▲

Mots clés : hypoxie, radiosensibilisation, synthase inductible de monoxide d'azote, infiltrat tumoral pro-inflammatoire, lipide A

Abstract. *Hypoxia is a common feature of the tumor microenvironment and a major cause of clinical radioresistance. During the last decades, several strategies to improve tumor oxygenation were developed such as breathing high oxygen content gas under hyperbaric conditions (3 atmosphere) and improving tumor perfusion by nicotinamide, in combination with carbogen breathing and accelerated radiotherapy to counteract tumor repopulation (ARCON). Other strategies to overcome hypoxia induced radioresistance are the use of hypoxic cell radiosensitizers, which mimic oxygen and enhance thereby radiation damage (e.g. the nitroimidazoles) and bioreductive drugs, which undergo intracellular reduction to form active cytotoxic species under low oxygen tension (e.g. mitomycin C and tirapazamine). A meta-analysis of all randomized trials in which some form of hypoxic modification was performed, showed an*

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improved local control and survival, especially in cervix and head-and-neck cancer. Nevertheless, none of the discussed strategies are used in clinical routine because of feasibility and toxicity issues. We developed an alternative strategy that takes advantage of the microenvironment of solid tumors for tumor specific radiosensitization. The inducible isoform of nitric oxide synthase (iNOS) may be induced by bacterial LPS or its derivate lipid A, is expressed by a variety of solid tumors and generates NO at high rates inside tumor cells. This local production of NO results in efficient hypoxic tumor cell radiosensitization, at non-toxic extracellular concentrations of NO. In addition, iNOS is transcriptionally upregulated by hypoxia and proinflammatory cytokines such as interferon-γ. Hence, we proposed the proinflammatory tumor infiltrate as a new target for radiosensitizing strategies and identified two mechanisms: First, tumor associated immune cells (macrophages, T/NK-cells) are a source of mediators that may induce the iNOS/NO pathway inside tumor cells. Second, tumor associated macrophages can produce high levels of NO that may radiosensitize bystander tumor cells. Our ongoing research is focused on combining immunostimulatory and radiosensitizing strategies. ▲

Key words: *hypoxia, radiosensitization, inducible isoform of nitric oxide synthase (iNOS), proinflammatory tumor infiltrate, tumor associated macrophages, lipid A*

Oxygen and tumor cell radioresponse

The response of cells to ionizing radiation is strongly dependent upon oxygen, as illustrated for EMT-6 mouse mammary carcinoma cells *(figure 1)*. In this figure, the cell surviving fraction is shown as a function of radiation dose, administered under aerated conditions and under anoxic conditions, which were achieved in a nitrogen based atmosphere. This allows us to determine the oxygen enhancement ratio (OER), which is classically calculated at a surviving fraction of 0.1 (OER = radiation dose in anoxia/radiation dose in air). For most mammalian cells, the OER for γ-irradiation is around 3. The mechanism responsible for the enhancement of radiation damage by oxygen is called "the oxygen fixation hypothesis". Briefly, when radiation is absorbed in a biological material, free radicals are produced *(figure 2)*. These radicals can be produced directly in the DNA, or indirectly in water molecules and diffuse far enough to damage the DNA. It is the fate of the free radicals produced in the DNA (DNA•) that determines the biological damage. In the presence of oxygen, DNA-OO• is produced and further processed to DNA-OOH. This results in a changed chemical composition of the DNA and thus fixation of the DNA damage. In the absence of oxygen, DNA• can react with H^+, restoring the DNA in its original form. To exert its effect on radiosensitivity, oxygen must be present during or within milliseconds after radiation [1, 2].

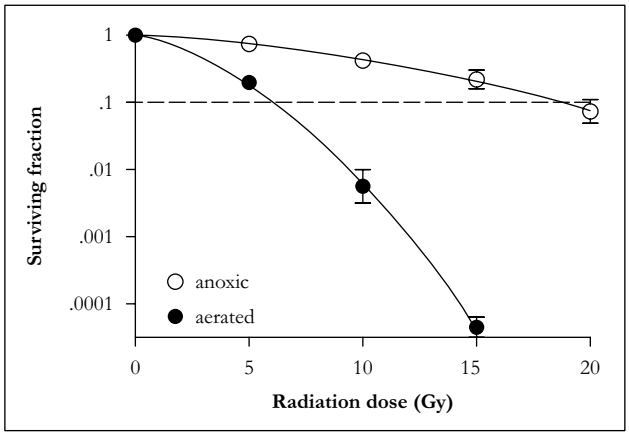

Figure 1. Survival cells of EMT6 cells in aerated and anoxic conditions. In order to obtain a surviving fraction of 0.1, a radiation dose of 18 Gy is required in anoxic and 6 Gy in aerated conditions, resulting in an OER of 3.

Tumor oxygenation and radiotherapy outcome

The most widely accepted technique for assessing tumor oxygenation is the measurement of tissue oxygen tension (pO_2) by polarographic needle electrodes (Eppendorf electrodes), which are introduced in the tumor and moved forward by an automatic stepping motor [3, 4]. This approach allows to measure the pO_2 along several electrode tracks. Generally, minimally 50 pO_2 readings are performed, from which the "median pO_2" is calculated. Tumor hypoxia may also be expressed as the "hypoxic proportion" (percentage of pO_2 readings < 2.5 or 5 mmHg) or as the "hypoxic subvolume" (percentage of pO_2 values below 2.5 or 5 mmHg multiplied by the total tumor volume). These parameters have been used to study the relation between tumor oxygenation on radiotherapy outcome in cervical carcinomas and head-and-neck tumors essentially (*table 1*). In summary, these studies show

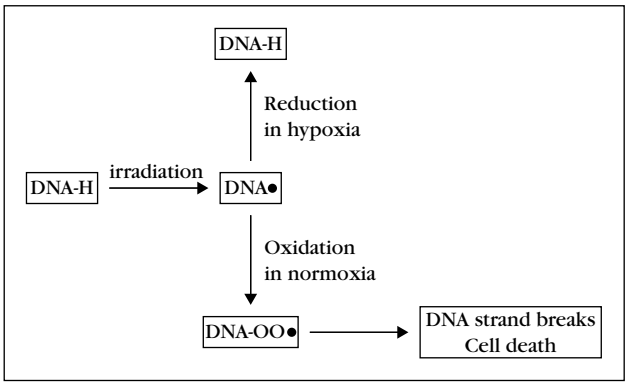

Figure 2. The oxygen fixation hypothesis.

Table 1. The prognostic significance of pre-treatment tumor oxygenation. All reported patients were treated by radiotherapy or chemoradiotherapy with curative intent. Hypoxia had a significant detrimental effect on the mentioned endpoints. S: survival; RFS: relapse free survival; DFS: disease free survival; LC: locoregional control) [73-82]

Publication	N	Parameter	pO_2 (mm Hg)	Follow up (months)	Endpoints
Cervix cancer					
Höckel et al., 1993	31	Median pO_2	< 10	19	S, RFS
Fyles et al, 1998	74	Hypoxic fraction	< 5	14	DFS
Knocke et al, 1999	51	Median pO_2	< 10	36	DFS, LC
Rofstad et al, 2000	32	Hypoxic subvolume	< 5		S, DFS, LC
Sundfor et al, 2000	40	Hypoxic subvolume	< 5	33	S, DFS, LC
Head-and-neck cancer					
Nordsmark et al, 1996, 2000	70	Hypoxic fraction	< 2.5	24	LC
Brizel et al, 1997	28	Median pO_2	< 10	12	DFS
Stadler et al, 1997	59	Hypoxic subvolume	< 5	8	S
Rudat et al, 2001	134	Hypoxic fraction	< 2.5	2	S

that cervix carcinomas and head-and-neck cancers are poorly oxygenated, with a median pO_2 of about 10 mmHg, and that low pO_2 before radiotherapy is the most significant adverse prognostic factor. Low and highly heterogeneous levels of oxygenation were found in other types of solid tumors as well, and are considered to be the most important signature of tumor microenvironment [5]. The etiology of tumor hypoxia will be discussed in the next paragraphs.

Pathophysiology of tumor hypoxia

The growth and survival of cells in solid tumors is dependent on the adequate supply of oxygen and nutrients, which diffuse from the blood vessels and are consumed by the tumor cells. In order to meet their increasing oxygen and nutrient demand, tumors develop their own blood supply. However, the neovasculature is morphologically and functionally abnormal, and is generally unable to meet the increasing demands, resulting in a diffusion limited chronic hypoxia. As a result we get a so-called "corded structure", which was first described by Thomlinson and Gray back in 1955, based on their observations of a metabolic oxygen gradient relative to blood vessels [6]. Tumor cells can roughly be divided in two categories with regard to their oxygenation and radiosensitivity. Cells lying near the capillaries, within the diffusion distance of oxygen (± 100 µm) are well oxygenated and radiosensitive. Cells lying at the edge and beyond the diffusion distance are hypoxic and radioresistant. We modelled this metabolic oxygen gradient in our radiosensitizing experiments by irradiating tumor cell in "micropellets". Essentially, $0{,}5 \times 10^6$ tumor cells in 100 µl of medium were placed in conical plastic tubes and pellets (± 300 µm thick) are produced by centrifugation at 300 g for 5 min, and kept on ice. Metabolic oxygen depletion in pellets is induced by a 3 min incubation at 37 °C prior to radiation. This model provides an oxygen enhancement ratio of at least 2.5, which indicates a mean level of oxygenation below 0.5 % [7].

In the late 1970s, Brown postulated that another type of hypoxia, being transient in nature, existed in solid tumors as well [8]. This was later confirmed and shown to result from temporary cessations in blood flow [9]. The mechanisms responsible for the intermittent closure of tumor blood vessels are not entirely understood yet. They may include plugging of blood vessels by blood cells or by circulating tumor cells, collapse of blood vessels in regions with high interstitial pressure, spasms and spontaneous vasomotion in incorporated host arterioles. This temporary closing of blood vessels results in perfusion limited acute hypoxia.

Strategies to overcome hypoxia induced radioresistance

Horsman and Overgaard performed a *meta-analysis* of all randomized trials in which some form of hypoxic modification was performed in solid tumors undergoing radiotherapy with curative intent [10]. They identified 91 trials, reporting more than 11 000 patients in total. The trials involved hypoxic cell radiosensitizers (n = 53), hyperbaric oxygen (n = 31), a combination of both (n =1), oxygen or carbogen breathing (n = 5) and blood transfusion (n = 1). Tumor sites were head-and-neck (n = 29), cervix (n = 20), bladder (n = 16), brain (n = 13), lung (n = 10), esophagus (n = 2) and mixed (n = 1). Overall, hypoxic modification significantly improved local control (odds ratio 1.29) and survival (odds ratio 1.19), without significantly affecting the rate of distant metastasis or radiotherapy related complications. The improvement remained significant when evaluating the trials with hypoxic cell radiosensitizers and hyperbaric oxygen separately. Analysis according to site showed only a significant improvement for head-and-neck and cervix cancer.

Improving tumor oxygenation

One of the earliest clinical attempts to eliminate hypoxia induced radioresistance involved patients breathing high oxygen content gas under hyperbaric conditions (3 atmosphere) [11]. The largest clinical trial with hyperbaric oxygen has been conducted by the British Medical Research Council, which randomized 1669 patients between radiotherapy with or without hyperbaric oxygen [12]. Hyperbaric oxygen significantly improved both survival and local control after radiotherapy for head-and-neck tumors and for advanced carcinomas of the cervix. In carcinomas of the bronchus there seemed to be some improvement in survival but this was not statistically significant. In carcinoma of the bladder hyperbaric oxygen showed no benefit.

Another way to improve the oxygen availability is to optimize the hemoglobin concentration. Indeed, it is well established that hemoglobin concentration is an important prognostic factor for the response to radiotherapy, especially in head-and-neck tumors, cervix carcinomas and bladder cancer [13, 14]. Generally, anemic patients have a reduced locoregional tumor control and survival. Based on these observations, the effect of transfusion in anemic patients was evaluated in the Danish head-and-neck cancer studies (Dahanca) 5, 6 and 7 [15, 16]. These trials confirmed the prognostic significance of the hemoglobin concentration. In addition, patients with low hemoglobin level were randomized to receive blood transfusions or not. Surprisingly, correction of anemia did not result in a significantly improved local control. This is supported by data from animal experiments, showing only a clear benefit of blood transfusion to mice when the interval between transfusion and irradiation is shorter than 24 h. This can be explained by the hypothesis that solid tumors posses adaptation mechanisms to anemia, which counteract the beneficial effects of transfusion on radiotherapy outcome [17]. The use of erythropoietin is another approach to correct anemia, and was evaluated in randomized placebo controlled trials in cervix and head-and-neck cancer [18, 19]. These trials demonstrated an impaired local control in the erythropoietin arm. Hence, we consider anemia to be a surrogate marker for poor prognosis rather than a pathophysiological cause of radioresistance. We do not support the general idea that non-symptomatic anemia should be corrected prior to radiotherapy.

Prevention of perfusion-limited acute hypoxia

Another way to improve tumor oxygenation is to prevent perfusion-limited acute hypoxia. Experimental studies showed that nicotinamide, a vitamine-B3 analog, can effectively radiosensitize murine tumors. In vitro studies suggested that this enhancement of radiation damage was the result of an inhibition of repair mechanisms. However, mouse studies demonstrated that the primary mode of action involves a reduction in tumor hypoxia. This was explained by a significant decrease in transient occlusions of blood vessels and thereby of perfusion-limited acute hypoxia. Nicotinamide was evaluated in clinical trials primarily in combination with carbogen breathing to decrease chronic hypoxia, and with accelerated radiotherapy to counteract tumor repopulation. Kaanders *et al*. recently reported such a phase II trial of *ARCON* (Accelerated Radiation CarbOgen and Nicotinamide) in 215 patients with advanced head-and-neck cancer. The 3-year locoregional control rates were excellent (e.g. 80 % for T3/4 carcinomas of the larynx), and the toxicity was acceptable [20]. Further evaluation of ARCON is necessary, to define in which clinical setting it might be beneficial.

Hypoxic cell radiosensitizers

In 1969, Adams and Cooke introduced the concept of hypoxic cell radiosensitizers, which are chemicals that mimic oxygen and enhance thereby radiation damage [21]. They demonstrated that the efficiency of radiosensitization is directly related to the electron affinity. These compounds are, in contrast to oxygen, not metabolized by tumor cells and can therefore diffuse further away from the capillaries than oxygen. Because these drugs mimic the effect of oxygen, they are supposed not to increase the toxic effects of radiotherapy to the well oxygenated normal tissues surrounding the tumor. The most widely studied group of hypoxic cell radiosensitizers are the nitroimidazoles. Laboratory experiments showed complete reversal of hypoxia induced tumor cell radioresistance by misonidazole without increasing the response of aerated tissues. These encouraging results led to a boom of clinical trials exploring the clinical radiosensitizing potential of misonidazole in the late 1970s. However, the results of these clinical trials have been generally disappointing. The most important factor underlying the failure of misonidazole to achieve useful advantage is undoubtedly the low plasma concentrations achievable with the permitted dose of this neurotoxic drug [22].

Nevertheless, a significant benefit was seen in some trials, such as the Dahanca 2 trial [23]. In this trial 626 patients with pharynx and larynx carcinoma were randomized to two different split-course radiation regimens and given either misonidazole or placebo during the initial 4 weeks of treatment. Overall, the misonidazole treated group did not have a significantly better control rate than the placebo group. However, a significant benefit was found in patients with pharynx carcinomas. Misonidazole induced significant peripheral neuropathy in 26 % of the treated patients, whereas other drug related side effects were minimal. In the Dahanca 5 trial, a less toxic nitroimidazole compound, nimorazole (Naxogin®), was used [15]. Briefly, 422 patients with invasive carcinoma of the supraglottic larynx and pharynx were randomized to receive nimorazole or placebo, in association with conventional primary radiotherapy. With a median follow up of 112 months, the nimorazole group showed a significantly better locoregional control rate than the placebo group and a lower cancer-related death rate, without major side-effects. As a consequence, nimorazole has become part of the standard treatment schedule for head-and-neck tumors in Denmark.

More recently, a number of nitroimidazole derivatives, such as pimonidazole, EF3 and EF5, were developed as hypoxia markers. These compounds undergo intracellular reduction, bind mainly to thiol containing proteins and are thereby trapped in hypoxic cells. Specific antibodies allow their detection by immunohistochemistry, immunofluorescence and flow cytometry. In head-and-neck squamous-cell carcinoma, piminidazole staining has been demonstrated to correlate with decreased 2-year local control rates after radiotherapy [24]. Evans *et al*. demonstrated that EF5 binding correlates with pO_2 measurements by Eppendorf needle electrodes, and may be predictive for recurrence in brain tumors treated by surgery and postoperative radiotherapy [25, 26]. Another possible method to detect nitroimidazole adducts is the use of radioactive tracers. This approach has some intrinsic advantages, including its non-invasiveness, the possibility to evaluate the whole tumor volume and its repeatability. In addition, image fusion techniques and the use of intensity modulated and image guided radiotherapy allow to delineate hypoxic radioresistant sub-target volumes for delivering a partial tumor boost [27, 28]. [^{18}F]EF3 is such a promising tracer. It can be detected by PET, and is currently being evaluated in a phase I clinical trial in head-and-neck cancer patients at the St-Luc University Hospital in Brussels [29].

Dynamic contrast enhanced MRI offers an alternative for delineating hypoxic subvolumes [30, 31].

Bioreductive drugs

Selective killing of hypoxic cells can also be achieved with bioreductive drugs, which undergo intracellular reduction to form active cytotoxic species under low oxygen tension. Two groups of bioreductive drugs, the quinines (e.g. mitomycin-C) and the organic nitroxides (e.g. tirapazamine), have been introduced in clinic.

Laboratory studies demonstrated that the activation of mitomycin-C by bioreduction under hypoxic conditions, leads to the formation of products that crosslink DNA and produce thereby cell killing. Animal studies indicated that mitomycin-C can be used in combination with radiotherapy to kill the hypoxic fraction of a solid tumor, while radiotherapy alone eradicates the oxygenated fraction. This led to the clinical evaluation of mitomycin-C in combination with radiotherapy in head-and-neck cancer patients [32]. Pilot studies reported an improvement in local tumor control, without an increase in normal tissue toxicity. However, these preliminary findings were not confirmed by larger clinical trials. There are two obvious reasons for this failure. First, mitomycin-C has a relative small differential killing effect between aerobic and hypoxic cells. Second, in most clinical trials mitomycin-C is only administered once or twice during the course of radiotherapy. Attempts to find more efficient quinones led to the development of porfiromycin and EO9, which are currently being evaluated.

The most promising group of bioreductive drugs are the organic nitroxides, of which tirapazamine is the lead compound. This molecule is metabolized by intracellular reductases to form a transient oxidizing radical that can be efficiently scavenged by molecular oxygen in aerated tissues to reform the parent compound. In the absence of oxygen, the oxidizing radicals abstract protons from the DNA to form DNA radicals and finally strand breaks. Tirapazamine shows substantial selective cytotoxicity for anoxic cells; it is approximately 100-fold more cytotoxic to anoxic than to oxygenated cell cultures. Animal studies showed a beneficial effect for combinations of tirapazamine with cisplatin and with radiation. Clinical phase II and III trials of tirapazamine combined with cisplatin in malignant melanoma and non-small cell lung cancer suggested a synergistic effect. Phase I and II trials of tirapazamine combined with radiation in patients with advanced head-and-neck cancer reported high local control rates and acceptable toxicity [33, 34]. The efficacy and toxicity of tirapazamine concurrent with radiotherapy is further being evaluated in several clinical trials.

NO-based radiosensitizing strategies

As early as 1957, Howard-Flanders showed that the authentic NO-gas is an efficient radiosensitizer of hypoxic bacteria, and postulated fixation of radiation induced DNA damage, thus mimicking the effects of oxygen on DNA lesions, as primary mechanism [35, 36]. An alternative mechanism might be interaction of NO with iron-sulphur containing enzymes, resulting in inhibition of mitochondrial respiration and sparing of the natural radiosensitizer oxygen [37].

In the early 1990s, Mitchell *et al.* evaluated the radiosensitizing potential of the NONOates, which have a X-[N(O)NO]-

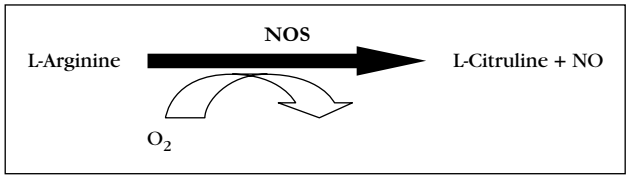

Figure 3. Conversion from L-arginine to L-citruline and NO by nitric oxide synthase (NOS).

structure. When X is a secondary amine group, 2 molecules of NO per molecule of NONOate are spontaneously generated when dissolved in aqueous media. The NO level produced by the NONOates can easily be controlled, since the generation of NO is constant, predictable and independent from the pO_2. Mitchell *et al.* demonstrated that spontaneous release of NO by diethylamine nonoate (DEA/NO) and dipropylamine nonoate (PAPA/NO) radiosensitizes hypoxic Chinese hamster V79 lung fibroblast to a similar extent as oxygen [36]. Griffin *et al.* reported comparable activity for DEA/NO and spermine nonoate (SPER/NO), with enhancement ratios of 2.8-3.0 in hypoxic SCK mammary carcinoma cultures [38]. However, the generation of NO by the NONOates is not tumor selective and their *in vivo* application would result in high NO levels in the circulation, causing vasoactive complications (septic-like shock).

To be able to decrease the concentration of NO in the circulation, our laboratory explored the possibility to exploit the hypoxic tumor microenvironment for selective generation of NO by bioreduction. We showed that bioreduction of sodium nitroprusside (SNP) and S-niroso-N-acetyl-penicillamine (SNAP) results in generation of NO and radiosensitization of hypoxic mouse mammary carcinoma and human pancreatic cancer cells [7, 39]. In our model of metabolic induced hypoxia, the radiosensitizing effect of NO was close to that of oxygen, while no radiosensitization was observed in aerobic cells. In an attempt to further decrease the extracellular concentration of NO, we decided to explore the possibility to endogenously generate NO inside tumor cells. Our laboratory was the first to demonstrate that the inducible isoform of nitric oxide synthase (iNOS), activated by cytokines (IL1β + IFNγ) in aerobic conditions, is capable of radiosensitizing tumor cells through endogenous production of NO *(figure 3)* [40]. We found that the iNOS pathway has a serious advantage over NO-donors, since a comparable level of radiosensitization was achieved at a 10 to 30-times lower extracellular NO background, which is favorable for *in vivo* applications.

Regulation of the iNOS promotor

To choose an optimal, clinically relevant iNOS induction schedule, we studied the transcriptional activation of iNOS in murine EMT-6 mammary carcinoma cells. It is well documented that full activation of the iNOS gene requires cooperation of two promoter regions, located from - 40 to - 300 bp (region I) and – 900 to – 1100 bp (region II) upstream of the TATA box *(figure 4)*. Standard combinations like IFNγ + IL1β, prime iNOS activation *via* an interferon-responsive element (ISRE) or an IFNγ activated site (GAS) in region II. To maintain a high iNOS transcription rate, a second stimulus toward region I is required and can be provided by IL1β or LPS via the NFκB signaling pathway [41]. This pathway involves kinase IKKα-

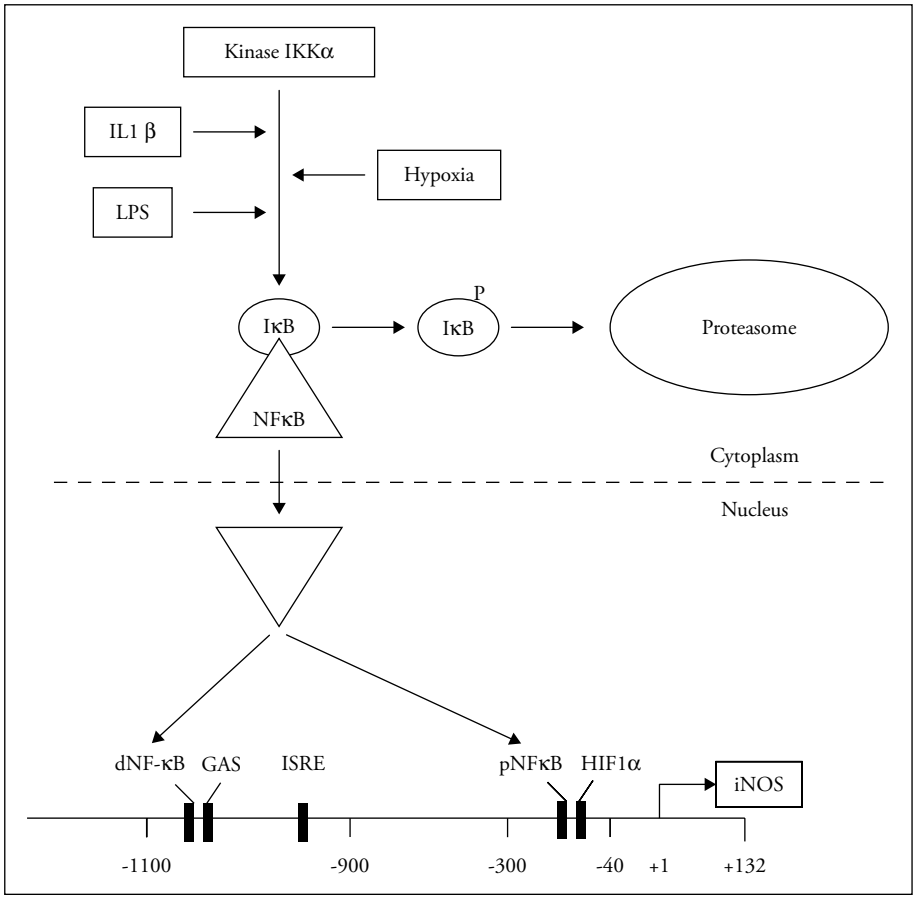

Figure 4. NFκB signaling towards the iNOS promoter.

induced phosphorylation and proteasome-mediated degradation of the inhibitory IκB subunit in the cytoplasm followed by translocation of the p50/p65 NFκB complex to the nucleus and its binding to the iNOS promoter. In macrophages, the second stimulus may be provided by hypoxia *via* hypoxia-inducible factor-1 (HIF1) that has a binding site in region I [42].

We identified a similar effect of hypoxia on iNOS induction in tumor cells. Indeed, 16 h pre-incubation of EMT6 cultures in 1% oxygen (average of the tumor microenvironment) indirectly modulated the radioresponse through cytokine-inducible iNOS. Low concentrations of cytokines, which were not active in aerobic cells became efficient inducers of iNOS in chronic hypoxia. This in turn drastically improved the radioresponse of EMT6 cells (up to 2.5-times), collected from chronically hypoxic cultures and dropped into micropellets, our model of metabolic hypoxia that was described above. Hence, hypoxia has a dual on the radioresponse of EMT6 tumor cells: it directly induces radioresistance, and may indirectly modulate radiosensitivity through up-regulation of the iNOS/NO pathway [43].

The dual role of NFκB in tumor cell radioresponse

It is time to face the possibility of conflicting roles of NFκB in the radioresponse of tumor cells, keeping in mind that NFκB signaling is triggered by diverse stimuli and involved in the regulation of multiple downstream genes. Extensive literature strongly suggested that dysregulation or constitutive activation of NFκB is linked to tumorigenesis, angiogenesis and metastasis, and that it protects tumor cells from radiation damage [44, 45]. Consistently, NFκB inhibition has been used as an approach to radiosensitize tumor cells, aiming at stimulating apoptosis and/or inhibiting DNA repair. This approach did work, but disruption of NFκB signaling by indomethacin, by the proteasome inhibitors MG132 and PS341 or by transfection with the super-repressor IκBα resulted in a moderate if not marginal radiosensitization by 1.2 to 1.4 times [46-48]. The range of radiosensitization covered by NFκB inhibition was not impressive but, we told, it might be amplified under hypoxic conditions, which were missing in all reports mentioned above. An idea behind was (a) it is difficult to further improve the radioresponse of already radiosensitive aerobic tumor cells, and (b) hypoxic rather than aerobic cells are an obstacle for radiotherapy. Therfore, we treated EMT6 cells with phenylarsine oxide (PAO) or lactacystin (a proteasome inhibitor) to inhibit NFκB, and afterwards analyzed their hypoxic cell radioresponse. Once again, the increase in radioresponse was rather moderate (up to 1.4-fold), but the picture turned upside down when NFκB was pre-activated by bacterial lipopolysaccharide (LPS) to imitate its increased basal activity in tumors. Instead of radiosensitization, PAO and lactacystin drastically impaired (by > 2 times) the radioresponse of hypoxic EMT6 cells through disruption of NFκB signaling towards the iNOS gene

[49]. Taken together, our findings suggest that the radiosensitizing effects of NFκB inhibitors may be seriously compromised through iNOS, one of its downstream targets. Secondly, this counteraction may be unmasked only in hypoxia, which is a major cause of tumor radioresistance. Finally, the benefit of radiosensitization obtained through activated NFκB signaling towards the iNOS gene (2 to 2.5-fold) is much more promising than that caused by NFκB inhibition (1.4-fold). This comparison encouraged us to look for a good preclinical candidate to activate the NFκB/iNOS/NO pathway.

Lipid A analogues and hypoxic tumor cell radiosensitization

During the last decade, the mechanism and profile of the NFκB signaling pathway has been clarified in many types of mammalian cells, activated by diverse stimuli such as TNFα, IL1β and LPS. Perhaps one of the best studied and widely used stimulus was and still remains to be LPS, the major component of the outer membrane of gram-negative bacteria. This endotoxin and immunostimulator is known to activate monocytes/macrophages through the TLR4/MyD88-receptor complex, which finally results in the NFκB controlled secretion of a variety of pro-inflammatory mediators including NO, TNFα and IL6 [50]. An attempt to use LPS in cancer immunotherapy was not successful, since strong septic-like toxicity could not be prevented at a dose as low as 4 ng/kg [51]. Because of that, the bioactive component of LPS, lipid A, was isolated and its simplified synthetic analogs MPL (monophosphoryl lipid A), ONO4007 and OM174 were developed and examined in preclinical and clinical trials. These derivatives appeared to be much better tolerated, while preserving the immunomodulating activities of LPS [52]. Although anticipated, the role of NFκB in lipid A signaling towards iNOS has not been studied in macrophages in detail, whilst tumor cells were simply out of the scope of interests. The questions we raised were: (1) Is lipid A as active as LPS in iNOS induction and radiosensitization of EMT6 tumor cells? (2) Does lipid A cause radiosensitization at plasma relevant concentrations? (3) Does lipid A activate iNOS in EMT6 tumor cells through NFκB signaling?

As we deduced from the LPS chemistry, lipid A did closely mimic the activity of LPS to induce iNOS in hypoxia but not in normoxia. What was similar and predictable, the range of radiosensitizing effects up to 2.5-fold observed in hypoxic but not aerobic EMT6 tumor cells [53]. What was different and worthy to mention, lipid A displayed a 30 to 100-times less potency and hence was routinely used at 3-30 µg/ml, compared with 0.1 µg/ml LPS. Such concentrations were, however, plasma achievable in cancer patients, as the phase I trials of ONO4007 has recently shown [54]. What was unclear and worthy to explore, the role the NFκB signaling pathway towards the iNOS gene, considering the considerable shift in active concentrations. Two conclusions emerged from our transfection experiments using an NFκB tandem and an array of plasmids containing either deletions or mutations at critical points in the two regions of the iNOS promoter that bind transcription factors. First, the role of NFκB is crucial in the lipid A-induced transcriptional activation of iNOS, since the activity of the NFκB tandem and the wild type iNOS promoter was significantly increased. Second, lipid A-driven signaling predominantly targets the proximal but not distal NFκB binding site of the iNOS promoter, which fits to the background of LPS [41, 55]. Therefore, the difference between LPS and lipid A observed in our experiments presumably reflected a modulated affinity of lipid A for the TLR4 receptor, as a result of the carbohydrate core removal from LPS. The similar if not identical profile of lipid A/LPS activities was very helpful for us to profit from the extensive background on the very toxic, and sometimes fatal, substance LPS in the domain of immunology. Why we turned to immunology being convinced radiobiologists is explained in the next paragraph.

Role of the proinflammatory tumor infiltrate in radioresponse

The tumor microenvironment has other particular aspects besides hypoxia. Indeed, solid tumors contain a complex network of inflammatory cells (e.g., macrophages, T/NK-cells), which are re-programmed to stimulate (rather than to inhibit) tumorigenesis, through the secretion of several growth and pro-angiogenic factors [56]. Such a mechanism was described for breast, cervix and bladder carcinomas, wherein an increased density of tumor-associated macrophages was correlated with poor prognosis. In the past, an idea to exploit inflammatory cells in tumor immunotherapy was regularly revisited but never realized. Surprisingly, an idea to exploit inflammatory cells in tumor radiotherapy was even never visited, although, the inflammatory mediators TNFα and NO have been repeatedly used as biochemicals to radiosensitize tumor cells [7, 36, 39, 57, 58]. We decided to explore whether the proinflammatory tumor infiltrate may be exploited for tumor cell radiosensitization.

As a first step, the macrophage-like RAW 264.7 cell line was used to model the interaction between pro-inflammatory and tumor cells [59]. RAW 264.7 macrophages were activated with plasma-relevant concentrations of lipid A, and the conditioned medium was screened for cytokine secretion by Elisa and for the oxidative NO metabolite nitrite. The use of RAW 264.7 cells appeared to be a valid experimental model, since the spectrum of released mediators was generally matching that of native macrophages [60]. Indeed, TNFα and IL6 were produced at the highest rates. Both cytokines are known to be co-expressed by monocytes/macrophages during the acute phase of inflammation and immunity. Second, IL12 and IL18 were produced as well, which illuminates paracrine signaling from macrophages to T/NK cells, leading to the secondary production of IFNγ [61, 62]. This cascade was omitted in our model, which was based on a pure macrophage like cell line. Two possible mechanisms of radiosensitization were identified: (a) indirect, through induction of the iNOS/NO pathway in EMT6 cells by macrophage-released mediators, and (b) direct, through induction of the iNOS/NO pathway in RAW 264.7 macrophages and diffusion of NO to bystander tumor cells *(figure 5)*.

Macrophages and hypoxia appear to be independent partners rather than occasional friends that normally co-localize in the same tumor regions. Indeed, hypoxia may up-regulate the iNOS expression in tumor cells only if a basal level of iNOS or a high transcriptional inducibility of iNOS is displayed, because of the genetic pattern of the tumor cells. Let us name such a pattern as iNOS-positive, which seems to be true for at least some types of tumors [63-71]. What about iNOS-negative tumors or tumor cell subpopulations, wherein the iNOS/NO pathway is genetically silenced? In such a case, neither hypoxia nor macrophage-released cytokines look

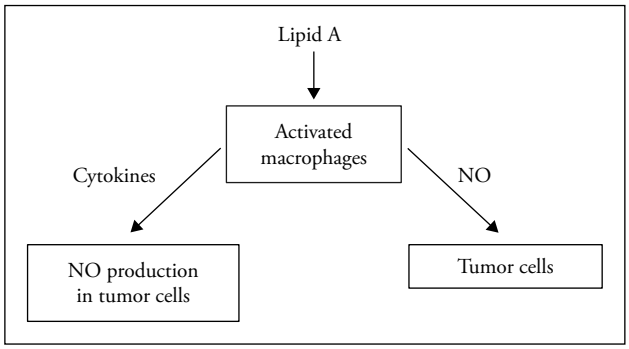

Figure 5. Activated macrophages may radiosensitize hypoxic tumor cells by two iNOS-dependent mechanisms: (a) directly through the release of NO, or (b) indirectly through the secretion of pro-inflammatory mediators which in turn activate NO production within tumor cells.

promising anymore. This is the situation wherein macrophages are supposed to make the difference, since they are an alternative source of NO. To our knowledge, an iNOS-negative pattern of macrophages has little if any chance to exist, regarding that macrophages are naturally programmed to deliver a high output of NO upon proper activation. It appears from our experimental data, that this activation may require IFNγ next to lipid A, to benefit from their synergism based on the cooperative signaling of NFκB and JAK/STAT towards the iNOS promoter [41]. To examine this, we modeled the immune cells in the proinflammatory infiltrate by splenocytes, which contain both macrophages and IFNγ producing T/NK-cells. Stimulation of splenocytes by the lipid A analogue OM174 resulted in the production of IL12, IL18 and IFNγ. This caused up to 2.1-fold radiosensitization of EMT6 tumor cells, which was associated with the iNOS-mediated production of NO. Both iNOS activation and radiosensitization were counteracted by neutralizing antibodies against IFNγ or against IL12/18 [72]. Lipid A analogues appear therefore be a potential new class of tumor cell radiosensitizers through activation of the IFNγ/iNOS pathway. This finding indicates a rationale for combining immunostimulatory and radiosensitizing strategies in the future. ▼

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