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Experimental radiobiology

Preclinical safety and antitumor efficacy of insulin combined with irradiation☆

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Abstract

***Background purpose*: We have previously reported that insulin significantly enhances tumor oxygenation (pO_2) and increases radiation-induced tumor regrowth delay in experimental models. Considering the large radiosensitizing effect, clinical trials might be envisioned. The aim of the present pre-clinical study was to obtain a more complete set of safety and efficacy data which would further justify the commencement of such clinical trials.**

***Material and methods*: Toxicity on normal (early and late-responding) tissues was measured by the intestinal crypt regeneration assay and the late leg contracture assay. Efficacy in terms of enhancement of pO_2 (measured by in vivo EPR oximetry) and increase in radiation-induced tumor regrowth delay was evaluated with a dose—response study on mice bearing FSaII fibrosarcoma.**

***Results*: The effect on regrowth delay was directly correlated with the effect on the tumor pO_2, with a maximal effect using 400 mU kg^{-1} insulin. Importantly, there was no increase in the radiation toxicity for normal tissues. Finally, we found that the hypoglycaemia induced by insulin can be corrected by simultaneous glucose infusion without modification of efficacy.**

***Conclusion*: Insulin here demonstrated a therapeutic gain and a lack of toxicity to normal tissues. The results of this study fully justify further larger preclinical assays such as the use of fractionated irradiation and a tumor control dose assay, before determining the utility of insulin as a radiosensitizer for human patients in the clinic.**
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Keywords: Insulin; Irradiation; Tumor; Oxygenation; Preclinical

Clinical investigations have clearly demonstrated that the prevalence of hypoxic tissue areas is a characteristic pathophysiological property of locally advanced solid tumors [1,2]. Oxygen is a key environmental factor in the development and growth of tumors, as well as their response to treatments such as chemotherapy, radiotherapy, and photodynamic therapy. Both oxygen diffusion and oxygen consumption by metabolism in tumor cells contribute to the occurrence of hypoxia. Numerous clinical studies suggest that tumor hypoxia is associated with a poor prognosis in different types of human cancers [3,4]. In addition, tumor hypoxia is considered to be a therapeutic problem, as it makes solid tumors resistant to sparsely ionizing radiation, some forms of chemotherapy and photodynamic therapy. It has been demonstrated that a *transient* increase in tumor oxygenation and/or perfusion may be beneficial when combined with radiotherapy or chemotherapy [5,6]. The tumor oxygenation may be modified by changing the oxygen supply (perfusion or haemoglobin saturation) [7—14] or by changing the local oxygen consumption [15,16].

We have previously reported that insulin significantly enhances tumor oxygenation as well as radiation-induced tumor regrowth delay in experimental models [17]. We provided evidence that the insulin-induced increase in oxygenation results from a decrease in tumor cell oxygen consumption rather than from an increase in tumor blood flow [18]. Moreover, we showed that this effect is mediated by a nitric oxide pathway [18]. Phosphorylation of eNOS after insulin treatment was also assayed to demonstrate the involvement of nitric oxide in the increase in tumor oxygenation. There is a great deal of evidence that NO regulates mitochondrial respiration by virtue of reversible interactions with cytochrome *c* oxidase [19]. We also

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demonstrated a direct correlation between the NO produced in the tumor after insulin treatment and the radiosensitizing effect [18]. Finally, we showed that NO is a complementary factor additive to oxygen in determining the sensitivity to irradiation, explaining the very high sensitization of insulin compared to carbogen [18].

A potential clinical advantage of the combination of a sensitizing agent and radiation can only be foreseen if the potentiation of radiation response is higher in tumors than in normal tissues in the radiation field, indicating that the therapeutic ratio of the combined treatment is above unity. Since insulin is widely used in humans, and since insulin was identified as a powerful radiosensitizing agent, it is certainly valid to envision clinical trials. For that purpose, it is necessary to obtain more pre-clinical data, which is the aim of the present study. We first tested the dose–response to insulin in terms of increase in tumor oxygenation and in terms of efficacy to sensitize radiation therapy. Second, we evaluated the potential toxicity of insulin when combined with radiation therapy. For this purpose, two normal tissue models were used to determine whether insulin was responsible for toxicity to early responding tissue (intestinal regenerated crypt assay) or late responding tissues (leg contracture assay). Finally, a potential concern when using infusion of insulin relates to a possible hypoglycaemia that this hormone could produce. Therefore, another aim was to assess the possibility of correcting the hypoglycaemia by simultaneous infusion of glucose, and the consequences of this correction on the tumor oxygenation and radiation sensitivity.

Methods

Models and treatments

NMRI mice were used for normal tissue response to irradiation assays and C3H/HeOuJIco mice bearing syngeneic FSaII fibrosarcomas were used to study the tumor response to irradiation. Anesthesia was first induced by an i.p. (intraperitoneal) injection of ketamine (80 mg/kg)/xylazine (8 mg/kg) and maintained with ketamine alone (20 mg/kg/10 min). Insulin (Actrapid HM, Novo Nordisk, Bagsvaerd, Denmark) was infused via a tail vein catheter. Irradiations were performed with X-rays (RT 250; Philips Medical Systems). When measured, glycaemia was determined with the Glucotest ''One Touch Ultra'' (Lifescan, Milipas, USA).

Tumor oxygenation and regrowth delays: dose–effect studies

Tumor oxygenation

Electronic paramagnetic resonance (EPR) Oximetry, using charcoal (CX0670-1, EM Science, Gibbstown, NJ) as the oxygen sensitive probe, was used to evaluate the tumor oxygenation [5]. EPR spectra were recorded using an EPR spectrometer (Magnettech, Berlin, Germany) with a low frequency microwave bridge operating at 1.2 GHz and extended loop resonator. Calibration curves were made by measuring the EPR line width as a function of the pO_2. For this purpose, the charcoal was suspended in a tumor homogenate, and EPR spectra were obtained on a Bruker EMX EPR spectrometer (9 GHz) between 0% and 21% O_2. Nitrogen and air were mixed in an Aalborg gas mixer (Monsey, NY), and the oxygen content was analyzed using a servomex oxygen analyzer OA540. Mice were injected in the centre of the tumor 1 day before measurement using the suspension of charcoal (100 mg/ml, 50 μl injected, 1–25 μm particle size). The localized EPR measurements correspond to an average of pO_2 values in a volume of ~10 mm^3. Four different doses of insulin from 100 to 400 mU kg^{-1} were tested. FSaII tumor-bearing mice were infused with insulin for 25 min and the tumor oxygenation was measured 35 min later, at $t = 1$ h, the time point of maximum increase in pO_2 [17].

Regrowth delays

When tumors reached 8.0 ± 0.5 mm in diameter, the mice were randomly assigned to a treatment group and irradiated. So, FSaII tumor-bearing mice were infused with insulin (8, 12, and 16 μU/kg/min) or with carrier for 25 min and the tumor-bearing leg was locally irradiated with a single dose of 25 Gy of 250 kV X-rays (RT 250; Philips Medical Systems). Mice were anesthetized, and the tumor was centred in a 3-cm diameter circular irradiation field. After treatment, tumors were measured every day until they reached a diameter of 16 mm, at which time the mice were sacrificed. For each tumor, transversal and anteroposterior measurements were obtained. An average tumor diameter was then calculated. A linear fit could be obtained between 8 and 16 mm, which allowed us to determine the time to reach a particular size for each mouse. Growth delay time (GD) was calculated as the time for treated tumors to reach 12 mm in diameter minus the time for control tumors to reach the same size. The regrowth delay factor (GDF) is the rd of a given treatment divided by the GD for X-rays alone.

Intestinal crypt regeneration assay

The jejunum crypt survival assay [20] was used to determine the radiation toxicity to mouse intestinal mucosa. Briefly, 7-week-old NMRI mice without tumors received whole-body irradiation with 250 kV X-rays at a dose rate of 84 cGy/min with prior insulin or carrier (control) infusion ($n = 4$ per group dose and treatment). Ten irradiation doses between 8 and 17 Gy were used ($n_{total} = 80$). Three days and 14 h after irradiation, mice were killed and a few centimetres of jejunum were removed from the angle of Treitz and fixed in 10% neutral-buffered formalin. After embedding, 4 μm transverse sections of the jejunum were cut and stained with hematoxylin and eosin. The number of regenerated crypts per jejunum circumference was counted in three different sections per mouse. Only crypts with 10 or more cells were counted.

Late leg contracture assay

The reduction in extension (contracture) of irradiated legs was determined in 12-week-old NMRI mice without tumors [21]. For each mouse, the right leg was centred in a 3-cm diameter circular irradiation field and locally irradiated with 250 kV X-rays at a dose rate of 1.2 Gy/min with prior insulin or carrier infusion. Four radiation doses between 20 and 50 Gy

were used ($n = 6$ mice per group dose and treatment, $n_{total} = 48$). At 120 days after irradiation, when the contraction reached a plateau, mice were positioned in a jig, and both irradiated and non-irradiated legs were extended over a millimetre scale. Readings were made at the ankle (detailed in ref [21]). The leg contracture was calculated by subtracting the length of the irradiated leg from that of the contralateral control leg and the difference was expressed as a percentage of the extension of the non-irradiated leg.

Statistics

Multiple comparison analysis with Dunnet's post hoc tests were performed to analyze the regrowth delay data. Dose modification factors (DMF) were compared with student's *t*-tests. *P* values under 0.05 were considered significant.

Results

Tumor oxygenation and regrowth delays: dose—response studies

Local tumor oxygenation was measured with EPR oximetry and the radiosensitization properties were determined with the regrowth delay assay after single dose irradiation. Insulin infusion significantly increased FSaII tumor oxygenation (measured 35 min after the end of the treatment) between 200 and 400 mU kg^{-1}, with the most efficient dose at 400 mU kg^{-1} (Fig. 1, top). The addition of glucose 5% to the saline vehicle did not alter the increase in tumor oxygenation induced by insulin. The improvement in tumor oxygenation translated into a significant increase in tumor regrowth delay after single dose irradiation in FSaII tumors (Fig. 1, bottom). We observed a regrowth delay (rd) of 5.9 ± 0.3 days for X-rays, 9.0 ± 0.8 days for X-rays combined with insulin at 200 mU kg^{-1}, 9.7 ± 0.2 days for X-rays combined with insulin at 300 mU kg^{-1}, and 10.3 ± 0.5 days for X-rays combined with insulin at 400 mU kg^{-1}. The rdf for the highest insulin dose (400 mU kg^{-1}) was 1.73, which was significantly greater than the rdf value of 1.43 for carbogen breathing (used as a positive control) ($P < 0.05$; Fig. 2). The addition of glucose prevented hypoglycaemia (Fig. 3) but did not significantly modify the increase in oxygenation or the radiosensitizing properties of insulin (rd of 9.6 ± 0.5 days). Thirty-five minutes after insulin infusion, glycaemia was decreased by 56 ± 5% (mean ± standard deviation, $n = 6$) for mice infused with insulin only, versus only 27 ± 5% for insulin combined with 5% glucose.

Intestinal crypt regeneration assay

The jejunum crypt survival assay established that insulin (400 mU kg^{-1}) did not potentiate radiation toxicity on mouse-jejunum tolerance after whole body single dose irradiation, as shown in Fig. 1. A dose of 10.85 Gy was required to reduce survival in the crypts by 50% for radiation alone, and 11.32 Gy for radiation combined with insulin, resulting in a non-significant DMF (dose modification factor) of 1.04 (*t*-test, $P > 0.05$) (Fig. 4). This was estimated using a sigmoïdal fit of the curves.

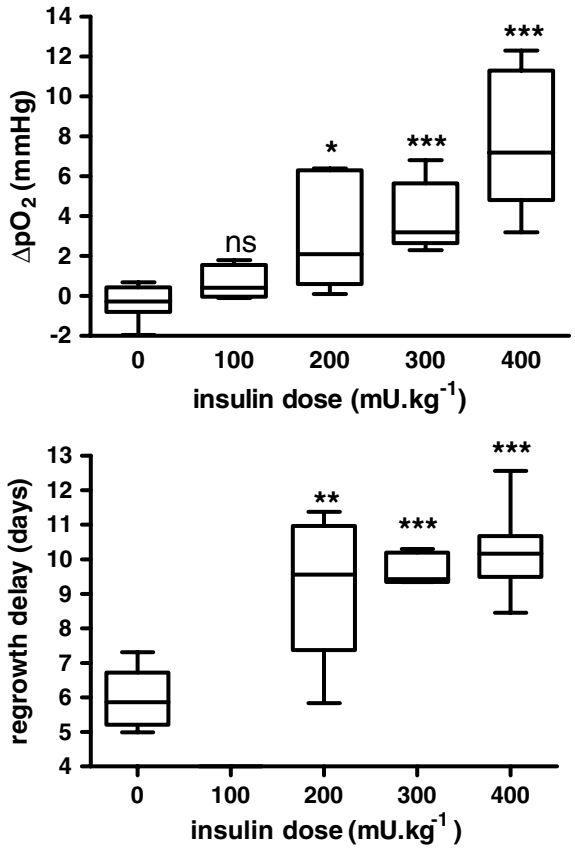

Fig. 1. Dose—response studies: (top) change in pO_2 (as measured with EPR oximetry) vs the dose of insulin. $N = 3$—7 mice/group. (bottom) Regrowth delay after irradiation (25 Gy of X-rays) (days) vs the dose of insulin. $N = 6$—7 mice/group. Ns, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The tumor oxygenation is significantly increased at doses between 200 and 400 mU kg^{-1}, with the most efficient dose at 400 mU kg^{-1}. Similar results were obtained in terms of regrowth delays.

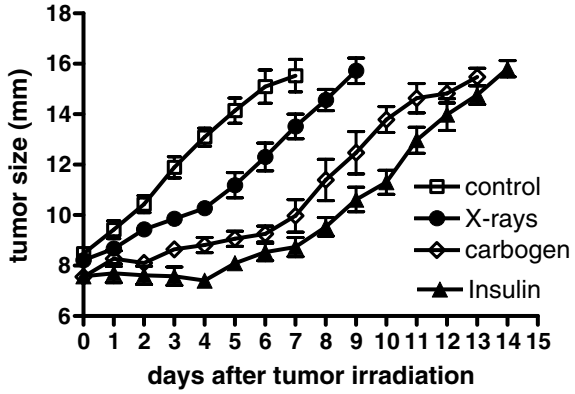

Fig. 2. Effect of insulin on tumor regrowth delay: tumor size is plotted vs time after irradiation. The control group ($n = 6$) was infused with saline. The X-rays group was infused with saline and irradiated with a single dose of 25 Gy ($n = 6$). The carbogen group was irradiated with a single dose of 25 Gy during gas breathing ($n = 6$). The insulin group was treated with 400 mU kg^{-1} and irradiated with a single dose of 25 Gy.

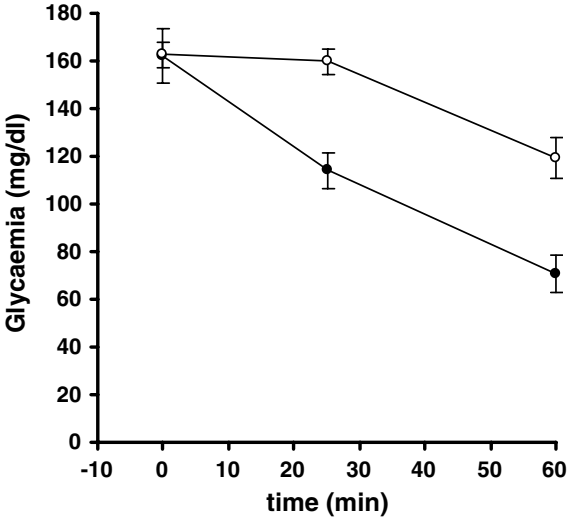

Fig. 3. Effect of glucose addition on the hypoglycaemia induced by insulin infusion: blood glucose level is plotted versus time after insulin infusion (in saline (○) or glucose 5% (●)).

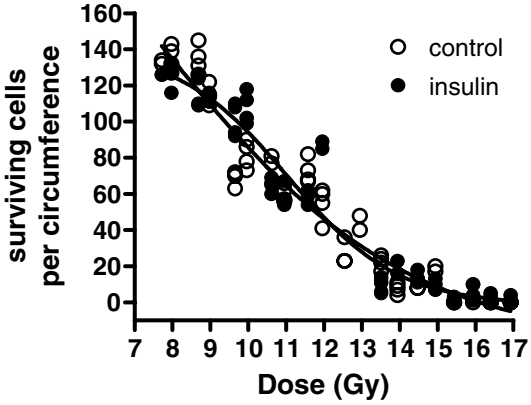

Fig. 4. Acute toxicity evaluation with the intestinal crypt regeneration assay. The number of regenerated crypts per jejunum circumference (at 86 h post-irradiation) is plotted versus the radiation dose (whole-body irradiation at a dose rate of 84 cGy/min). Treated mice were pre-injected with insulin (400 mU kg^{-1}) while control mice were injected with saline.

Late leg contracture assay

The late leg fibrosis assay established that late radiation damage in musculo-connective tissues was not affected by insulin (i.e. there was no significant change in the contracture of irradiated legs at 120 days after irradiation alone or irradiation combined with insulin, Fig. 5). The radiation dose required to achieve a leg contracture of 15% was 36.1 Gy for X-rays and 33.3 Gy for X-rays combined with insulin (400 mU kg^{-1}), resulting in a non-significant DMF of 0.92 (*t*-test, $P > 0.05$). This was estimated using a linear fit of the data.

Discussion

The principal findings of the present study are that: (a) insulin increases the tumor oxygenation and radiation

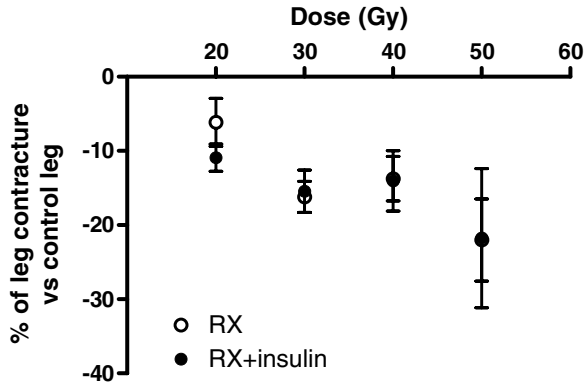

Fig. 5. Late toxicity evaluation with the leg contracture assay: reduction in extension (contracture) of irradiated legs vs control legs (%) at 120 days post-irradiation is plotted versus the radiation dose (local irradiation at a dose rate of 1.2 Gy/min). Treated mice were pre-injected with insulin (400 mU kg^{-1}) while control mice were injected with saline. $N = 6$ mice/dose/treatment ($n_{total} = 48$).

sensitivity; (b) there was no increase in the radiation toxicity for normal (early and late-responding) tissues; (c) the hypoglycaemia induced by insulin can be corrected by simultaneous glucose infusion without modification of tumor oxygenation or radiosensitization.

The fact that tumor hypoxia is a determining factor in the response of tumors to cytotoxic treatments is well established [22–24]. Nevertheless, only a small number of the numerous preclinical studies trying to target tumor hypoxia were already translated into the clinic [6]. Presently, the most advanced radiation therapy clinical trials employ tirapazamine [25] to kill hypoxic tumor cells directly or ARCON (combination of carbogen and nicotinamide) [26] to increase the supply of oxygen to the tumor. With respect to modifiers of hypoxia, it has been predicted theoretically that modification of oxygen consumption is much more efficient at affecting oxygen transport than modification of delivery [15]. Drugs that inhibit respiration, such as metaiodobenzylguanidine (MIBG) [27] or COX-2 inhibitors [28], have been characterized for their potential radiosensitizing ability. In this class of inhibitors of oxygen consumption, we previously found that insulin was able to radiosensitize tumors significantly through an increase in tumor oxygen and nitric oxide [17,18], two compounds which are known as radiosensitizers.

In view of the large radiosensitizing effect that was measured here with insulin, it is certainly valid to envision further preclinical studies as well as clinical trials. The present preclinical study shows significant improvements in tumor oxygenation and tumor growth delay at different insulin doses. Interestingly, we found that there was a direct relationship between the increase in tumor oxygenation and the increase in radiosensitizing effect induced by insulin. However, further preclinical tests would be of particular interest before translating to the clinic. Since the oxygen effect is typically reduced for fractionated courses, and since fractionated schemes are used in the clinical setting, it seems necessary to test the effect of insulin in a fractionated irradiation protocol. Furthermore, a tumor control dose assay to determine a TCD50 would further

document the radiosensitizing properties of insulin since it is considered as the most relevant endpoint for curative radiotherapy. Then, if this type of study were transferred into the clinic, the choice insulin dose could be determined on the basis of the effect on tumor oxygenation. This could be done using EPR, since this tool is now becoming available in the clinic [29]. It may be possible to use other tumor oximetry techniques as well, such as polarographic oxygen electrodes or PET labeled nitroimidazole tracers. Using 400 mU/kg of insulin for this current study, the effect on the tumor growth delay was significantly higher than using carbogen. Therefore, all further efficacy and safety studies were carried out using this dosage.

Importantly, the radiosensitization effect appears to be tumor specific. Acute and chronic toxicity of irradiation assessed on fast and slow critical responding tissues, with the intestinal crypt regeneration assay and the late leg contracture assay, respectively, were not modified by pre-treatment with insulin. This is consistent with the concept that oxygen-mediated radiosensitization may only occur in tissues that start off with a pO_2 less than the threshold value ($\sim$5 to 10 mmHg) which delimits ineffective and effective radiation cell killing. Since normal tissues are already well oxygenated (pO_2 > 10 mmHg), the increase in radiosensitivity is negligible in these tissues.

One possible limitation of an insulin treatment could be an induced hypoglycaemia. We indeed found that there was a significant drop in glycaemia (up to 56%) 35 min after insulin infusion. However, we demonstrated that we could ameliorate this hypoglycaemia in mice by mixing the insulin with 5% glucose (drop in glycaemia of only 27%). More importantly, we found that this treatment was able to maintain the oxygenating and radiosensitizing properties of insulin. A decrease of 27% in glycaemia is certainly tolerable in the clinic. Moreover, it could be further corrected with existing clinical protocols that are based on the correction of glycaemia induced by the administration of insulin (''insulin-clamps'').

In conclusion, the therapeutic gain of insulin is here demonstrated in a preclinical setting. The results of this study fully justify further larger preclinical assays such as the use of fractionated irradiation and a tumor control dose assay, before determining the utility of insulin as a radiosensitizer for human patients in the clinic.

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