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The radiobiology of laser-driven particle beams: focus on sub-lethal responses of normal human cells

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Abstract: Accelerated proton beams have become increasingly common for treating cancer. The need for cost and size reduction of particle accelerating machines has led to the pioneering investigation of optical ion acceleration techniques based on laser-plasma interactions as a possible alternative. Laser-matter interaction can produce extremely pulsed particle bursts of ultra-high dose rates ($\geq 10^9$ Gy/s), largely exceeding those currently used in conventional proton therapy. Since biological effects of ionizing radiation are strongly affected by the spatio-temporal distribution of DNA-damaging events, the unprecedented physical features of such beams may modify cellular and tissue radiosensitivity to unexplored extents. Hence, clinical applications of laser-generated particles need thorough assessment of their radiobiological effectiveness. To date, the majority of studies have either used rodent cell lines or have focussed on cancer cell killing being local tumour control the main objective of radiotherapy. Conversely, very little data exist on sub-lethal cellular effects, of relevance to normal tissue integrity and secondary cancers, such as premature cellular senescence. Here, we discuss ultra-high dose rate radiobiology and present preliminary

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data obtained in normal human cells following irradiation by laser-accelerated protons at the LULI PICO2000 facility at Laser Lab Europe, France.

KEYWORDS: Accelerator Applications; Instrumentation for hadron therapy; Plasma generation (laser-produced, RF, x ray-produced)

Contents

1 The rationale for laser-driven cancer hadrontherapy

Hadrontherapy, the clinical exploitation of accelerated charged particles for cancer treatment, has become an increasingly common therapeutic modality [1], because of several advantages it offers compared to conventional radiotherapy with megavoltage photon or electron beams. The use of charged particles significantly reduces the dose absorbed by normal tissues due to their inverse dose-depth deposition profile as described by the Bragg curve. Their superior ballistic properties are thus the physical pillar justifying hadrontherapy as the eligible treatment for deep-seated tumours and/or close to organs at risk [2]. Heavier charged particles such as carbon ions exhibit a higher radiobiological effectiveness than protons or photons, due to their denser ionization event pattern through matter, whereby highly clustered DNA lesion sites arise. Such damage complexity is believed to confer high-LET radiations their radiobiological advantage at cell killing [3], hence making carbon-ion based radiotherapy best suited to treat cancers with intrinsic or acquired radioresistance [4]. However, a wider adoption of hadrontherapy is hampered by the high installation and running costs of the accelerating facilities [5]. This has fuelled efforts in developing novel approaches towards compact, flexible and cost-effective solutions to accelerate particles for medical applications. One viable solution may be represented by laser-based optical ion acceleration techniques [6]. Clinically amenable laser-driven beams require specific constraints to be met, such as beam reproducibility, homogeneity and stability. More importantly, the radiobiological effectiveness of these beams must be assessed. For they exhibit unprecedented spatio-temporal physical features [7]. The physical pattern of the energy deposition events at nanometric scale and their very early-stage (10^{-12}–10^{-6} s) radiochemical evolution determines the biological outcome, shaping the role of biochemical repair processes [8]. The ultra-intense ($\geq 10^{19}$ W/cm^2) nature of the laser-target interaction foreseen to produce proton beams of energies useful for hadrontherapy gives rise to ultra-short (from fs to ps) proton bursts at ultra-high dose rates ($\geq 10^9$ Gy/s), i.e. several orders of magnitude larger than those from current therapeutic regimes [9]. While laser-driven particle beams can thus be seen as a valuable probe to investigate the core mechanisms of radiation action at the sub-nanometer

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and femtosecond scales [10, 11], it has been suggested that the extreme spatio-temporal nature of laser-driven particle beams may ensue unknown biological responses [7, 12, 13]. Conversely, elegant theoretical work by Kreipl et al. [14] investigating the impact of multiple radiation tracks in close spatio-temporal proximity on the yields of chemical species and DNA damage patterns argues against clinically relevant influence on biological effectiveness by ultra-short pulses. Hence, research in ultra-fast high-energy particle radiobiology is needed to ascertain whether and to what extent such peculiar irradiation conditions influence cellular radiosensitivity. Whereas local tumour control is the mainstay of any curative radiotherapy strategy, late-occurring effects such as recurrence or metastatization represent its most feared consequence. These may stem from cells that are sub-lethally damaged by irradiation and by the interplay between these and neighbouring cancer cells: the former can harbour tumorigenic lesions inheritable by their progeny while the tumour microenvironment may be modified by acquiring a more aggressive phenotype via paracrine interaction with such damaged normal cells [15]. In particular, very little experimental data exist on the induction of sub-lethal cytogenetic damage along the Bragg curve, adding to the uncertainties deriving from the still scant clinical follow-up data on sequelae incurred by hadrontherapy patients. *A fortiori*, the dependence of sub-lethal effects on unprecedented factors, such as the exceedingly high dose rates and/or the pulsed nature of beams originated by laser interaction with target materials, needs investigation prior to actual laser-driven cancer hadrontherapy. Here we present work carried out at LULI $PICO_{2000}$ laser facility focusing on cellular premature senescence in a normal human cell line.

2 The radiobiology of ultra-fast high-energy particles

Because of the increasing attention towards alternative modalities for cancer hadrontherapy, there has been a revival in recent years in the field of ultra-high dose-rate (UHDR) radiobiology. Historically, an ample spectrum of dose rate values has been used in experimental radiobiology and clinical radiotherapy [16], extending from a few cGy per day to hundreds of Gy in a fraction of a second (figure 1). The extremes of the spectrum have been largely considered of mere academic interest as has been the case for the low end of the spectrum, i.e. chronic exposure regimes typical of interstitial brachytherapy, also of relevance to environmental exposure.

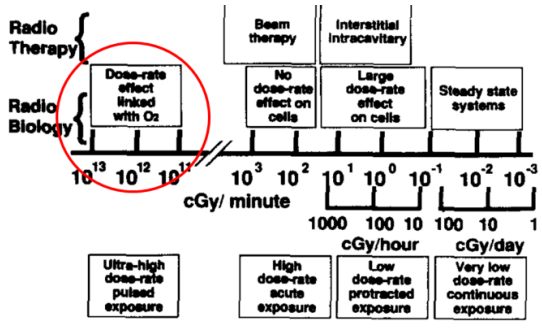

Figure 1. A sketch of the dose-rate spectrum of interest in radiobiology and radiotherapy: highlighted is the mechanistic basis hypothesized for biological effects due to UHDR and/or highly pulsed radiation regimes (adapted from [16]).

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In fact, exploration of the top range of the dose-rate spectrum was long hampered by the technical difficulty of achieving such extreme conditions. The most complete set of data concerning the dose-rate effect has been accumulated *in vitro* for a number of biological systems in the range between 0.1 Gy/hr and 10 Gy/min, demonstrating the principle of repair of sub-lethal damage [17, 18]. Oxygen concentration was thought to be critical as an increase in cell survival with decreasing dose rates was seen to be much reduced under hypoxic conditions [19], pointing to less recoverable, intra-track lethal damage. At the opposite end of the dose-rate range, early studies invoked oxygen consumption by radiation concomitantly with an increased likelihood of recombination during free radical formation to explain a diminished biological effectiveness under UHDR [20]. UHDR were attained by discharge machines or electron linear accelerators: by both methods, estimated instantaneous dose rates of 10^{11} Gy/min could be obtained [16]. If both dose and instantaneous dose rate are sufficiently high, the rapid deposition of radiant energy depletes oxygen too quickly for diffusion to maintain an adequate level of oxygenation, and dose-response curves are those characteristic of hypoxia, i.e. lacking the shoulder at low dose where cell killing is inefficient because of sub-lethal damage repair. The promise that these findings held for radiotherapy was based on the view that UHDR would constitute a means of killing cells independent of oxygen tension since at sufficiently high dose rates even aerated cells would behave as if they were anoxic. This would represent an ideal radiation modality for radiotherapy since the protection afforded to some tumour cells by their hypoxia would be completely eliminated. For bacteria, a firmly established dose-rate effect in the UHDR region exists as first demonstrated by an anoxic-like response found in bacteria irradiated in 1% oxygen [21]. This biphasic type of response was confirmed by others [22] irradiating mammalian cells equilibrated in air (figure 2). Nias et al. showed instead lack of anoxic-like response and no differences in radiosensitivity after irradiation with highly pulsed electrons of oxyc cells [23]. Further studied failed to clarify the influence of UHDR and pulsed irradiation modality on cellular response. Schulz et al. [24] found a pulsed

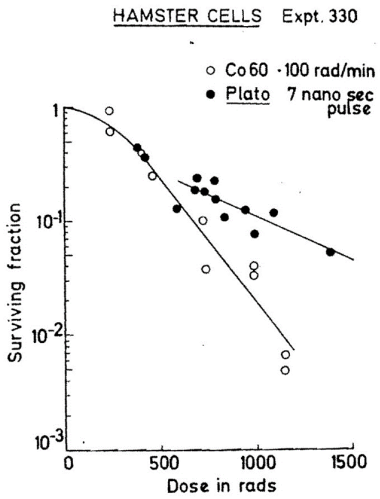

Figure 2. The typical "hockey-stick" type shape of the clonogenic survival dose response curves from γ-rays at conventional dose rates (1 Gy min^{-1}) or a 7-ns pulse of electrons (from [22]).

30-MeV electron beam at 0.5–5 10^8 Gy/s to be less effective at cell killing compared to 250 kV X-rays, but they could not relate their results to oxygen consumption. Purdie et al. [25] found opposite results using similar experimental conditions. These conflicting results, together with the consideration that total doses required to use up local oxygen are far too high, and the instantaneous dose-rates enormous, led to a decline of interest in this development for the clinic. At least, prior to the advent of laser-driven accelerating techniques.

Several groups have started preliminary work using laser-driven ion sources for cell irradiation experiments, mainly aiming at establishing procedures for cell handling, irradiation and dosimetry compatible with a laser-plasma interaction environment. Activities in this area have been carried out in Germany [26–28] and Japan [29], where, using Ti:Sa lasers, lethal effects on cells have been investigated by accumulating dose on cells over several laser shots. An UHDR radiobiology program was initiated on the TARANIS and GEMINI laser systems in the U.K., employing a single-exposure approach, which is believed to be more relevant to isolate effects associated to short burst deposition, as the dose required to induce biological effects is deposited in a single, ultrashort burst [30, 31]. Neither approach did find significant radiobiological differences between laser-driven electrons or protons and conventionally accelerated beams or reference photon radiations. In fact, the majority of *in vitro* studies making use of different sources confirm that in the therapeutically relevant dose range of a few Gy, even if applied in a single pulse of only few nanoseconds duration, non-linear radiobiological effects due to simultaneous multiple damages in cells and, thus, below any timescale of repair mechanisms, are unlikely to arise [32–37]. Only two relevant exceptions exist: Achayra et al. [38], who reported a decrease in genetic damage measured as micronucleus formation after a single pulse of electrons but not after multiple pulses (10^6 to 10^8 Gy/s), hypothesising more efficient radical recombination; Schmid et al. [39], who found a slight decrease in effectiveness at causing (some types of) chromosome aberrations after nanopulsed protons (conventionally accelerated). Of fundamental importance to gain insights into laser-driven particle biological effectiveness is, of course, *in vivo* work. From these studies, mainly limited to tumour growth delay as endpoint of clinical relevance and still too scarce for firm conclusions to be drawn, no enhancing effects have emerged for both protons and electrons [40–42].

3 Sub-lethal cellular damage and particle therapy

Despite allowing considerable normal tissue sparing, a main health concern of hadrontherapy remains the onset of secondary cancers [43]. The tumorigenic potential of ionizing radiation has been long known as well as its dependence on dose, dose rate and radiation quality [44]. However, non-cancer late effects may also undermine overall treatment success. Recent evidence suggests a significantly higher risk of developing cardiovascular disease in patients cured for breast cancer, in which heart and/or blood vessel are deemed to receive some dose, in line with the results from the epidemiological studies on the Japanese atomic bombs survivors [45]. Stress-Induced Premature Senescence (SIPS), a process whereby sub-lethally damaged cells enter a permanent state of quiescence, has attracted growing interest for its possible long-term health consequences [46, 47]. SIPS is opposed to the well-known phenomenon of *in vitro* replicative senescence, which is the physiological exhaustion of a cell's ability to proliferate [48]. This is mechanistically linked with the progressive shortening of end-chromosome sequences (telomeres) [49] and is

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universally recognised as a natural barrier to tumorigenesis *in vivo* [50, 51]. In particular, ionizing radiation is capable of inducing SIPS; more interestingly, charged particles have proven particularly effective at eliciting a senescence response at very low dose and dose rates [52, 53]. Ectopic *in vivo* accumulation of senescing cells may adversely affect normal tissue/organ integrity and/or function resulting in genomic instability and increased risk of cancer [54–56]. In hadrontherapy patients, the role of SIPS is still undetermined [57, 58]. In addition, because senescing cells remain metabolically active [48], a Senescence-Associated Secretory Phenotype (SASP) has been identified whose effect on neighbouring primary cancer cells is controversial as secretion of factors released by cells undergoing SIPS has been associated with either the inhibition or the promotion of proliferation/invasiveness [59–61]. Therefore, sub-lethal effects are clearly as important as tumor local control in determining the successful outcome of cancer radiation treatment. To date, there is no knowledge on the influence that very high dose rates or pulsed time-structure in dose delivery may have on such effects.

4 Radiobiological studies with laser-accelerated protons at the PICO$_{2000}$ facility

4.1 Experimental setup

Protons were generated from the interaction of laser bursts focused on 25 and 50 μm Au foil targets. Laser energy was, on average, of (100 ± 10) J with a pulse duration of 1.3 ps (mean laser intensity of approximately 5×10^{19} W/cm^2). In an initial phase of the experiment, a stack of HD-V2 radiochromic films (RCF) were positioned inside the target chamber at about 5 cm from the proton source in order to characterize the beam input properties, providing information on the energy content and angular divergence. The use of RCF stacks for obtaining coarse spectral information on the broadband laser-accelerated proton beams is a well-established procedure [62, 63] and requiring a prior calibration of the film using suitable proton sources [64]. Non-linearities in RCF film response to lower energy, higher LET protons (in the MeV region) [65–67] were included in the analysis by means of a correction factor which had been experimentally validated against Markus Chamber dose measurements [64]. In the cell irradiation set-up a dipole magnet deflected the plasma-accelerated protons to angularly disperse them according to their energy, after an appropriate angular selection employing a suitable aperture (as in [30]). Parasitic X-ray radiation was suppressed by appropriate lead shielding in the chamber, while X-rays were spatially separated from the deflected protons. A purposely designed cell dish was used for biological sample irradiation, with cells growing on its 3-μm mylar basis, using as a template a prototype originally used for microbeam studies [68]. To optimize sample irradiation, we simultaneously exposed three cell dots in one shot (on the same dish), each at a specific energy and dose (figure 3).

After exposure, cells from each dot were treated independently. RCF, image plates (IP) and nuclear track detectors (CR39) were used for dosimetry. High-sensitivity EBT3 RCFs were placed outside the target chamber, just behind the biological sample, in an *ad hoc* frame, as to provide a shot-to-shot characterization of the dose across the whole irradiated area. IP were employed, in dedicated shots, to directly assess the energy distribution and its spatial uniformity at the sample position. CR39, instead, were used to estimate particle fluence and homogeneity, and to cross-calibrate the IP.

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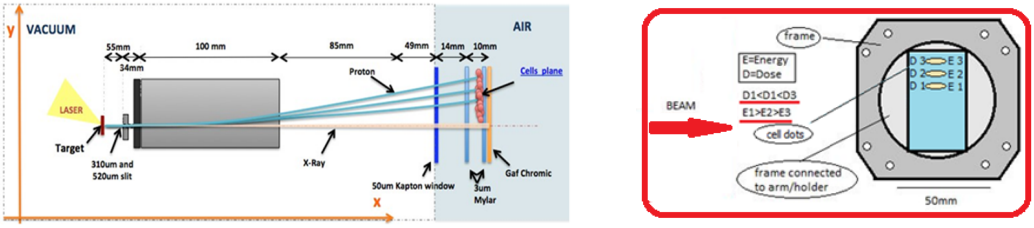

Figure 3. Proton beam transport and delivery system (left); scheme of the cell dish and cell positioning (right).

The energy differences within the dot-containing dish region varied in a range of 6 to 14 MeV, with a 10% dispersion approximately, corresponding to a minimal LET variation (5 ± 2 keV/μm). Hence, it was possible for the radiobiological data to be grouped by dose. Human Umbilical Vein Endothelial (HUVEC) cells, a widely used *in vitro* system to investigate cellular senescence [69], were aliquoted as three dots of 20 μl each of cell suspension, corresponding approximately to 15,000 cells, and seeded onto the cell dish 24 h before exposure. Twelve shots were fired with a total of 8 dishes irradiated (each containing three cell dots treated separately), of which 4 at the time were processed at different time points to improve the statistical robustness of the analysis. For senescence data, in particular, between 500 and 1,000 cells were counted for each dose and time point. The three mean doses estimated at the cell positions were (0.6 ± 0.2) Gy, (1.8 ± 0.6) Gy and (4.5 ± 0.8) Gy. The onset of cellular senescence in irradiated cells was assessed by affinity for β-galactosidase expression as a function of time post-irradiation. Clonogenic survival was also studied being the standard measure for cell radiosensitivity. Results were compared with data from experiments with conventionally accelerated protons (mid-SOBP, LET = 5 keV/μm) and X-rays carried out at INFN-LNS and University of Naples, respectively.

4.2 Results

Surviving fractions (SF) were fitted according to the linear-quadratic model by the equation $\mathrm{SF} = \exp -(\alpha D - \beta D^2)$. No statistically significant difference between cellular survival to laser-driven and conventionally accelerated proton beams was observed (figure 4). An RBE of 1.1 was found between conventional proton irradiation and X-rays: such a value is traditionally used in current protontherapy planning treatments, although experimental evidence points to a significant increase in proton RBE at the distal end of the SOBP [70]. Figure 5 illustrates the time course of radiation-induced cellular senescence. All irradiation conditions resulted in an increase in the fraction of senescing cells. In particular, proton irradiations were generally more effective than X-rays. As for laser-driven and conventionally accelerated protons, the former appeared less effective at the lowest dose, of relevance for healthy tissues in a radiotherapy scenario. Conversely, at the intermediate dose used in our experiments (1.5 Gy for conventionally accelerated protons, 1.8 ± 0.5 Gy for laser-driven protons), i.e. a value close to the fractionated therapeutic dose of 2 Gy administered to the tumor volume, occurrence of senescence induced by laser-driven protons was at all times greater than that by conventionally accelerated protons.

At the highest dose used (4 Gy or 4.5 ± 0.8 Gy for conventionally- and laser-accelerated protons, respectively), no particular trend was observed with minor differences between radiation types.

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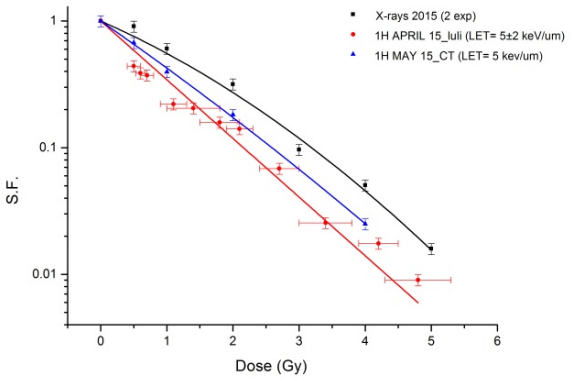

Figure 4. HUVEC clonogenic survival curves following laser-driven (red symbols) and conventionally accelerated proton beams (blue symbols). Results of X-ray irradiation (black symbols) are also shown.

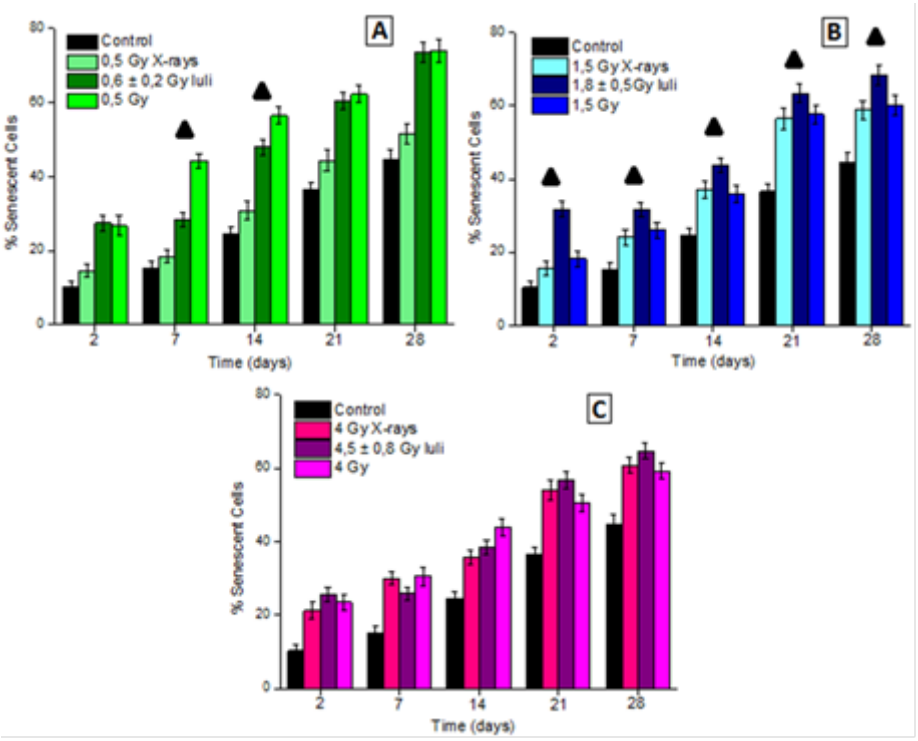

Figure 5. Time course of senescence induction as grouped by doses. Black triangles highlight the statistically significant differences between laser- and conventionally accelerated protons ($p < 0.05$).

4.3 Conclusions

To our knowledge, our data represent the first experimental results on radiation-induced premature senescence, an important sub-lethal effect of potential clinical relevance [71], in normal cells exposed to laser-accelerated particles. Laser-driven proton beams were found to yield a higher percentage of senescing cells at quasi-therapeutic doses while causing a lower percentage of cells

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to enter prematurely senescence at doses typical of healthy tissues. Conversely, no significant effect was observed in the induction of cell death. No straightforward explanation is currently available. However, the potential clinical implications are intriguing since a greater effect at the tumor level (*in vivo* senescing endothelial cells may translate in disruption of the blood vessels feeding the tumor) seems to be accompanied by a lessened effect on normal tissues. Given that the very mechanisms underlying radiation-induced cellular senescence *per se* are in general poorly understood but known to be influenced by epigenetic alterations rather than directly induced DNA damage as is the case for cell death, a differential action on the former due to the peculiar temporal scale of laser-driven proton acceleration may be one possibility. Therefore, further experiments are mandatory to confirm these preliminary data.

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