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BIOLOGY CONTRIBUTION

LOCAL CONTROL OF LUNG DERIVED TUMORS BY DIFFUSING ALPHA-EMITTING ATOMS RELEASED FROM INTRATUMORAL WIRES LOADED WITH RADIUM-224

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Purpose: Diffusing alpha-emitters radiation therapy (DART) is a new form of brachytherapy enabling the treatment of solid tumors with alpha radiation. The present study examines the antitumoral effects resulting from the release of alpha emitting radioisotopes into solid lung carcinoma (LL2, A427, and NCI-H520).

Methods and Materials: An *in vitro* setup tested the dose-dependent killing of tumor cells exposed to alpha particles. In *in viv*o studies, radioactive wires (0.3 mm diameter, 5 mm long) with ^{224}Ra activities in the range of 21-38 kBq were inserted into LL/2 tumors in C57BL/6 mice and into human-derived A427 or NCI-H520 tumors in athymic mice. The efficacy of the short-lived daughters of ^{224}Ra to produce tumor growth retardation and prolong life was assessed, and the spread of radioisotopes inside tumors was measured using autoradiography.

Results: The insertion of a single DART wire into the center of 6- to 7-mm tumors had a pronounced retardation effect on tumor growth, leading to a significant inhibition of 49% (LL2) and 93% (A427) in tumor development and prolongations of 48% (LL2) in life expectancy. In the human model, more than 80% of the treated tumors disappeared or shrunk. Autoradiographic analysis of the treated sectioned tissue revealed the intratumoral distribution of the radioisotopes, and histological analysis showed corresponding areas of necrosis. *In vitro* experiments demonstrated a dose-dependent killing of tumors cells exposed to alpha particles.

Conclusions: Short-lived diffusing alpha-emitters produced tumor growth retardation and increased survival in mice bearing lung tumor implants. These results justify further investigations with improved dose distributions. © **2009 Elsevier Inc.**

Alpha radiation, Lung carcinoma, Radiotherapy, Interstitial, Brachytherapy.

INTRODUCTION

Bronchogenic carcinoma remains the leading cause of cancer-related mortality worldwide. In the United States alone, this disease is the source of 31% of all cancer deaths among men (89,510 cases annually) and 26% (70,880 cases annually) of cancer deaths among women (1, 2).

Even though surgery is the mainstay of treatment, only one quarter of the patients undergo successful resection, with a recurrence rate of 50%. Therefore, a constant effort to develop novel therapeutic approaches for advanced disease is being made.

Along with the development of newer chemotherapeutic agents and targeted therapies for treating lung cancer, radiotherapeutic techniques have evolved and have contributed to improved survival rates and decreased treatment toxicity (3).

To date, radiation therapy for lung cancer has been based primarily on the use of low linear energy transfer (LET) radiation, namely x- and gamma-rays. Whereas high-LET radiation has many radiobiological benefits, which stem from the high ionization density along its path, its use against lung cancer has so far been limited to clinical trials involving external irradiation with fast neutrons (4).

Alpha particles have long been known to be highly effective against cancer cells. With a high LET of $\sim$100 keV/μm,

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no more than a few alpha particle tracks passing through the nucleus are required for cell inactivation. DNA lesions induced by alpha particles are significantly more complex and difficult to repair than those arising from low-LET irradiation (5, 6). As with all high-LET radiations, the biological effect of alpha particles does not require the action of free radicals and is thus largely independent of the oxygenation state of the cell, making hypoxic cells nearly as sensitive to irradiation as cells with normal oxygen levels (7, 8). In contrast to low-LET radiation, the biological effect of alpha radiation is largely independent of the dose rate and is significantly less sensitive to variation in the cell age in the mitotic cell cycle (9, 10). Finally, the short range of alpha particles (less than 100 μm in tissue) may—in principle—guarantee that healthy tissue lying outside of the target zone is spared.

In spite of their many radiobiological merits, when it comes to practical utilization in cancer treatment, the short range of alpha particles has constituted an Achilles heel. The range problem has so far limited the utilization of alpha particles (in preclinical and clinical investigations) to the treatment of malignancies characterized by single cells, micrometastases, or microscopic cell clusters, using monoclonal antibodies or peptides tagged with alpha emitters as targeting vectors (11). Until recently, the treatment of solid tumors was considered to lie outside of the scope of alpha particle irradiation.

In previous articles (12–14), we described a new form of brachytherapy, which allows the treatment of solid tumors by alpha particles. The method, named diffusing alpha-emitters radiation therapy (DART), relies on the utilization of implantable radioactive sources that carry small activities of radium-224 (^{224}Ra, 3.7 days half-life), incorporated into their surface. When ^{224}Ra decays, it emits into the tumor its short-lived progeny: radon-220 (^{220}Rn, 55.6-s half-life), polonium-216 (^{216}Po, 0.15-s half-life), and lead-212 (^{212}Pb, 10.64-h half-life), which leave the source by virtue of their recoil energy. ^{224}Ra itself remains fixed to the source at a typical depth of a few nanometers. The radionuclides released from the source disperse in its vicinity by diffusive and convective processes, leading to the formation of a high-dose region through their alpha decays. The typical size of this region, as observed in squamous cell carcinoma tumors (12), is approximately 5–6 mm for sources carrying a few dozen kBq of ^{224}Ra, indicating that tumor coverage may be achieved by an arrangement of sources placed a few mm apart. The rapid falloff of the dose field suggests that the dose will be highly localized, with minimal irradiation to adjacent healthy tissue. It was shown that although some of the lead-212 activity leaves the tumor through the blood, the resulting dose to all organs can be expected to be low enough to enable a safe treatment of tumors.

The application of DART against experimental squamous cell carcinoma tumors has spurred us to investigate its efficacy in experimental models of lung carcinoma. Proving that alpha particles introduced into lung tumors by this method could eradicate the malignant tissue is a vital stage in the development of a clinical tool, which would involve this high-LET–based technology with advanced navigation and source placement techniques.

METHODS AND MATERIALS

Cell culture

The Lewis lung carcinoma (LL2) cell line was provided by Prof. E. Flescher from the Department of Clinical Microbiology & Immunology in Tel Aviv University. A427 (cat. no. HTB-53) and NCI-H520 (cat. no. HTB-182) cell lines were purchased from the American Type Culture Collection (Manassas, VA).

All cultures were grown in RPMI-1640 media (Gibco, Israel) 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/mLE). (All supplemental materials purchased from Biological Industries, Beit Haemek, Israel.)

Radioactive microplates

An *in vitro* irradiation setup was developed, in which LL2 or A427 cells seeded in a 96-well microplate (Corning, Corning, PA) were allowed to grow under continuous irradiation by alpha particles, emitted along the decay chain of ^{224}Ra atoms implanted on the well bottoms. ^{224}Ra implantation took place before cells were seeded and was carried out inside a vacuum chamber, using a column of eight unsealed surface sources of ^{228}Th, fitting a single column of the microplate. The ^{224}Ra activity densities on the well bottoms were set by the duration of exposure to the ^{228}Th sources.

Cell proliferation assay

The antiproliferative effects of alpha particles on cell cultures were determined using the XTT assay (Cell Proliferation Kit, Biological industries, Beit-haemek, Israel). Cells were seeded in 96-well microplates implanted with escalating ^{224}Ra activities (radioactive microplates), ranging from 0.06 to 2 Bq/mm^2. Cells were allowed to grow for 24 h, 48 h, or 72 h, after which the activated XTT mixture was added to a final concentration of 0.33 mg/mL according to the manufacturer's instructions. After 2 h of incubation, absorbance was analyzed using spectrophotometer (VersaMax, Molecular Devices, Sunnyvale, CA) at 475 nm. Viability was expressed as the ratio between the measured optical density of irradiated cells and the average optical density of the nonirradiated controls.

Kapton wells irradiation setup

A second setup for *in vitro* irradiation by alpha particles relied on short exposures of cells, seeded on a 7.5-μm thick Kapton (polyamide) foil (Dupont, Luxembourg), to alpha particles emitted by a sealed ^{228}Th source (14). Cells were seeded on the Kapton foil at a density of 3×10^5 cells/well and exposed to the alpha particle flux 24 h later. Exposure was performed by positioning the cells seeded on the foil 10 mm above the ^{228}Th source in air. Exposure times were 0, 1, 2, 3, 4, and 6 min, with an average flux of 1.9×10^4 alpha particles/mm^2/min^{-1} across the exposed area (as determined by an alpha-spectroscopy setup based on an EG&G alpha particle detector). The calculated average dose rate, based on a Monte Carlo calculation (not shown) performed using the SRIM-2003 code (15), was 0.5 Gy/min.

Colony formation assay

Immediately after being irradiated in the Kapton wells, the cells were harvested and plated sparsely in 10-cm culture dishes

(Corning), followed by a 6-day incubation to allow for colony formation. The colonies were then fixed in methanol and stained using the Hemacolor method (16). Cells were counted manually in a double-blind manner and colonies were determined as positive when they contained more than 50 cells. The surviving fraction was calculated as the ratio between the number of viable colonies in a given Petri dish (containing irradiated cells) and the average number of colonies in the control dishes. The data was fitted by a pure exponential function $S(D) = e^{-D/D_0}$ in a least-squares procedure.

Tumor cell inoculation

Male C57BL/6 mice (8–12 weeks old) were obtained from the breeding colony of Tel-Aviv University, Israel. Athymic nude mice were obtained from Harlan (Rehovot, Israel). Animal care and experimentation was carried out in accordance with Tel-Aviv University guidelines. All surgical and invasive procedures were held under anesthesia using Ketamin (100 mg/kg, Fort Dodge, IA) and xylazine hydrochloride (10 mg/kg, VMD, Arendonk, Belgium) solution in 0.25 mL of phosphate-buffered saline. Animals were inoculated subcutaneously with 5×10^5 LL2 cells (C57Bl/6 mice) or 5×10^6 A427 / NCI-H520 cells (nude mice), in 0.2 mL HBSS (Biological industries, Beit Haemek, Israel) into the low lateral side of the back. Local tumor growth was determined by measuring three mutually orthogonal tumor diameters with a digital caliper (Mitutoyo, Onomy, Japan). The volume of tumor was calculated using the formula: $V = (\pi/6) \cdot D_1 D_2 D_3$, where D_1, D_2, D_3 stand for the measured diameters

^{224}Ra-loaded wire (DART wire) preparation

^{224}Ra-loaded wires were prepared using a ^{228}Th generator as described elsewhere (12). Positive ^{224}Ra ions emitted by recoil from a surface layer containing ^{228}Th, were electrostatically collected near the tip of a thin stainless steel wire (0.3 mm in diameter) (Golden Needle, China). The wires were then heat-treated to induce radium diffusion to a typical depth of 10–20 nm. The ^{224}Ra-impregnated wires were characterized by an alpha particle detector to determine their ^{224}Ra activity and release rate of ^{220}Rn. The wires used in the *in vivo* experiments had ^{224}Ra activities in the range of 21.3–35.7 kBq, with ^{220}Rn desorption probabilities of 31–38%.

Wire insertion

Wires, either loaded with ^{224}Ra or inert, cut to a length of 5–6 mm, were placed near the tip of a 23G 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.

^{212}Pb autoradiography

The size of the high-dose region created by the DART source is determined by the dispersion of the recoiling daughters of ^{224}Ra—^{220}Rn, ^{216}Po, and ^{212}Pb—inside the tumor. The spread of radioactivity was measured by an autoradiography technique, as described elsewhere (12). Briefly, several days after a ^{224}Ra-loaded source was inserted into the center of a given tumor, the tumor was excised (as a whole), the source was extracted and the tumor was fixed in 4% formaldehyde (for 24–48 h). Histological sections (5–10 μm thick) were then cut and placed on glass slides. These were laid on a phosphor-imaging plate (BAS-TR2040S, Fujifilm, Japan), for 10–15 h. The phosphor-imaging plate was then scanned by an image reader (BAS-2500, Fujifilm, Japan). The recorded intensity patterns were converted to ^{212}Pb activity using ^{212}Pb calibration samples, which were laid along with the tumor sections on the imaging plate. ^{212}Pb activity was then converted to dose estimates. The same histological sections measured on the imaging plate were later stained with hematoxylin-eosin (Surgipath, Richmond, IL) for tissue damage detection, to be correlated with the activity distribution measurements.

Statistical analysis

The statistical significance (p value) of the differences between tumor volumes in the various groups was assessed by applying Student's two-sided t-test and repeated measures analysis of variance. Survival analysis (Mantel-Cox test) was carried out using Statsoft Statistica 7.0.

RESULTS

Inhibition of cell proliferation and colony formation ability by alpha particles

A427 and LL2 cells were exposed to alpha particles in radioactive microplates. The results of these experiments indicated that cell proliferation was progressively inhibited by exposure to higher activity levels and longer exposure periods. Figure 1 describes the dependence of cell viability on both the ^{224}Ra activity and duration of exposure. For example, exposure to 2 Bq/mm^2 for 24 h resulted in toxicity levels of 37% (LL2, Fig. 1A) and 61% (A427, Fig. 1B) (with toxicity defined as one minus the viability). This effect increased to 83% (Fig. 1A, B) when cells were incubated for 72 h.

The colony formation assay described in the material and methods was used to calculate the mean lethal dose D_0 for LL2 (0.8 Gy) and NCI-H520 (1.5 Gy) cells (Fig. 2A). In Fig. 2B, there is an example of the colonies formed by LL2 cells exposed to escalating doses.

Spatial dose pattern spreading of radioactive atoms from ^{224}Ra wires inserted into lung tumors

The spatial ^{212}Pb activity distribution and corresponding estimated dose was determined in seven LL2 tumors. Figure 3 shows the calculated dose map after 4 days from source insertion to tumor excision, and hematoxylin-eosin staining of a section taken from a tumor treated by a source carrying 32.7 kBq ^{224}Ra (with a ^{220}Rn desorption probability of 34%). The resulting dose maps were used to calculate the effective diameter of the region for which the asymptotic alpha particle dose (from source insertion to infinity) delivered by ^{212}Bi and ^{212}Po exceeds 5, 10, and 20 Gy. For tumors treated by sources with initial ^{220}Rn release rate of 0.22–0.6 (expressed in μCi), the effective diameter corresponding to 10 Gy/μCi was in the range of 4.3–4.7 mm. The asymptotic effective doses for 5 Gy and 20 Gy were 4.8 mm and 4.05 mm, respectively.

Effect of a single ^{224}Ra wire on the development of murine lung carcinoma tumors in C57BL/6 mice

LL2-bearing mice were treated using a single interstitial ^{224}Ra wire (DART wire) per animal. Wire insertion was performed when tumors reached an average diameter of 6–7 mm (longest diameter) and subsequent monitoring of both tumor volume and survival was conducted 3 times per week. Mice were divided randomly to three treatment group as follows:

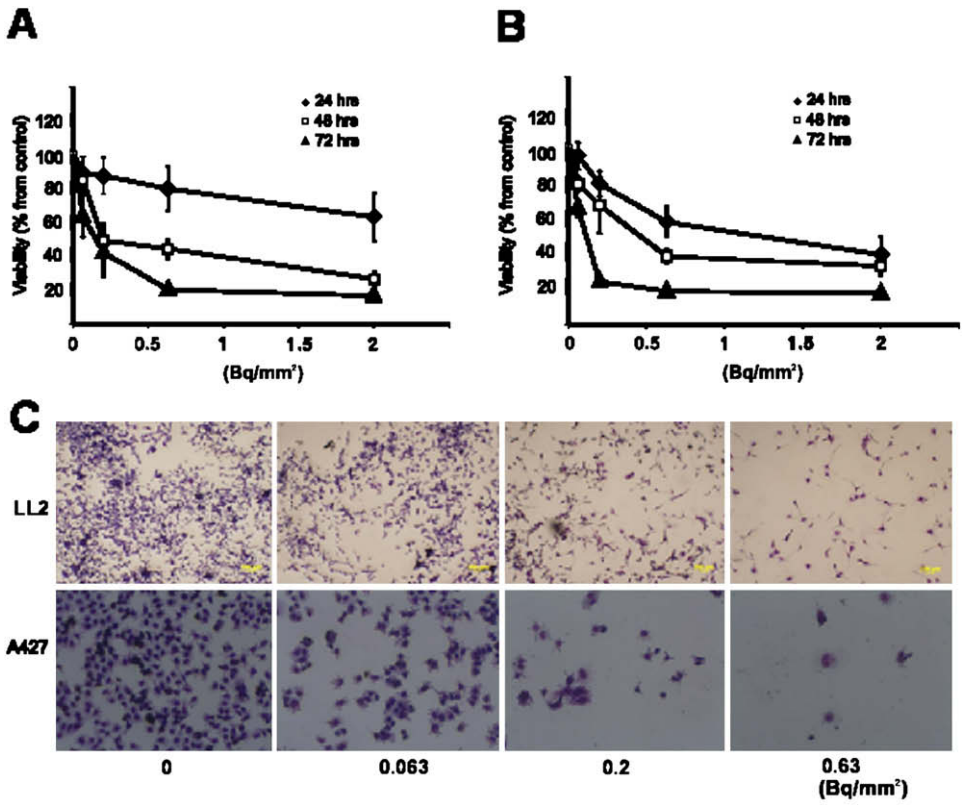

Fig. 1. (A) LL2 cells irradiated using ^{224}Ra implanted microplates for different durations. (B) A427 cells irradiated using ^{224}Ra-implanted microplates for different durations. (C) Photos of both cell lines: LL2 (×4) and A427 (×10).

not-treated group, consisting of tumor-bearing mice (n = 7) that received no treatment throughout the experiment; *inert* group consisting of tumor-bearing mice (n = 20) that were treated with non-radioactive wires identical in shape to the DART wires; *^{224}Ra wire* group consisting of tumor bearing mice (n = 16) treated with a single DART wire each. A significant tumor growth arrest was detected in the ^{224}Ra wire treatment group compared with both control groups. Twenty-one days posttreatment, the average tumor volumes of ^{224}Ra wire treated mice, was at least 39% lower then both inert and not-treated groups (Fig. 4A) with statistical significance Pv lower than 0.01. The ^{224}Ra wire treatment also affected life expectancy: Figure 4B reveals that the mean survival of animals treated with ^{224}Ra wires was increased by 31% and 44% compared with not-treated and inert groups, respectively. The statistical analysis comparing the ^{224}Ra wire group with each of the controls led to Pv < 0.05, whereas this value was 0.53 between inert and not-treated.

Effect of a single ^{224}Ra wire on the development of human lung carcinoma tumors in nude mice

After the assessment of the efficacy of a single DART wire in a murine model of lung carcinoma, a line of experiments was dedicated to a tumor derived from a human malignant tissue. We transplanted either A427 or NCI-H520 tumor cells in athymic nude mice and when tumors reached 6 mm (average diameter) we treated them with a single radioactive wire (^{224}Ra wire group) and monitored tumor development and survival compared to a group treated with inert wires (inert group). For the A427 model, the difference in tumor growth rate between the two treatment groups existed since the day of the treatment and increased with time. Whereas in the Inert treated group (n = 15), tumor volumes grew bigger in all the animals, 100% of the tumors receiving a ^{224}Ra wire (n = 14) shrunk at the initial phase of the experiment (first 7 days). Furthermore, 57% of the ^{224}Ra wire–treated tumors were completely eradicated during 120 days of inspection (experimental end point) as could be seen in Fig. 5A (Pv < 0.001). The difference between both groups remained robust when survival was examined (Fig. 5B). Eighty-five days after tumor transplantation, 53% of the animals in the inert group died, whereas all of the ^{224}Ra wire–treated mice were still alive. When all inert group mice died (after 106 days), 65% of the ^{224}Ra wire treatment group survived (Pv < 0.001). Figure 6 presents photos of nude mice 29 days after treatment with either an inert wire (a) or a ^{224}Ra wire (b). When mice bearing the NCI-H520 tumors were treated (n = 6 for each group), the effect was more moderate and the most prominent effect was recorded 20 days after the treatment, because ^{224}Ra wire–treated tumors showed growth inhibition of 42% compared with the inert group (Fig. 5C) with Pv = 0.106.

DISCUSSION

It was commonly hypothesized that alpha particles fail to serve as a suitable radiation modality for solid tumors from

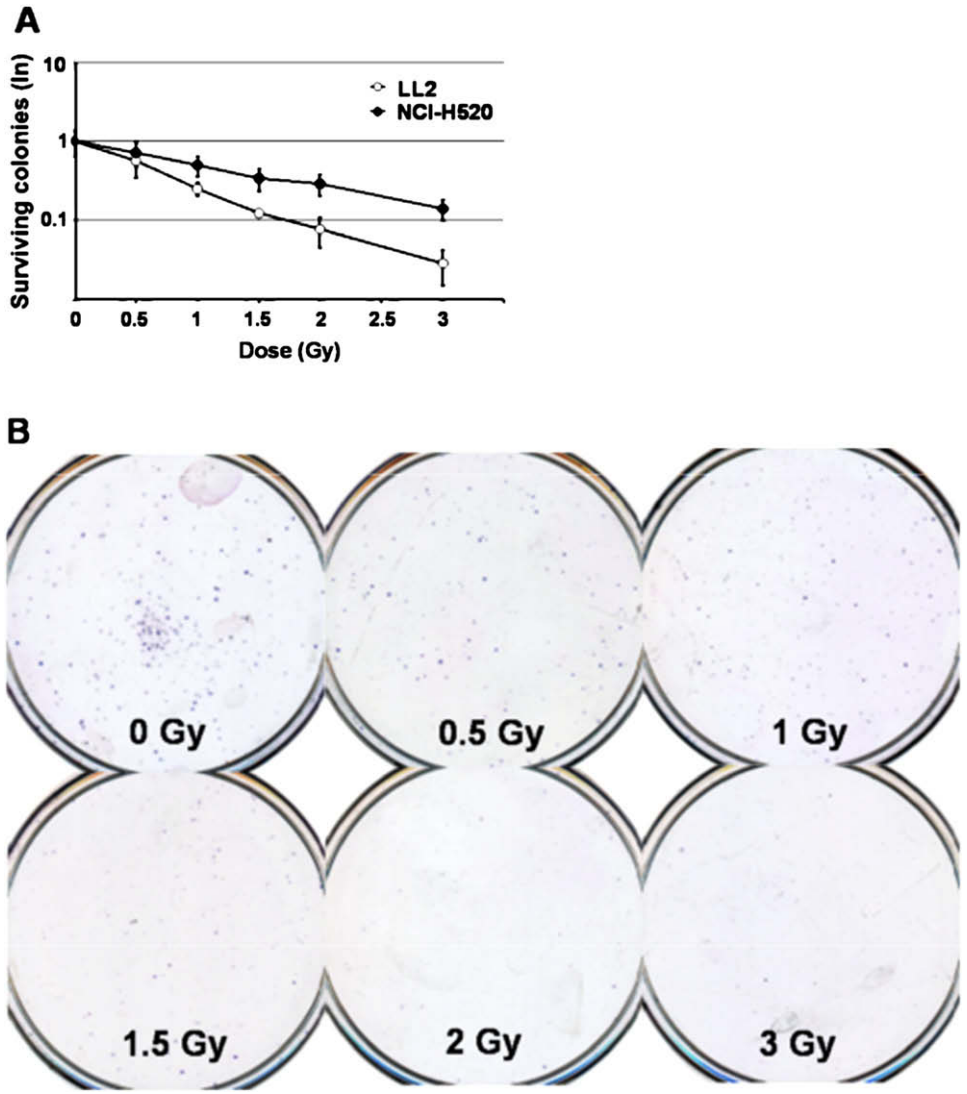

Fig. 2. Colony formation of cells irradiated with escalating intensities of alpha particles fluxes. (A) The logarithmic fraction of formed colonies (LL2 compared with NCI-H520) in respect of nonirradiated controls. (B) Stained LL2 colonies in 10-cm Petri dishes: 0, 0.5, 1, 1.5, 2, and 3 Gy.

their inability to travel considerable distances in tissues. In this study, we investigated the manner in which the daughters of ^{224}Ra fixed to a thin wire can spread through a lung malignancy, killing tumor cells through their alpha decays.

The ability of increasing doses of alpha radiation to affect the survival of cultured lung cancer cells was measured, demonstrating a clear dose response which increased with alpha particle flux and exposure duration. Proliferation rates dropped to 17% of the control in both examined cell lines (LL2, A427) after exposure for 3 days to an initial ^{224}Ra activity of 2 Bq/mm^2. Cells were also exposed to higher doses, which were applied for shorter periods. It was found that the calculated D_0 value differed substantially between the examined cell lines—0.8 Gy for LL2 cells compared with 1.5 Gy for NCI-H520.

We then examined whether the progenies of the utilized decay chain are practically able to disperses in the tumor and introduce their alpha particles to cancerous cells over therapeutically significant distances. For this purpose, we analyzed the activity profiles recorded on histological sections of treated tumors. The analysis demonstrated a rapid falloff of the dose with the distance from the source—from several thousand Gray near the source to ~10 Gy at a radial distance of 2–3 mm. The average diameter of the region subject to therapeutic dose levels (>10–15 Gy per 1 μCi of ^{220}Rn released from the source) was 4–5 mm. Hematoxylin-eosin staining after autoradiography demonstrated tissue necrosis over regions of comparable size.

In light of these results, we decided to study the effect that ^{224}Ra wires might have on the primary tumor volume as well as on mice survival. Murine tumors induced in C57BL/6 mice, were treated by radioactive wires and compared with two control groups, either untreated or treated with an inert wire. The ^{224}Ra wire treatment group showed significantly better performance in all measured parameters (*i.e.*, slower tumor growth rate and increased survival). LL2 tumors

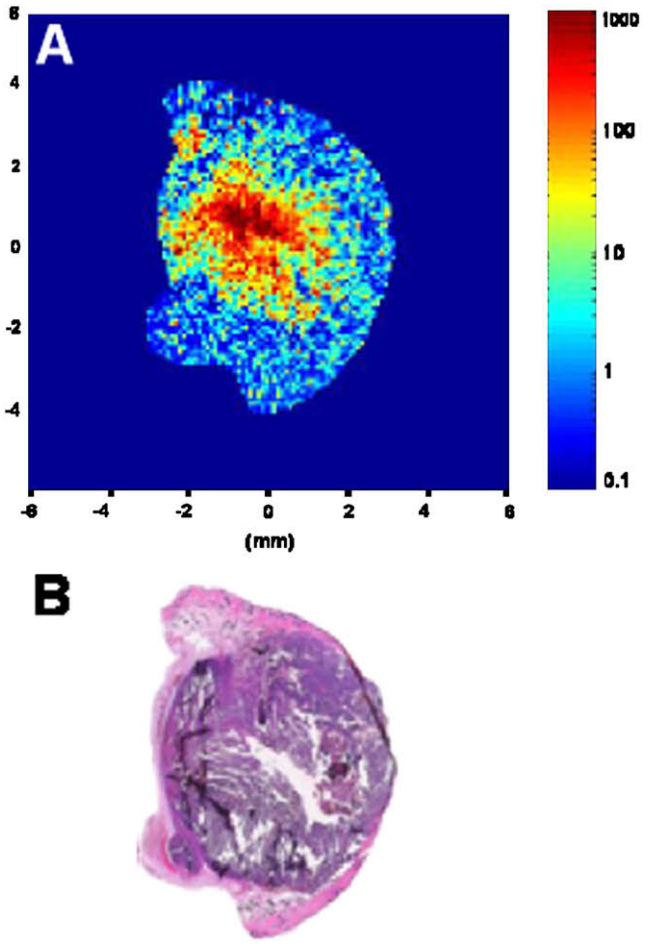

Fig. 3. Histological section of a LL2 tumor (492 mg) treated with a ^{224}Ra wire characterized with an initial ^{220}Rn release of 8.14 kBq. (A) Normalized asymptotic dose. (B) Hematoxin and eosin staining.

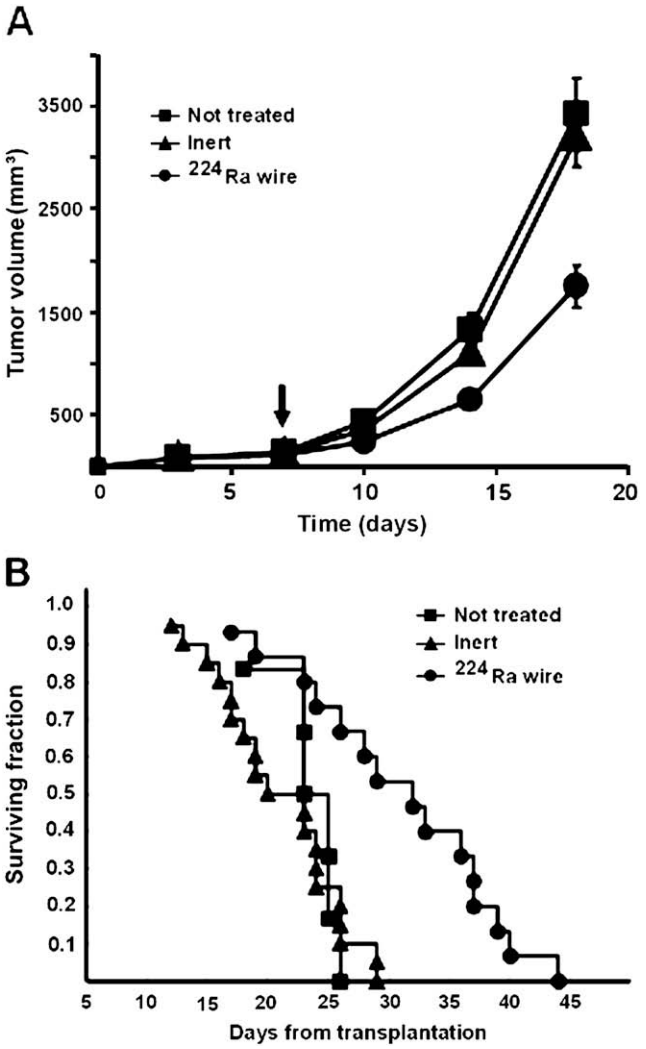

Fig. 4. Single ^{224}Ra wire intratumoral insertion for murine tumors. C57BL mice bearing LL2 tumors treated with a single ^{224}Ra wire and monitored for tumor growth and survival. Tumors were treated 7 days after tumor inoculation by the time tumors reached the size of 6–7 mm (average diameter). (A) Tumor development (standard errors are distinguished by bars). (B) Survival curve.

induced in mice are aggressive and very rapidly growing, causing death as early as 14 days posttumor inoculation). Hence, improving mean survival time from 21 days (Inert group) to 30.4 days (^{224}Ra wire group) could translate into a clinically meaningful effect.

In addition, the effect in two different human models was examined. A427-derived tumors were very responsive to the intratumoral alpha irradiation. One week after the treatment, tumors were 60% smaller in the radioactive treated group compared with the control—a difference that grew larger with time. Moreover, 8 of 14 mice receiving a single ^{224}Ra wire, were completely cured as opposed to none of the inert treated mice (Fig. 5A). The NCI-H520 tumors were more resistant to the treatment, and although growth rates of tumors treated with radioactive sources were somewhat lower, no statistically significant differences were measured (Fig. 5C). These findings were consistent with the outcomes of the above-mentioned *in vitro* examinations (Fig. 2) showing that NCI-H520 cells were clearly less sensitive to irradiation by alpha particles.

The factors determining the relative responsiveness of a given tumor model are to be more thoroughly investigated. On the cell level, microarray analyses gathered along recent years provide insights regarding genes determining the relative radiosensitivity of a given culture. The vast majority of these genes are associated with DNA repair, apoptosis, growth factor, signal transduction, cell cycle, and cell adhesion (17). A comparative study including cell lines of different alpha-radiation responsiveness is being conducted by our group examining the possible role of these factors. Another major point to be addressed is what can influence the dispersion pattern of the decay products of ^{224}Ra inside the tumor. Elucidating the role of parameters such as tissue fluidity (18) or generic vascularity (19) may help predict in the future whether a given tumor is a suitable candidate for DART. Furthermore, insights into the mechanisms controlling the radioactive spread and its effects in the malignant tissue might lead to the improvement of this technique.

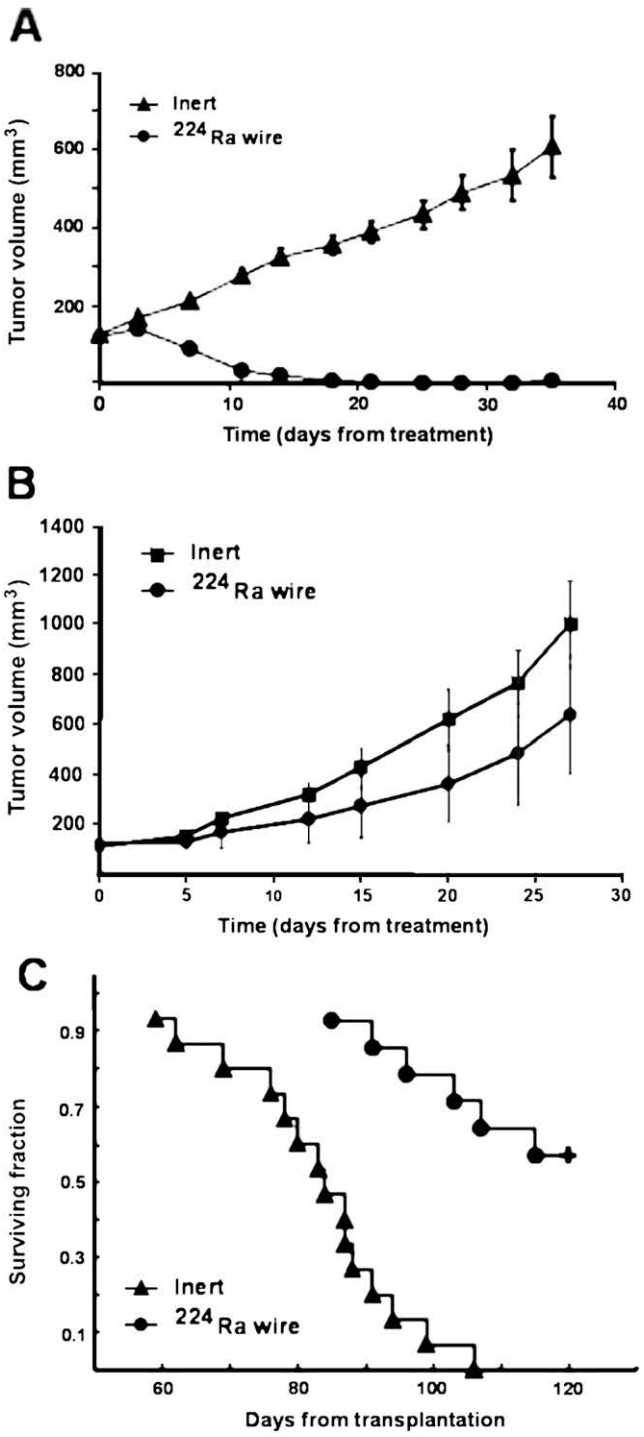

Fig. 5. Single ^{224}Ra wire intratumoral treatment of human derived tumors. Nude mice bearing A427 or NCI-H520 tumors treated with a single ^{224}Ra wire and monitored for tumor growth and survival. Tumors were treated when they reached the size of 5–6 mm (average diameter). (A) A427 tumor development (standard errors are represented by bars). (B) NCI-H520 tumor development (standard errors are represented by bars). (C) A427 survival curve.

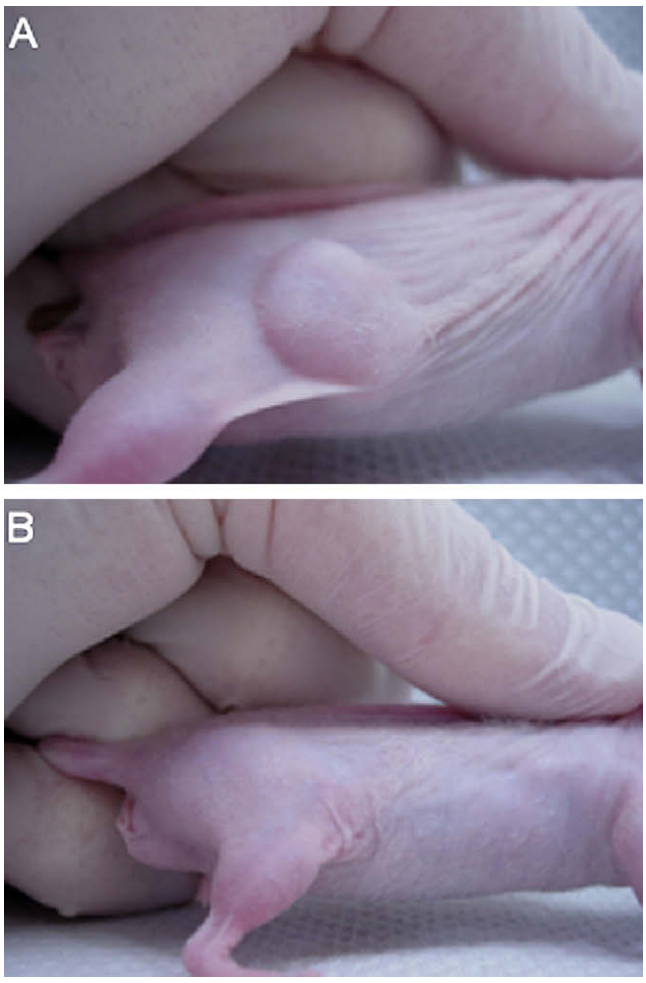

Fig. 6. (A) Inert treated mouse 29 days after tumor transplantation. (B) ^{224}Ra-wire treated mouse 29 days after tumor transplantation (27.2 kBq).

In most cases, lung cancer is incurable, even though current treatments can prolong life and enhance its quality (20, 21). Most of the efforts are dedicated to avoiding normal lung irradiation and allowing increasing dose and decreasing normal tissue toxicity. A major problem remains local tumor control and therefore new methods of delivering radiotherapy have been sought, including protons and carbon ions (22–24). Treating lung tumors with alpha particles should help to minimize the extent and severity of pulmonary injury. Such treatment could be delivered as a boost treatment to residual disease to enhance the chance of local control without increasing the risks of late radiation damage. It could also be proposed to patients with localized lung cancer, not amenable to surgery because of severe comorbidities.

To conclude, our findings show that alpha-emitting daughter atoms of ^{224}Ra were able to travel considerable distances inside lung carcinoma derived tumors, forming a region of destructive high-LET dose through their alpha decays and inducing local tumor control. The practical utilization of DART in the treatment of lung cancer evidently calls for the development of suitable delivery mechanisms that will allow for the placement of multiple sources inside the target region without the risk of lung puncture and collapse. Although technically challenging, such delivery schemes can rely on existing medical navigation technologies.

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