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Enhanced response to C225 of A431 tumor xenografts growing in irradiated tumor bed ☆

Oliver Riesterer [a,d,*], Kathryn A. Mason [a], Uma Raju [a], Qiuan Yang [a,e], Li Wang [a], Walter N. Hittelman [c], K. Kian Ang [b], Luka Milas [a]

[a] Department of Experimental Radiation Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, USA
[b] Department of Radiation Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, USA
[c] Department of Experimental Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, USA
[d] Department of Radiation Oncology, University Hospital Zurich, Switzerland
[e] Cancer Center, Qilu Hospital of Shandong University, Jinan, China

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ABSTRACT

Background and purpose: We recently demonstrated that C225 maintenance therapy after completion of radiotherapy further increased tumor radiocurability. The present study assessed mechanisms underlying the observed improvement in C225 efficacy in pre-irradiated tissue (tumor bed).
Materials and methods: A431 xenografts growing in pre-irradiated and non-irradiated tissue were treated with C225. Tumors were assessed for growth delay, cell proliferation, hypoxia, EGFR and VEGF expressions. *In vitro* clonogenic survival of cells derived from these tumors was also assayed.
Results: Pre-irradiation of tumor bed induced growth retardation, reduction in Ki-67 labeling, and overexpression of HIF-1α, CA IX, EGFR and VEGF biomarkers. C225 treatment dramatically inhibited tumor growth in the irradiated tumor bed ($P < 0.0001$), which was associated with further reduction in Ki-67 labeling, and reduced expression of HIF-1α, CA IX, EGFR and VEGF. Cells derived from tumors in the pre-irradiated bed showed increased sensitivity to C225. C225 was more cytotoxic against hypoxic than well-oxygenated A431 cells grown *in vitro*.
Conclusion: A431 xenografts growing in pre-irradiated tumor bed exhibit enhanced sensitivity to C225. Pre-irradiated tissue microenvironment seems to render tumor cells more susceptible to C225 cytostatic and cytotoxic actions. If confirmed in other tumor models these findings support the use of C225 maintenance therapy after completion of radiotherapy.
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C225 improves radiotherapy by augmenting susceptibility of tumor cells to radiation damage as well as by altering tumor microenvironmental factors [1,2]. The clinical proof of principle came from the randomized clinical trial by Bonner et al. [3] demonstrating that C225, when given concurrently with radiotherapy, significantly improved loco-regional tumor control and overall survival in head and neck cancer patients. However, in spite of the increased therapeutic benefit more than 50% of patients still developed loco-regional recurrences. Using A433 xenografts we recently demonstrated that a 7-day course of fractionated radiotherapy was enhanced by a factor of 1.8 when C225 was administered 3 times concurrent with radiotherapy and by a substantially larger factor of 2.7 when 3 additional doses were administered after completion of radiotherapy [4]. In addition, administration of C225 after radiotherapy improved not only tumor cure, but also significantly delayed local tumor recurrence in mice not cured of their primary tumor.

Mechanisms for this therapeutic improvement may include a number of possibilities. The minimal disease setting following definitive radiotherapy resembles that of tumor cell inoculations into an irradiated tissue environment. Prior studies suggested that irradiating the host tissue site increases the efficacy of tumor take [5,6], and the tumors that do grow have an increased propensity for metastatic spread [7,8] and resistance to treatment with chemotherapy and radiotherapy [9,10]. At the same time, tumors growing in irradiated sites exhibit a retarded growth rate, a phenomenon termed tumor bed effect (TBE) [6,11,12]. Prior studies have suggested that mechanisms underlying the tumor bed effect include compromised vascularization [13–15] resulting in increased proportions of hypoxic cells and increased necrosis [8,9,14].

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* Corresponding author. Address: Department of Radiation Oncology, University Hospital Zurich, 8091 Zurich, Switzerland.
E-mail address: oliver.riesterer@usz.ch (O. Riesterer).

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This study was undertaken to investigate whether tumors growing in the irradiated tissue bed exhibit enhanced sensitivity to C225 and to examine possible underlying mechanisms.

Material and methods

Mice, tumors and irradiation

Solitary tumors were produced by inoculation of 10^6 A431 tumor cells subcutaneously into the right hind leg of 3–5-month-old male nude (Ncr nu/nu) mice. The mice were irradiated using a small-animal irradiator with two parallel-opposed ^{137}Cs sources. Unanaesthetized mice were mechanically immobilized in a jig and the leg tumors were centered in a 3-cm-diameter circular field. Tumor bed irradiation was performed with 20 Gy single doses at 24 h before tumor cell inoculation.

Monoclonal antibody

Human mouse chimeric anti-EGFR mAb C225 was provided by ImClone Systems, Inc. (New York, NY). For *in vivo* experiments, three ip injections of 1 mg per mouse were given three days apart. For *in vitro* experiments, A431 cells were exposed to C225 at a concentration of 30 nM.

Cells, culture medium and immunohistochemistry

Human A431 epidermoid carcinoma cells were purchased from ATCC and maintained in Dulbecco's-Modified Eagle's Medium (DMEM/F12) supplemented with fetal bovine serum to a final concentration of 10%.

For immunohistochemical analysis three tumors for each condition were excised three days after the second dose of C225. Primary antibodies used were mouse anti-human Ki-67 antibody (clone B56, BD Pharmingen, San Diego, CA; 1:30), mouse anti-human EGFR antibody (clone 31G7, ZYMED, South San Francisco, CA), mouse monoclonal anti-HIF-1α antibody (Novus Biologicals, Littleton, CO), rabbit anti-human CA IX antibody (Abcam, Cambridge, MA; 1:500) and rabbit anti-human anti-VEGF antibody (A-20, Santa Cruz Biotechnologies, Santa Cruz, CA; 1:100). Secondary antibodies used were fluorescein (FITC)-conjugated donkey anti-rabbit IgG, biotinylated anti-mouse IgG and biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). The Ki-67 labeling index was defined as the percentage of positive nuclei out of at least 10^3 cells counted per tumor.

Clonogenic cell survival assays

Tumor cell suspensions from A431 tumor xenografts were prepared as described previously (15). Viable tumor cells in the cell suspension were determined by trypan blue exclusion. Defined numbers of viable tumor cells were plated in 100 mm dishes. After 24 h C225 was added to half of the dishes and the cells were allowed to grow to colonies for an additional 8 days.

For comparison of hypoxic versus normoxic culture conditions cells from *in vitro* culture were plated in 6-well plates and allowed to attach over night before half of the plates were transferred to a hypoxia chamber. The plates were incubated either in humidified air, 5% CO_2 (normoxia) or in 1% O_2, 5% CO_2, 94% N_2 (hypoxia) using an InVivo2 Hypoxia Workstation 400 (Ruskinn Technology Ltd., Pencoed, UK). After 24 h in hypoxic conditions the cells were treated with C225 and after an additional 24 h they were returned to a normoxic incubator.

Clonogenic survival curves were constructed from at least three independent experiments by fitting the average survival levels using least squares regression by the linear quadratic model. Colonies with more than 50 cells were counted under a dissection microscope.

Statistical analysis

Statistical analysis was done using GraphPad Software. In all cases the unpaired *t*-test was used with the two-sided significance level set at $P < 0.05$.

Results

Irradiated tumor bed enhanced tumor response to treatment with C225

Tumor bed irradiation caused a delay in tumor appearance of several days; it took 17.5 ± 1.1 days for tumors to grow to 5 mm in diameter in irradiated tumor bed as compared to 10.3 ± 0.3 days for tumors to reach the same size in non-irradiated tumor bed ($P = 0.001$).

Irradiated tumor bed strongly influenced the growth of established tumors on its own as well as response to C225 (Fig. 1). Tumors in non-irradiated tissue needed 10.3 ± 1.0 days to grow from 5 mm to 12 mm, whereas tumors in the pre-irradiated tissue required a highly significantly longer time, 60.3 ± 7.2 days, to grow to the same size ($P < 0.0001$), indicating that A431 xenografts exhibited a very strong TBE. While C225 slowed the growth of tumors in non-irradiated tumor bed for only a few days (13.4 ± 1.1), it dramatically slowed the growth of tumors in pre-irradiated tissue (112.6 ± 15.3 days, $P < 0.0001$), an increased growth delay of nearly 10-fold.

Increased inhibition of cell proliferation in tumors growing in irradiated bed

The Ki-67 labeling index was 82.8 ± 1.8% in tumors growing in non-irradiated tissue and 53.4 ± 5.0% in tumors growing in pre-irradiated tissue ($P = 0.005$) (Fig. 2). Treatment with C225 inhibited cell proliferation in tumors growing in both non-irradiated [45.2 ± 3.9% ($P = 0.0006$)] and pre-irradiated tumor beds [13.2 ± 2.4% ($P < 0.0001$)]. This effect of C225 was greater in pre-irradiated tumor bed where labeling index was reduced by 4-fold as compared to the reduction of 1.8-fold in tumors growing in non-irradiated tumor bed.

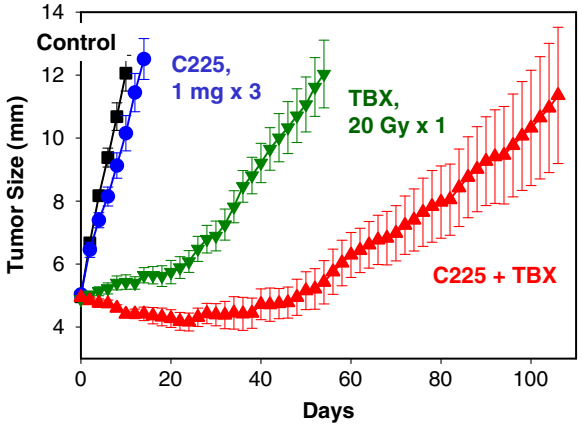

Fig. 1. Effect of C225 on the growth of A431 xenografts in irradiated tumor bed. The error bars show mean values of 10–11 mice per group ± SE.