Intratumoral ^{224}Ra-Loaded Wires Spread Alpha-Emitters Inside Solid Human Tumors in Athymic Mice Achieving Tumor Control

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Abstract. *Background: We developed a new method of brachytherapy, termed diffusing alpha-emitters radiation therapy (DaRT), based on the use of intratumoral ^{224}Ra-loaded wires, which release short-lived alpha-emitting atoms by recoil. Here, we examined their ability to destroy and control the development of several human-derived tumors implanted in athymic mice. Materials and Methods: The experiments were performed on athymic mice bearing malignant human-derived tumors including prostate (PC-3), glioblastoma (GBM, U87-MG), colon (HCT15), squamous cell carcinoma (FaDu) and melanoma (C32). One or more ^{224}Ra-loaded wires were inserted into the tumors, and mice were assessed for tumor growth rate and survival. Results: In vivo studies showed that DaRT can effectively destroy the tumors, and in vitro tests confirmed the sensitivity of the studied cells to alpha particles. While the C32 cells were relatively resistant, other tumor types (e.g. HCT15) exhibited sensitivity in both measured aspects. Conclusion: DaRT could potentially be combined with chemotherapy or other treatment modalities to effectively treat non-resectable tumors.*

The efficacy of an anticancer treatment, in general, depends on the sensitivity of the treated tumor to the treatment. Specifically in the field of radiotherapy, many factors might determine the response of a given malignant tissue. Tumor site and origin, cell-cycle stage and the oxygenation state of the cell are considered the most influential parameters on the degree of radiosensitivity or radioresistance. Hence, the ability to predict tumor response to radiotherapy may indicate the outcome of the treatment (1-4).

Due to the fact that the vast majority of methods involving ionizing radiation utilize low-linear energy transfer (LET) radiation (x- and gamma-rays, and electrons), the accumulated experimental data are almost entirely related to this form of energy transfer (5, 6). There are significantly fewer data about the effect of high-LET radiation on different malignancies and in particular about the effect of alpha particles on solid tumors.

Alpha radiation is generally more effective against cancer cells than is low-LET radiation, achieving a higher degree of cell killing probability for a given absorbed dose, and forming complex DNA lesions (7, 8). The biological effect of alpha irradiation has the further advantage of being largely insensitive to oxygen deficiency which is a frequent condition in solid tumors that dramatically reduces the efficacy of radiation treatments based on photons and electrons (9).

The most prominent obstacle that has so far prevented the use of alpha radiation from becoming therapeutically efficient against solid tumors is its range in tissue. The difficulty in creating conditions that would allow effective coverage of the entire tumor volume with alpha particles has been considered too large a hindrance (10, 11).

In previous studies, we described a new form of brachytherapy allowing the treatment of solid tumors by alpha particles (12-15). The method, named diffusing alpha-emitter radiation therapy (DaRT), is based on the utilization of implantable radioactive sources which carry small amounts of ^{224}Ra incorporated into their surface. When ^{224}Ra decays it emits into the tumor its short-lived progeny: ^{220}Rn, ^{216}Po and ^{212}Pb, which leave the source by virtue of their recoil energy. The basic claim of DaRT, namely that the short-lived alpha emitters released from the source can lead

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to cell death over therapeutically significant distances (while the parent ^{224}Ra remains fixed on the source), was demonstrated in three different experimental models.

The need to evaluate the consistency of the DaRT method and its efficacy in other malignancies has brought us to challenge the treatment in additional experimental models of solid tumor. Since the power of alpha particles to destroy tumor cells is not limited to a specific site or origin, it needs to be applied to various types of malignancies in order to strengthen the understanding of its efficacy under different conditions of tumor microenvironments. The focus on human-derived tumors in this work is evidently an essential step in promoting the method towards clinical trials.

Materials and Methods

Cells and tumors. HCT15, FaDu and PC3 cell lines were purchased from the American type culture collection (ATCC) and their use begun within six months. The U87 cell line was kindly provided by Professor Yoel Kloog (Life-Sciences Faculty, Tel-Aviv University). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) in 5% CO_2 at a humidified incubator with 37˚C. All media were supplemented with 10% fetal calf serum, sodium pyruvate (1 mM), non-essential amino acids (1%), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (all obtained from Biological Industries, Beit-Haemek, Israel). Athymic nude mice (8 to 12 weeks old) were obtained from Harlan (Rehovot, Israel). Animal care and experiments were carried out in accordance with the guidelines of the Israeli National Council for Animal Experimentation (permits no. M-06-015, M-06-070 and M-07-060). All surgical and invasive procedures were conducted under anesthesia by intraperitoneal inoculation of Imalgen (100 mg/kg; Fort Dodge Laboratories, Germantown Hills, IL, USA) and xylazine hydrochloride (10 mg/kg; VMD, Arendonk, Belgium) solution in 0.25 ml of phosphate buffers solution (PBS). Xenografts were transplanted subcutaneously into the low lateral side of the back. Local tumor growth was determined by measuring 3 mutually orthogonal tumor dimensions with a digital caliper (Mitutoyo, Onomy, Japan). The volume of tumor was calculated using the approximate formula: $V=\pi/6\ D_1D_2D_3$ where D_1, D_2, and D_3 are the measured dimensions.

Colony formation assay. The *in vitro* effect of alpha particles traversing various cell lines was studied using a ^{228}Th source (half life 1.91 years) in a setup described previously (15). It consists of a small deposit of ^{228}Th in secular equilibrium with its daughters on a silicon substrate. The surface was coated with a 4.0-μm layer of silver to prevent the release of the recoiling daughters of ^{228}Th. The irradiation setup comprised a 7.5-μm thick Kapton (polyimide) foil (Dupont, Manon, Luxembourg) held between two cylindrical stainless steel parts, forming a well with an inner diameter of 9 mm. Cells were placed in the wells and were incubated for 24 h to form a confluent monolayer, after which the wells were exposed to alpha particles for 0, 1, 2, 3, 4 and 6 min, at an average dose rate of approximately 0.5 Gy/min. The dose rate was estimated by a measurement of the total flux of alpha particles crossing the Kapton foil using an alpha particle detector, and a Monte Carlo simulation of the passage of alpha particles through the foil using the 'stopping and range of ions in matter' (SRIM) code. Immediately following irradiation, cells were harvested and sparsely seeded in 10-cm culture dishes (Corning, Corning, NY, USA) with 15 ml of medium, to allow the formation of well-separated colonies. Colony formation was monitored daily, and typically 6-9 days post irradiation colonies were fixed in methanol and stained with Hemacolor (Millipore, Dubllin, Ireland). The colonies were counted manually and were considered viable when containing more than 50 cells.

High-resolution autoradiography (HRA). The radionuclide spread inside treated tumors was studied by an autoradiographic method utilizing a phosphor-imaging plate, as described in a previous publication (12). Briefly, tumors were treated with a single ^{224}Ra-loaded wire each and were excised four days later, with the wire extracted within ~10 min. The excised tumors were then placed in 4% formaldehyde for 24 h and the preserved specimens were subsequently processed and embedded in paraffin following standard procedures. Histological sections (10-μm thick) were cut and placed on glass slides. The slides were then laid on a Fuji imaging plate (BAS-TR2040S, Fujifilm, Tokyo, Japan) for 10-15 h, to measure the ^{212}Pb spread throughout the tumor sections. Following each measurement, the plate was scanned by a Fuji FLA-9000 system with a pixel size of 100 μm. The measured ^{212}Pb activity was then used to estimate the absorbed dose and the asymptotic dose was further normalized by the initial rate of ^{220}Rn release from the source. The same histological specimens were stained with hematoxylin-eosin (H&E) (Surgipath, Richmond, IL, USA) for tissue damage detection, to be correlated with the activity distribution measurements.

^{224}Ra-loaded wire preparation and insertion. ^{224}Ra-loaded wires were prepared using a ^{228}Th generator [as described in detail in (12)]. In this set-up, positive ^{224}Ra ions emitted by recoil from a surface layer containing ^{228}Th are collected electrostatically on a thin stainless stee wire (0.3 mm in diameter; Golden Needle, Suzhou, China). The wires are subsequently heat-treated to induce radium diffusion away from the surface to a typical depth of 5-10 nm. The ^{224}Ra-impregnated wires are characterized by an alpha particle detector to account for their ^{224}Ra activity and the release rate of ^{220}Rn. Wires, either loaded with ^{224}Ra or inert, cut to a length of 4-6 mm were placed near the tip of a 21-gauge needle attached to a 2.5 ml syringe (Picindolor, Rome, Italy) and inserted into the tumor by a plunger placed internally along the syringe axis. Typical wire activities were in the range of several dozen kBq ^{224}Ra.

Results

Variable cell-dependent sensitivity to alpha particles. A set of experiments testing cell lines derived from different human solid malignant tissues was performed in order to compare the relative sensitivity of each cell line to measured fluxes of alpha particles. Escalating doses of radiation were delivered through cell-containing Kapton wells and a comparative analysis was conducted.

For each cell line, two colony formation experiments were performed, with three to five wells irradiated in each experiment for each dose level. The surviving cell fraction was calculated as the ratio of the number of viable colonies in a given culture dish (containing irradiated cells) to the

average number of colonies in the unexposed control dishes. Survival curves obtained for C32, HCT15, PC3 and FaDu cells were drawn and the data in each experiment were fitted with the function $f(D)=e^{-D/D_0}$ using Matlab's curve fitting tool to estimate the mean lethal dose (D_0). The resulting average values for D_0 are given in Table I.

Dose distribution inside treated tumors from different human histotypes. The dispersion of radioisotopes released from the ^{224}Ra wire to further emit alpha particles throughout the malignant tissue was evaluated using an HRA system. We examined tumors treated by an intra-tumoral insertion of a single ^{224}Ra wire and compared the dimensions of the region inside the tumors in which an effective therapeutic dose of alpha radiation (10 Gy) was achieved. The choice of 10 Gy as a marker dose was derived from estimations of low-LET effective doses of 20 Gy and considered a relative biological effect (RBE) of two-fold in the case of alpha particles. The following human derived malignancies were tested: C32 melanoma cell line (n=4), FaDu squamous cell carcinoma (SCC) (n=4), colon carcinoma HCT15 (n=5), U87 (n=4), head and neck SCC CAL27 (n=3), and prostate carcinoma PC3 (n=2). Autoradiography indicated that there are significant differences in the distribution pattern between tumors of different histotypes (the findings derived from our analysis are summarized in Table I). The average area of the region subjected to a normalized asymptotic dose of 10 Gy (per 1 μCi of ^{220}Rn released from the source at insertion) was consistent across the tumors of given cell lines, but varied from 9 to 21 mm^2 across different cell lines (with the minimum spread detected in the C32 melanoma model and the maximal spread in FaDu and U87) (Figure 1). The corresponding average diameters of the 10 Gy region varied, accordingly, from 3.5 to 5.2 mm.

Tumor growth retardation. Colon: We investigated the effect of ^{224}Ra wires on colonic tumors derived from a human cell line. Randomized athymic mice inoculated with HCT15 cells were treated with either ^{224}Ra loaded or inert wires when tumors reached 5 mm (average lateral diameter). Figure 2A shows a pronounced effect of the radioactive wires on tumor development monitored three times a week. Thirty-eight days post-treatment, the average volume of the ^{224}Ra-treated tumors (n=7) was 4.7 times smaller than that of the inert group (n=8). The photographs shown in Figure 2 were taken 33 days after treatment, emphasizing the difference between a mouse treated with a single ^{224}Ra wire (Figure 2D), in which tumor suppression was observed, as opposed to an untreated mouse (Figure 2E).

Prostate: PC3 tumors were either treated with radioactive wires or inert wires. Twenty-nine days post treatment, the average volume of the ^{224}Ra-treated tumors (n=6) was three times smaller than that of the inert group (n=5) as can be seen in Figure 2B. Furthermore, one case of complete cure with no tumor recurrence was observed in the group treated with the radioactive wires.

Table I. *Relative sensitivity to alpha particles by histotype.*

Tumor	n	10 Gy measured diameter (mm)	10 Gy measured area (mm^2)	$D_{(0)}$
C32	4	3.42±0.5	9.45±2.43	1.17±0.09
PC3	2	3.95±0.02	12.23±0.12	0.86±0.11
HCT15	5	4.33±0.53	14.96±3.69	1.12±0.1
U87	4	4.93±1.37	20.09±10.82	–
FaDu	4	5.24±0.2	21.6±1.7	0.64±0.06

Glioblastoma: Athymic mice inoculated with human derived U87 tumor cells (4 mm in average diameter) were treated with either ^{224}Ra or inert wires. The results shown in Figure 2C indicate that after 24 days, the average tumor volume of the ^{224}Ra-treated group was 10 times smaller compared to that of the inert group. There was one case of complete regression of the tumor in the ^{224}Ra wire-treated group and in the inert –wire treated group.

Melanoma: C32 human melanoma tumors induced in nude mice were treated at an average lateral diameter of 8 mm with either ^{224}Ra-loaded (n=8) or inert (n=8) wires. An interesting phenomenon was observed, as the radioactive treatment did not significantly affect tumor growth. We noticed that untreated animals died when tumors reached an approximate volume of 1000 mm^3. We therefore determined 1000 mm^3 as an end-point value for the C32-transplanted mouse experiments. A statistically significant difference (χ^2=0.002) between the inert-treated group and the ^{224}Ra wire-treated group was shown as six out of eight animals in the inert group had died when tumors reached 1000 mm^3 compared to none in the radioactive treatment group. These results were consistent with an additional experiment utilizing a double wire insertion method (data not shown).

Discussion

This study examined several types of solid human tumors differing in site for the treatment with alpha-emitting atoms released interstitially. The choice of malignancies took into consideration sites, which may potentially become clinical candidates for this radiotherapy method. Effective treatment of GBM is complicated by multiple factors, including the difficulty in overcoming the blood-brain barrier and the diffusely infiltrative nature of the disease, which limits complete surgical resection (16, 17). Brachytherapy has been demonstrated in clinical trials to be effective for relapsed GBM and the concept of using alpha radiation may give rise to a new clinical tool. For prostate cancer, where brachytherapy is

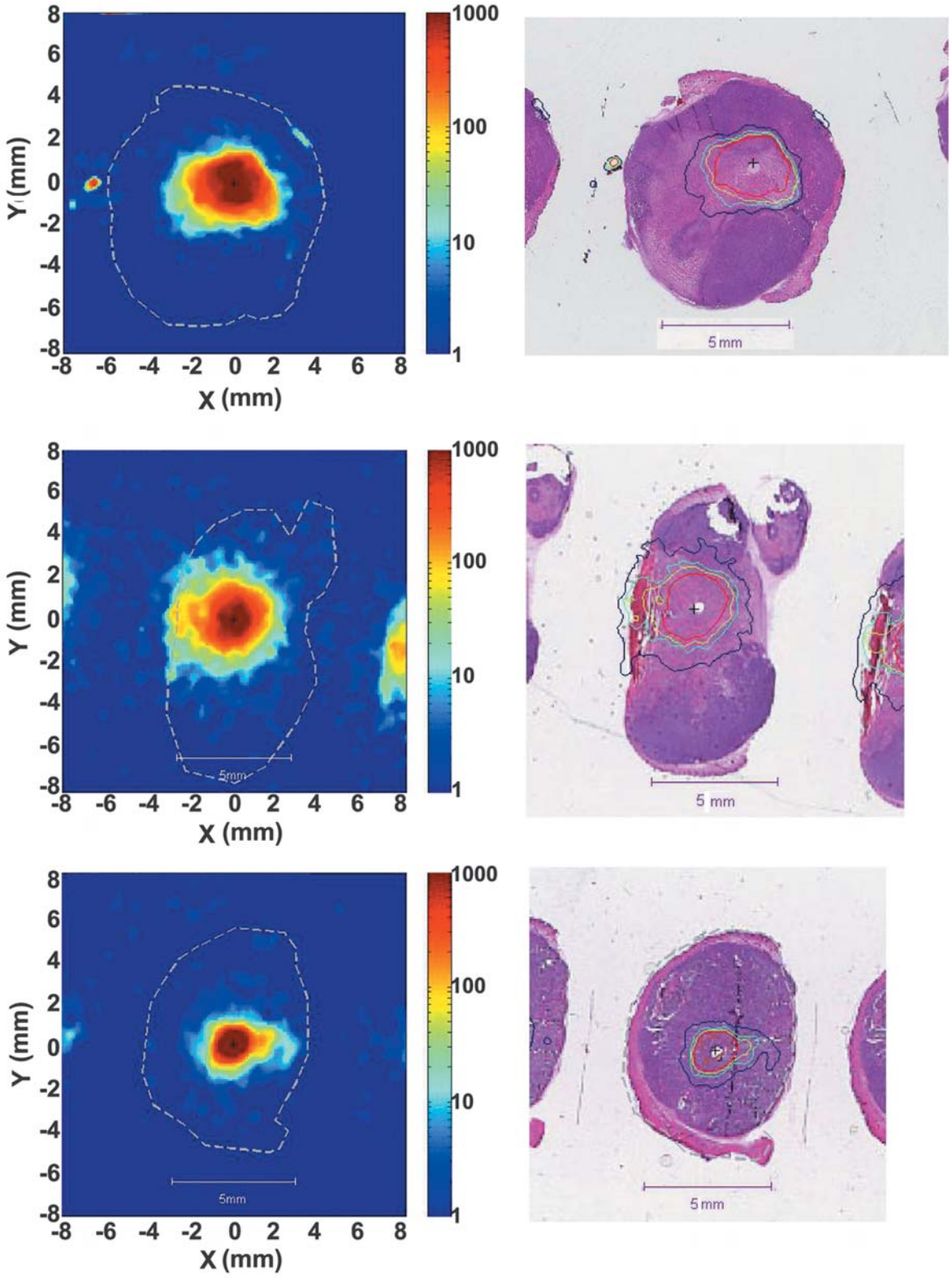

Figure 1. *Normalized asymptotic dose formed by interstitial ^{224}Ra wires in different human solid tumors. High-resolution autoradiography (HRA) analysis of tumors from different histotypes was performed in order to evaluate the average spread of ^{224}Ra progeny (namely ^{212}Pb) inside the malignant tissue. Average values measured in all tested models are presented in Table I. Representative distributions subsequently stained with H&E to correlate with necrotic domains are presented from HCT15 (A), U87 (B) and C32 (C) models.*

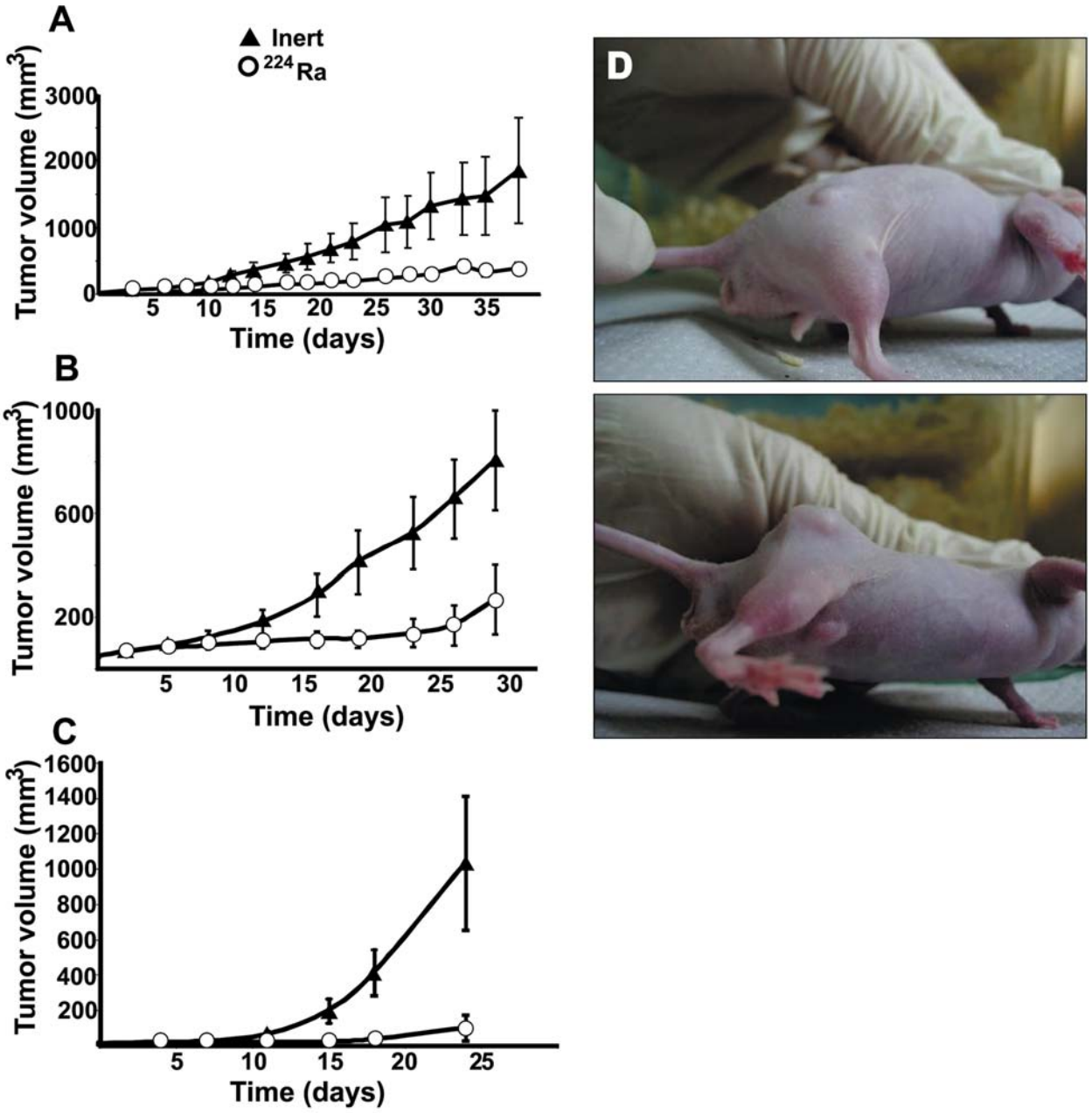

Figure 2. *Human colonic (A) prostatic (B) or glioblastoma tumors (C) treated by a single ^{224}Ra wire applied to athymic mice bearing tumors and monitored for tumor growth. Control group compared to tumor-bearing mice treated with inert wires. A: HCT15: tumor sizes (average diameter) on treatment day were 5 mm. The ^{224}Ra wire carried activities ranging from 20-24.2 kBq. Inert n=8; ^{224}Ra wire n=7. B: PC3: tumor sizes (average diameter) on treatment day were 3 mm. The ^{224}Ra wire carried activities ranging from 15.3-25.4 kBq. Inert n=5; ^{224}Ra wire n=6. C: U87: tumor sizes (average diameter) on treatment day were 4 mm. The ^{224}Ra wire carried activities ranging from 21.3-24.3 kBq. Inert n=7; ^{224}Ra wire n=7. Standard deviations are represented by bars. Representative photographs of Inert-treated animal (E) and ^{224}Ra wire-treated animal (D) bearing HCT15 tumors 33 days post-treatment are displayed.*

already well-established but still not free of complications, a potential advantage of using DaRT (in conjunction with other methods) may be the ability to deliver a highly localized dose to the region surrounding the urethra while sparing the urethra itself (18, 19). The standard treatment for colonic carcinoma and melanoma, on the other hand, does not include any interstitial irradiation at present. Nevertheless, the relative accessibility of these tumors, together with the fact that surgical

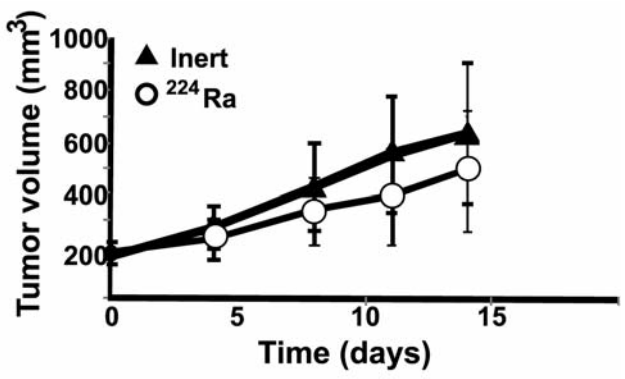

Figure 3. *Human melanoma tumors treated by a single ^{224}Ra wire-treatment applied to athymic mice bearing tumors and monitored for tumor growth. ^{224}Ra wire (n=8). Tumor-bearing mice treated with ^{224}Ra wires. The control group was referred to as Inert (n=8) tumor-bearing mice treated with inert wires.*

procedures fail to yield satisfying cure rates for advanced stages of these diseases, have brought us to investigate the response of these tumors to DaRT application.

We report here that when wires impregnated with small amounts of ^{224}Ra (in the range of a few dozens of kBq) are inserted into different solid human tumors, they may lead to considerable local tumor growth inhibition. The efficacy of the treatment varies, to some extent, across cell lines, and might be affected by several factors including, in particular, the effective diffusion rate of the radionuclides released from the source in each histotype and the relative sensitivity at the cellular level.

When alpha particles traversed cell monolayers *in vitro*, a scope of responses ranged between the relatively resistant cell lines (C32) to the most alpha radiation sensitive cells (FaDu). The differences, which were significant, implied that the effect caused by this high LET radiation is cellular dependent. The further comparison between histotypes, in regard of the formation of an effective dose region inside the treated tumor, gave rise to yet another range of values. These two tested elements may suggest diversity in the responses to DaRT at two separate levels, so that if the cells inside a bulky tumor require larger doses in order to be eradicated and additionally the spread of the atoms inside the tissue is relatively small, the tumor will only be moderately affected and *vice versa*. The assumption about the linkage between cell sensitivity and spread must be further examined since it may hold the key for understanding what controls the basic principles of this technique. As already demonstrated both *in vitro* and by histological means, the effect of DaRT on primary tumors differs across cell lines. Here, again, the results from the *in vitro* cytotoxicity experiments and HRA experiments were mostly correlative with the effects on tumor volume. The most prominent example is the C32 melanoma model, where we observed that cells were alpha radiation-resistant and the perimeter of the effective dose was relatively small and, indeed, tumor progression was only moderately reduced (Figure 3). On the other hand, it was demonstrated that U87 tumors could be sufficiently controlled by the interstitial ^{224}Ra wires as the radioactive treatment yielded a reduction of over 90% in tumor volume compared to the control animals at 24 days post- wire insertion (Figure 2C). Both the colonic carcinoma model and the prostate carcinoma model were also affected significantly by the intra-tumoral irradiation giving rise to growth arrest of a 4-fold decrease for the PC3 tumors (Figure 2B) to a 4.7-fold decrease for the HCT15 tumors (Figure 2A). In order to rule out any additive damaging effects of non-alpha progeny in this decay chain (*i.e.* electrons and photons), we used sealed DaRT wires ensuring nothing but alpha-emitting daughters infiltrated the tumor (data not shown). Despite the fact that C32 melanoma primary tumors did not respond to the treatment, the phenomenon in which animals treated with inert wires were highly affected by tumor size, while DaRT wire-treated animals survived, indicated an involvement in the metastatic process (which eventually leads to morbidity). The manner by which the interstitial irradiation using alpha-emitting atoms may promote anti-metastatic processes should be thoroughly investigated.

The probability of eradicating a single cultured cell with alpha particles may vary and be dependent on several characteristics such as the ability to initiate DNA repair mechanisms and recover from complex double-strand breaks (20, 21). Inducing cell death would be even more complicated when the irradiation is aimed against cells dwelling inside a bulky solid tumor due to the fact that the tumor microenvironment becomes a key factor in determining radiosensitivity and a large diversity may be expected between malignancies not sharing the same kind of vasculature, stroma or density for instance (22, 23).

Intensive research efforts must be dedicated to recognizing the factors tilting the balance from radioresistance to radiosensitivity when an alpha particle intercepts a given tumor cell *in situ*. The pre-clinical results presented here may in the future help predict if DaRT will serve as a good practice option for a given tumor.

Conflict of Interests

The Authors declare conflict of interests as detailed in the acknowledgment section.

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