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Béregère Piron, Salomé Paillas, Vincent Boudousq, André Pèlerin, Caroline Bascoul-Mollevi, et al.. DNA damage-centered signaling pathways are effectively activated during low dose-rate Auger radioimmunotherapy.: Signaling pathways induced by 125I-RIT. Nuclear Medicine and Biology, Elsevier, 2014, 41 Suppl, pp.e75-83. 10.1016/j.nucmedbio.2014.01.012 . inserm-00981411

**HAL Id: inserm-00981411**

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Submitted on 22 Apr 2014

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**DNA damage-centered signaling pathways are effectively activated during low dose-rate Auger radioimmunotherapy**

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Running title: Signaling pathways induced by <sup>125</sup>I-RIT

Key words: Radioimmunotherapy, Auger electrons, signaling pathways, DNA damage, <sup>125</sup>I-mAbs

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*Introduction:* Low dose-rate radioimmunotherapy (RIT) using  $^{125}\text{I}$ -labelled monoclonal antibodies ( $^{125}\text{I}$ -mAbs) is associated with unexpected high cytotoxicity per Gy. *Methods:* We investigated whether this hypersensitivity was due to lack of detection of DNA damage by the targeted cells. DNA damage were measured with the alkaline comet assay, gamma-H2AX foci and the micronucleus test in  $p53^{-/-}$  and  $p53^{+/+}$  HCT116 cells exposed to increasing activities of internalizing anti-HER1  $^{125}\text{I}$ -mAbs or non-internalizing anti-CEA  $^{125}\text{I}$ -mAbs. The expression of proteins involved in radiation response and progression of cells through the cycle were determined. *Results:* Cell hypersensitivity to low absorbed doses of anti-CEA  $^{125}\text{I}$ -mAbs was not due to defect in DNA damage detection, since ATM (ataxia telangiectasia mutated gene), gamma-H2AX, p53 and p21 were activated in RIT-treated HCT116 cells and G2/M cell cycle arrest was observed. Moreover, the alkaline comet assay showed that DNA breaks accumulated when cells were placed at  $4^{\circ}\text{C}$  during exposure but were repaired under standard RIT conditions ( $37^{\circ}\text{C}$ ), suggesting that lesions detected under alkaline conditions (mostly DNA single strand breaks and alkali-labile sites) are efficiently repaired in treated cells. The level of gamma-H2AX protein corroborated by the level of foci measured in nuclei of treated cells was shown to accumulate with time thereby suggesting the continuous presence of DNA double strand breaks. This was accompanied by the formation of micronuclei. *Conclusion:* Hypersensitivity to non-internalizing  $^{125}\text{I}$ -mAbs is not due to lack of detection of DNA damage after low absorbed dose-rates. However, DNA double strand breaks accumulate in cells exposed both to internalizing and non-internalizing  $^{125}\text{I}$ -mAbs and lead to micronuclei formation. These results suggest impairment in DNA double strand breaks repair after low absorbed doses of  $^{125}\text{I}$ -mAbs.

## INTRODUCTION

Auger electrons are produced in cascades during electronic shell rearrangement consecutive to electronic capture and/or conversion processes in unstable atoms [1]. They have a very low energy (from few eV to few keV) and are considered as high LET particles (from 4 to 26 keV/ $\mu\text{m}$ ) when their energy is less than 1 keV. Consequently, their path length in biological matter is very short, between about 2 nm and 500 nm for most of them (for reviews see [2-8]). Due to their physical characteristics, efforts have been done to bring Auger electrons in the cell nucleus to obtain the highest cytotoxicity (for review [5]).

However, although clinical radioimmunotherapy (RIT) studies using Auger electrons labeled to monoclonal antibodies (mAbs) have not given clear-cut results [9-12], we and others have shown that, in mice, Auger electrons could efficiently delay growth of small solid tumors [13-18]. This efficacy remains to be elucidated because the final localization of  $^{125}\text{I}$ -mAbs (and the subsequent energy deposit [19]) upon binding to their receptors is the cell membrane (non-internalizing mAbs) or the cytoplasm (internalizing mAbs) after a receptor-mediated internalizing process. Therefore, for both types of mAbs, most of the energy is released in extra-nuclear compartments. Moreover, we previously reported in several cell lines that non-internalizing  $^{125}\text{I}$ -mAbs are more toxic than internalizing  $^{125}\text{I}$ -mAbs [19, 20]. This higher cytotoxicity is p53-independent (differently from internalizing  $^{125}\text{I}$ -mAbs) and does not involve cell membrane-mediated apoptotic mechanisms [19].

In conventional external beam radiation therapy (CEBRT), the nucleus is the key target of ionizing irradiation. However, the comparison between CEBRT and RIT is not straightforward. Indeed, CEBRT delivers high radiation doses (between 40 and 80 Gy) at high dose-rate (1—2 Gy.min<sup>-1</sup>) that are fractionated in daily 2 Gy doses and the biological response to CEBRT is correlated with both dose and dose-rate [21, 22]. The cell response to radiation is mostly triggered by DNA double strand breaks (DSBs) the occurrence of which is strictly

proportional to the mean nucleus absorbed dose [21]. DSBs initiate different signaling pathways that involve ATM, a DNA damage sensor molecule. In turn, ATM activates proteins (particularly, H2AX and p53) that participate in cell cycle arrest, apoptosis and DNA repair (for reviews see [23-26]). Conversely, RIT is characterized by protracted exposure (hours to days), resulting in low total doses (10—30 Gy compared to 40 —80 Gy in EBRT) delivered at low dose-rates ( $<1\text{Gy}\cdot\text{h}^{-1}$ ), and is accompanied by a strong heterogeneity in subcellular energy deposits. These physical features provide cancer cells with an opportunity for DNA repair during RIT. However, several studies in mice have shown that RIT cytotoxicity per unit of absorbed dose is higher than that of a single fraction of radiation delivered at higher dose-rate in EBRT [27, 28]. Hypotheses have been proposed for explaining such increased efficacy per Gy (for review [29]).

Here, we investigated whether the higher RIT cytotoxicity per unit of absorbed dose was due to lack of detection of DNA damage induced by low dose-rate RIT in  $p53^{-/-}$  and  $p53^{+/+}$  HCT116 cells targeted with  $^{125}\text{I}$ -mAbs.

## **MATERIALS AND METHODS**

### **Cell lines**

HCT116 ( $p53^{+/+}$ ) human colorectal cancer cells were from ATCC. The  $p53^{-/-}$  HCT116 cell line was a gift from Professor Bert Vogelstein (Johns Hopkins University). HCT116 cells express HER1 and basal level of CEA receptors. They were grown in RPMI supplemented with 10% heat-inactivated FBS, 100  $\mu\text{g}/\text{mL}$  L-glutamine and antibiotics (0.1 unit/mL penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin). A-431 and SK-OV-3 cells were previously described in [20].

### **Antibodies and radiolabelling**

The internalizing m225 mAb [19, 20] was used for targeting the Epidermal Growth Factor Receptor (EGFR/HER1) expressed in HCT116 and A-431 cells. The internalizing Trastuzumab (Herceptin<sup>®</sup>, Genentech Incorporated, San Francisco CA, USA) binds to Epidermal Receptor type 2 (HER2) expressed in SK-OV-3 cells [20] and the non-internalizing murine IgG1k mAb 35A7 targets CEA in all the cell lines [19, 20]. The non-targeting IgG1 mAb PX was used as control [19, 20]. MAbs were radiolabelled with <sup>125</sup>I using the conventional IODO-GEN method (1,3,4,6-tetrachloro-3 $\alpha$ , 6 $\alpha$ -diphenylglycoluryl) described in [20].

### **Cellular dosimetry**

The mean nucleus absorbed dose was previously calculated for HCT116 cells exposed to increasing activities (0—4MBq/ml) of <sup>125</sup>I-mAbs [19]. Briefly, the MIRD cellular approach [30] that requires the determination of the total cumulative number of decays ( $\tilde{A}_{rs}$ ) occurring in cells and the *S*-values, was used. Then, for each test activity, radioactivity uptake per cell (Bq/cell) was determined as described in [20], and used to estimate  $\tilde{A}_{rs}$ . For all targeting models,  $\tilde{A}_{rs}$  was then multiplied by the corresponding *S*-value to obtain the mean nucleus absorbed dose. For *S*-value calculations, three sources of irradiation were considered: self-irradiation from radiolabelled vectors that were internalized in the cytoplasm (anti-HER1 <sup>125</sup>I-mAbs) or bound to the cell membrane (anti-CEA <sup>125</sup>I-mAbs); culture medium irradiation from unbound radiolabelled vectors; and cross-fire irradiation. Cell size was measured by fluorescence microscopy after propidium iodide staining and the cell radius (distributed normally within the HCT116 cell population) was 5.6 $\pm$ 1.0  $\mu$ m when the nucleo-cytoplasmic ratio was about 0.65. The cellular radioactivity was assumed to be uniformly distributed within cytoplasm and at cell surface for internalizing and non-internalizing <sup>125</sup>I-mAbs, respectively.

### **Alkaline single-cell gel electrophoresis**

The alkaline single-cell gel electrophoresis assay (comet assay) was used to measure DNA damage in  $3 \times 10^5$  HCT116 cells grown in 6-well plates and exposed to 0 and 4 MBq/mL of  $^{125}\text{I}$ -mAbs. The comet assay was carried out either i) under standard RIT conditions (i.e., cells at  $37^\circ\text{C}$  for the entire RIT duration) at different time points (15 min, 1h, 2h, 3h, 6h, 12h, 24h and 48h), or ii) following pre-incubation of cells in cold medium at  $4^\circ\text{C}$  for 1h and RIT at  $4^\circ\text{C}$  for 4h. Samples were collected at 15 min, 30 min, 1h, 2h, 3h and 4h after RIT onset. For both conditions ( $37^\circ\text{C}$  and  $4^\circ\text{C}$ ), the methodology described in [31] was used. The tail moment (TM) was the parameter retained for analysis. The mean TM, expressed in arbitrary units (a.u.), was measured in 100 randomly chosen cells per slide for each time point and was calculated with the Comet Imager 2.0 software (Metasystems, Hamburg, Germany). Three slides for each condition and each time point were analyzed per experiment and experiments were repeated three times.

### **Micronucleus assay**

$3 \times 10^4$  HCT116 cells were seeded in 24-well plates. HCT116 cells exposed to activities between 0 and 4MBq/mL of  $^{125}\text{I}$ -mAbs for 2 days. Twenty four hours before selected time points (day 1, day 2 and 3 post onset of RIT), cytochalasin B was added to the culture medium at a final concentration of  $5 \mu\text{g/mL}$  in order to block cytokinesis and was maintained for 24 hours. Cells were then harvested and centrifuged. Supernatant was carefully discarded and the pellet was treated with KCl 125 mM under constant shaking for hypotonic chock. Cells were then fixed three times in acetic acid:ethanol (1:6), dropped onto slides under humidified atmosphere and air dried. Before analysis, slides were stained with propidium iodide ( $500 \mu\text{g/mL}$ ). Experiments were repeated three times in triplicate.

### **Protein extraction and western blotting**

$5 \times 10^5$  HCT116 and  $4 \times 10^5$  A-431 and SK-OV-3 cells were grown in 6-well plates and exposed to 0 and 4 MBq/mL of  $^{125}\text{I}$ -mAbs for 2 days. Proteins were extracted as described in [19]. Membranes were pre-incubated with 5% milk in PBS/0.1% Tween 20 and then with anti-p53 (1:10,000), -p21 (1:500) (Santa-Cruz Biotechnology, CA, USA), -gamma-H2AX (1:1000) (Merck Millipore, Guyancourt, France) or p-ATM (1:1000) (Cell Signaling Technology, Danvers, MA, USA) primary antibodies, followed by horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:10,000; Sigma-Aldrich, Saint-Louis, MO, USA). GAPDH level was used to evaluate protein loading. Levels of protein expression were quantified using the G-Box system (Syngene; Cambridge UK).

### **Gamma-H2AX immunofluorescent measurement**

For double strand breaks formation,  $2 \times 10^4$  cells were grown on coverslip and were exposed for 2 days to 0 and 4 MBq/mL of  $^{125}\text{I}$ -mAbs. Cells were washed twice with PBS and fixed in 3.7% formaldehyde in PBS for 30 min. They were then permeabilized at room temperature for 30 min using PBS/Triton (0.5%). Cells were next washed twice with PBS, saturated with PBS/BSA (1mg/ml) for 1 hour before incubation overnight with anti-gamma-H2AX (1:200 PBS/BSA; Merck Millipore). Next, coverslips were incubated for 1h in the dark with FITC-labeled goat anti-mouse Ig (Sigma) in PBS-BSA and next washed three times with PBS-BSA and once with PBS before analysis.

### **Cell cycle**

$20 \times 10^4$  HCT116 cells were grown in 6-well plates one day before RIT. Cells were harvested at day 0, 1, 2, 3, 4 and 7 of RIT by trypsinization and washed twice with PBS. They were then fixed in 70% ethanol at  $-20^\circ\text{C}$  for 3 hours and stained with cell cycle kit reagent from Merck Millipore (Merck Millipore, Guyancourt, France) in the dark for 30 min at room temperature before analysis using an Muse<sup>®</sup> flow cytometer (Merck Millipore, Guyancourt, France). The



percentage of cells in G0/G1, S and G2/M phases was then calculated (mean of three experiments in triplicate).

### Statistical analysis

The linear mixed regression model (LMRM) [20] was used for statistical analysis of the data.

## RESULTS

### DNA damage formation and repair occur simultaneously during RIT

We investigated the occurrence of DNA single (SSBs) and double (DSBs) strand breaks and alkali-labile sites (ALSs) in  $p53^{+/+}$  and  $p53^{-/-}$  HCT116 cells upon exposure to 4 MBq/mL of anti-CEA (35A7), anti-HER1 (m225) or non-targeting (PX)  $^{125}\text{I}$ -mAbs for 2 days. The mean nucleus doses were calculated [19] using the MIRD cellular formalism. Under standard RIT conditions (at 37°C), occurrence of DNA damage (as indicated by the TM parameter) was not significantly different in HCT116 cells treated with anti-HER1 or anti-CEA  $^{125}\text{I}$ -mAbs in comparison to untreated cells ( $p=0.85$  and  $p=0.72$ , respectively) (Fig. 1).

This finding suggests that radiation-induced DNA damage formation was compensated by DNA repair during RIT. In order to confirm this hypothesis, we investigated the level of DNA breaks in cells incubated with  $^{125}\text{I}$ -mAbs at 4°C for 4h (instead of 48h at 37°C as used under standard RIT). Temperature of 4°C is known to block enzymatic systems involved in DNA breaks repair. The 4h exposure time was determined as the longest time of exposure at 4°C producing no cytotoxicity. We observed under these conditions a progressive increase in DNA damage in  $p53^{+/+}$  and  $p53^{-/-}$  HCT116 cells exposed to anti-CEA and anti-HER1  $^{125}\text{I}$ -mAbs in comparison to those treated with non-targeting  $^{125}\text{I}$ -mAbs (Fig. 2A and B). These data confirmed that DNA repair occurred during standard RIT while DNA breaks accumulated at 4°C. We next calculated, the corresponding absorbed doses delivered to the nucleus by 4 MBq/mL  $^{125}\text{I}$ -mAbs. We thus considered the cumulative number of decays ( $\tilde{A}_{rs}$ ) calculated over 4h (and not 48h like under standard RIT). We also considered that

internalization of mAbs was blocked at 4°C such that *S*-values for cell surface localization were used for internalizing mAbs. Absorbed doses were then shown to be lower than under standard RIT (anti-HER1 <sup>125</sup>I-mAbs: from 43 Gy at 37°C to 1.92 Gy at 4°C; anti-CEA <sup>125</sup>I-mAbs: from 1.20 Gy to 0.1 Gy; non-targeting <sup>125</sup>I-mAbs from 0.27 Gy to 0.015). Corresponding highest TM values were 12.8 (anti-HER1 <sup>125</sup>I-mAbs), 6.0 (anti-CEA <sup>125</sup>I-mAbs) and 2.8 (non-targeting <sup>125</sup>I-mAbs) a.u. in *p53*<sup>+/+</sup> HCT116 cells and 10.4 (anti-HER1 <sup>125</sup>I-mAbs), 5.9 (anti-CEA <sup>125</sup>I-mAbs) and 4.5 (non-targeting <sup>125</sup>I-mAbs) a.u. in *p53*<sup>-/-</sup> cells. In cells exposed to anti-HER1 <sup>125</sup>I-mAbs, TM increased linearly as a function of the absorbed dose in both *p53*<sup>+/+</sup> and *p53*<sup>-/-</sup> HCT116 cells (p<0.001). Following exposure to anti-CEA <sup>125</sup>I-mAbs, TM increase was linear in *p53*<sup>+/+</sup> HCT116 cells (p<0.007), but not in *p53*<sup>-/-</sup> cells (p=0.103), where it exhibited a larger standard error (Fig. 2A and B). However, comparison of the efficacy of anti-CEA and anti-HER1 <sup>125</sup>I-mAbs showed that, overall, they produced quantitatively similar effects in both *p53*<sup>-/-</sup> (p=0.251) and *p53*<sup>+/+</sup> (p=0.172) HCT116 cells, because anti-CEA <sup>125</sup>I-mAbs (with lower mean nucleus absorbed doses) were more efficient per Gy than anti-HER1 <sup>125</sup>I-mAbs (with higher mean absorbed doses) in producing DNA damage. Moreover, the effect of each antibody was similar in the two cell lines (p=0.681 for anti-CEA <sup>125</sup>I-mAbs and p=0.372 for anti-HER1 <sup>125</sup>I-mAbs).

### **DNA damage-mediated signaling pathways are efficiently activated in HCT116 cells**

Under standard RIT conditions, the comet assay results suggested activation of DNA repair mechanisms. We thus assessed whether such low levels of RIT-induced DNA damage could activate ATM, H2AX and p53 (one of the key proteins of the radiation response) (Fig. 3). Western blot analysis revealed that phosphorylated ATM (p-ATM) and p53 level increased in *p53*<sup>+/+</sup> HCT116 cells exposed to RIT at 37°C and were associated with p21 up-regulation (Fig. 3A). The extent of their induction was not correlated with the nucleus absorbed dose because it was only slightly higher upon exposure to anti-HER1 <sup>125</sup>I-mAbs than to anti-CEA

$^{125}\text{I}$ -mAbs, although much different doses were delivered to the nucleus (43 Gy versus 1.2 Gy). Detection of gamma-H2AX indicated that DNA DSBs were continuously produced under standard RIT conditions (Fig. 3A), while no increase could be observed using the comet assay which detects all kind of breaks (DSBs + SSBs + ALSs). Similar results were obtained in  $p53^{-/-}$  HCT116 cells for p-ATM, gamma-H2AX, and p21 (Fig. 3B).

Activation of p53 was also observed in A-431 ( $p53^{+/+}$ ), but not in SK-OV-3 ( $p53^{-/-}$ ) cells exposed to  $^{125}\text{I}$ -mAbs (Supplementary Fig. 1). These two cell lines are more sensitive to non-internalizing anti-CEA than to internalizing anti-HER1/HER2  $^{125}\text{I}$ -mAbs [20]. In HCT116 and A-431 cells, p53 up-regulation is followed by induction of apoptosis at day 2 post-RIT [19]. These results suggest that the DNA damage induced by mean nucleus absorbed doses as low as 1.2 Gy can be efficiently detected in cells exposed to RIT.

The p21 protein up-regulation measured in HCT 116 cells was accompanied by cell cycle arrest in G2/M phase in both  $p53^{+/+}$  and  $p53^{-/-}$  cells upon exposure to anti-CEA and anti-HER1  $^{125}\text{I}$ -mAbs (Fig. 6). In both cell lines, the % of cells in G2/M was shown to increase significantly at day 1 post onset of RIT up to day 7.

### **Double strand breaks detection**

Double strand breaks (DSBs) as revealed by gamma-H2AX foci detection were produced in  $p53^{-/-}$  and  $p53^{+/+}$  HCT 116 cells exposed for 2 days to 4MBq/mL of either internalizing anti-HER1 or non-internalizing anti-CEA  $^{125}\text{I}$ -mAbs (Fig.4). DSBs were detected as early as 6h post incubation with radiolabeled mAbs and their yield was continuously increasing in a similar way for both types of mAbs, independently of the mean nucleus absorbed dose. Moreover, they were still detected 24h after the radioactivity was removed, namely 72 h post onset of RIT. These data suggest that DSBs are continuously formed under RIT using  $^{125}\text{I}$ -mAbs and that they are not accurately repaired and accumulate with time.

### **Yield of micronuclei as a function of the mean nucleus absorbed dose**

Micronuclei per binucleated cells (MN/BN) were produced in both  $p53^{+/+}$  and  $p53^{-/-}$  HCT116 cells during RIT using  $^{125}\text{I}$ -mAbs, suggesting error-prone DNA repair. In  $p53^{+/+}$  HCT116 cells, their formation at 24h post onset of RIT using either internalizing anti-HER1 or non-internalizing anti-CEA  $^{125}\text{I}$ -mAbs was increasing with the test activity. For non-internalizing  $^{125}\text{I}$ -mAbs, yield of MN at 4MBq/mL was higher at 24h ( $0.34\pm 0.06$  MN/BN versus  $0.14\pm 0.06$  MN/BN at 0MBq/mL) than at 48h ( $0.20\pm 0.11$  MN/BN) and it decreased to background level at 72h ( $0.12\pm 0.08$ ). Similar observation could be done with internalizing  $^{125}\text{I}$ -mAbs ( $0.53\pm 0.02$  MN/BN at 24h,  $0.22\pm 0.04$  MN/BN at 48h,  $0.14\pm 0.12$  MN/BN at 72h). It must be noted that yield of MN/BN in cells treated with non-specific  $^{125}\text{I}$ -PX mAbs remained at the background level (between  $0.08\pm 0.03$  and  $0.14\pm 0.06$  MN/BN).

In  $p53^{-/-}$  HCT116 cells, relationship between MN yield and test activity was less pronounced (Fig. 5). This can be partly due to a higher background level in non-treated cells (around between 0.15 and 0.27 MN/BN) and to large uncertainties associated to MN measurement. Similarly, no clear trend of a decrease between 24h and 48h in MN yield was observed.

### **DISCUSSION**

We showed previously in HCT116 cells targeted by 4MBq/mL of  $^{125}\text{I}$ -mAbs (Fig. 7 and [19]) that a mean nucleus absorbed dose of 1.2 Gy of non-internalizing anti-CEA  $^{125}\text{I}$ -mAbs is as effective as 43 Gy of internalizing anti-HER1  $^{125}\text{I}$ -mAbs. As the cell response to ionizing radiation is generally, in CEBRT, triggered by nuclear DNA damage, we thus investigated the relationship between the energy deposited in the nucleus (*i.e.*, the mean nucleus absorbed dose) and several biological endpoints during RIT with  $^{125}\text{I}$ -mAbs. Non-internalizing anti-CEA  $^{125}\text{I}$ -mAbs and, to a lower extent, internalizing anti-HER1  $^{125}\text{I}$ -mAbs, were very efficient in killing cells [19] and also in producing DNA damage despite lower dose-rates (about 0.025

Gy.h<sup>-1</sup> for anti-CEA <sup>125</sup>I-mAbs and 0.9 Gy.h<sup>-1</sup> for anti-HER1 <sup>125</sup>I-mAbs) than in CEBRT (about 2 Gy.min<sup>-1</sup>). The high efficiency of <sup>125</sup>I-mAbs, particularly of anti-CEA <sup>125</sup>I-mAbs per unit-dose, is reminiscent of low-dose hyper-radiosensitivity and of the inverse dose-rate effect. These two phenomena, which were reported in CEBRT (for review [32]) after low dose (below 0.5 Gy) or low dose-rate (0.02–1.00 Gy.h<sup>-1</sup>) exposure, lead to increased effectiveness of radiation per unit-dose beyond what could be predicted by the conventional linear and linear quadratic radio-biologic models ([33] and for review [32]). It has been proposed that they are the result of decreased sensing of DNA damage by signaling proteins and of the consequent reduced activation of the early DNA damage response through ATM and p53 or the G2 checkpoint ([34] and for review [29]). Our findings do not support this hypothesis. Indeed, ATM, H2AX and p21 were efficiently activated in *p53*<sup>-/-</sup> and *p53*<sup>+/+</sup> HCT116 cells and p53 expression was also increased (in *p53*<sup>+/+</sup> HCT116 cells and A-431 cells) during RIT with <sup>125</sup>I-mAbs. Moreover, most of the DNA breaks were immediately repaired under standard RIT conditions (comet assay data, Fig. 1 and 2). However, the significant increase in expression and accumulation of gamma-H2AX, due to phosphorylation of H2AX at DSB sites [35] in cells treated with anti-CEA or anti-HER1 <sup>125</sup>I-mAbs indicates that DSBs were continuously formed and not repaired. Consequently, mis- or un-repaired DSBs might have led to micronuclei formation (Fig. 4) and to mitotic death of damaged cells. These data indicate that acute cell death associated with low doses and low dose-rate of <sup>125</sup>I-mAbs (particularly of non-internalizing anti-CEA <sup>125</sup>I-mAbs) was not due to defective detection of DNA damage by the cells. Impaired repair of double strand breaks would be involved in low dose rate efficacy of RIT using <sup>125</sup>I-mAbs in a non-dependent dose-effect relationship.

Another intriguing observation is the lack of relationship between the mean nucleus absorbed doses (in Gy) and these biological endpoints when comparing the efficacy/Gy of anti-CEA

and anti-HER1  $^{125}\text{I}$ -mAbs. In CEBRT, the biological effects and, consequently, the survival of irradiated cells are correlated with the mean nucleus absorbed dose [36], according to a linear or linear quadratic relationship depending on whether low-LET or high-LET radiations are used. Here, no linear dose-effect relationship was observed when the effects per Gy of absorbed dose of anti-CEA and anti-HER1  $^{125}\text{I}$ -mAbs are considered. Indeed, the biological response per Gy of non-internalizing anti-CEA  $^{125}\text{I}$ -mAbs was much higher than that of internalizing anti-HER1  $^{125}\text{I}$ -mAbs. Hence, quantitatively similar effects were produced by  $^{125}\text{I}$ -mAbs with much different nucleus absorbed doses. This is true for clonogenic survival [27], frequency of DNA breaks and micronuclei formation, induction of DNA damage response and effects on cell cycle progression. Based on the localization of the energy deposits due to decay of anti-CEA  $^{125}\text{I}$ -mAbs at the cell membrane [19] and the lack of correlation between absorbed dose and biological effects we hypothesize that cell membrane-mediated  $^{125}\text{I}$ -induced bystander effect, as described in CEBRT ([37-39] and for reviews [29, 40]), could play a significant role in the higher efficacy of non-internalizing  $^{125}\text{I}$ -mAbs [41-43] and should be further investigated.

We would like to highlight that the hypersensitivity of HCT116 cells to non-internalizing  $^{125}\text{I}$ -mAbs is not dependent on our dosimetric approach based on MIRD formalism since we showed that it was still observed when survival was expressed as a function of cumulated uptake of radioactivity (Figure 7A). Moreover, cytoplasmic and cell surface localizations of m225 and 35A7, respectively, were confirmed in several cell lines using cell fractionation assays and immunofluorescence approaches (unpublished data). However, it must be kept in mind that Bousis *et al.* showed that for the case where the radiopharmaceutical are either internalized into the cytoplasm or remained bound onto the cell surface (non-internalized), the dose to the cell nucleus determined using Monte Carlo code was found to differ significantly

from the MIRD values. Then, use of MIRD formalism with short range Auger electrons emitters must be done carefully [44].

In conclusion, this study shows that DNA damage produced by low dose of  $^{125}\text{I}$ -mAbs is efficiently detected by targeted cells even after very low absorbed dose, but is associated to accumulation of DNA double strand breaks. G2/M cell cycle arrest does not prevent damaged cells still proceed through cell cycle and to undergo mitotic death. The lack of dose-effect relationship between mean nucleus absorbed dose and several biological endpoints and the property of non-internalizing  $^{125}\text{I}$ -mAbs to deliver localized doses at the cell membrane suggest that cell membrane-mediated bystander effects might be involved in  $^{125}\text{I}$ -mAbs cytotoxicity.

#### **ACKNOWLEDGEMENT**

This work was supported by the Institut National du Cancer, grant R09080FF / RPT09005FFA and by Action Nu1.1 of Plan Cancer 2009–2013 (ASC 13038FSA).

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## FIGURE LEGENDS

**FIG. 1. DNA damage in  $p53^{+/+}$  (panel A) and  $p53^{-/-}$  (panel B) HCT116 cells after RIT at 37°C.** The alkaline comet assay was used to measure DNA damage at various time points (15 min and then 1, 2, 3, 6, 12, 24 and 48 h) in HCT116 cells targeted with 0 or 4MBq/mL of internalizing anti-HER1 (m225), non-internalizing anti-CEA (35A7) or non-targeting (PX)  $^{125}\text{I}$ -mAbs at 37°C (standard RIT conditions). The mean TM of 100 randomly chosen cells per slide was calculated using the Comet Imager 2.0 software. Three slides were analyzed per each condition and time point and experiments were repeated three times.

**FIG. 2. DNA damage in  $p53^{+/+}$  (panel A) and  $p53^{-/-}$  (panel B) HCT116 after RIT at 4°C.** The alkaline comet assay was used to measure DNA damage at various time points (15 min, 30 min and then 1, 2, 3 and 4 h) in HCT116 cells exposed to 4MBq/mL of internalizing anti-HER1 (m225), non-internalizing anti-CEA (35A7) or non-targeting (PX)  $^{125}\text{I}$ -mAbs at 4°C. TM was measured as a function of the mean nucleus absorbed dose, but only during 4 hours in order to keep cells alive. The mean TM of 100 randomly chosen cells per slide was calculated using the Comet Imager 2.0 software. Three slides were analyzed per each condition and time point and experiments were repeated three times.

**FIG. 3. Activation of DNA damage-related signaling proteins.** The level of phosphorylated ATM (p-ATM) and H2AX (gamma-H2AX), p53 and p21 proteins was measured at the indicated time points in HCT116 cells exposed to 0 and 4 MBq/mL of internalizing anti-HER1 (m225), non-internalizing anti-CEA (35A7)  $^{125}\text{I}$ -mAbs. GAPDH served as loading control (bottom). Panel A:  $p53^{+/+}$  HCT116 cells and panel B:  $p53^{-/-}$  HCT116 cells.

**FIG.4. Immunofluorescent detection of gamma-H2AX in cells.** Cells were grown on coverslip and exposed for 2 days to 0 and 4 MBq/mL of  $^{125}\text{I}$ -mAbs. Gamma-H2AX foci

were next detected at various time in (A)  $p53^{+/+}$  and (B)  $p53^{-/-}$  following onset of exposure to  $^{125}\text{I}$ -mAbs.

**FIG. 5. Micronucleus (MN) frequency.**  $p53^{+/+}$  (panel A) and  $p53^{-/-}$  (panel B) HCT116 cells were exposed to 0, 1, 2 and 4 MBq/mL of internalizing anti-HER1 (m225), non-internalizing anti-CEA (35A7) or non-targeting (PX)  $^{125}\text{I}$ -mAbs. Micronuclei (MN) occurrence was determined in 500 binucleated cells at day 1, 2 and 3 of incubation and the cumulative MN frequency during the three days was determined.

**FIG. 6. Cell cycle analysis.**  $p53^{+/+}$  (panel A) and  $p53^{-/-}$  (panel B) HCT116 cells were exposed to 0 or 4 MBq/mL of internalizing anti-HER1 (m225), non-internalizing anti-CEA (35A7) for several days. Cells were collected at day 0, 1, 2, 3, 4 and 7 of RIT, fixed in 70% ethanol for 3 hours and stained with Cell Cycle Kit reagent for analysis by flow cytometry. Experiments were repeated in triplicate.

**FIG. 7. Survival.** Clonogenic survival was assessed in (A)  $p53^{+/+}$  and (B)  $p53^{-/-}$  HCT116 cells following exposure to test activities of  $^{125}\text{I}$ -mAbs that gradually increased from 0 to 4 MBq/mL for 2 days. Cells were targeted with internalizing anti-HER1 (m225), non-internalizing anti-CEA (35A7)  $^{125}\text{I}$ -mAbs. Survival was expressed as a function of (A) the total cumulative radioactivity uptake  $\tilde{A}_{rs}$  (B) the mean nucleus absorbed dose. Results are the mean of four experiments done in triplicate (from Paillas *et al.* [19]).

**Supplementary FIG. 1. Activation of p53 in A-431 and SK-OV-3 cells.** The level of p53 was measured at the indicated time points in A-431 ( $p53^{+/+}$ ) and SK-OV-3 ( $p53^{-/-}$ ) cells exposed to 0 or 4 MBq/mL of internalizing anti-HER1 (m225 for A-431 cells), internalizing anti HER2 (Trastuzumab for SK-OV-3 cells) or non-internalizing anti-CEA  $^{125}\text{I}$ -mAbs. GAPDH served as loading control (bottom).