Localized Irradiation of Cell Membrane by Auger Electrons Is Cytotoxic Through Oxidative Stress-Mediated Nontargeted Effects

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Abstract

Aims: We investigated whether radiation-induced nontargeted effects are involved in the cytotoxic effects of anticell surface monoclonal antibodies labeled with Auger electron emitters, such as iodine 125 (monoclonal antibodies labeled with125I [125I-mAbs]). Results: We showed that the cytotoxicity of125I-mAbs targeting the cell membrane of p53+/+ HCT116 colon cancer cells is mainly due to nontargeted effects. Targeted and nontargeted cytotoxicities were inhibited in vitro following lipid raft disruption with Methyl-β-cyclodextrin (MBCD) or filipin or use of radical oxygen species scavengers.125I-mAb efficacy was associated with acid sphingomyelinase activation and modulated through activation of the AKT, extracellular signal-related kinase ½ (ERK1/2), p38 kinase, c-Jun N-terminal kinase (JNK) signaling pathways, and also of phospholipase C-c (PLC-c), proline-rich tyrosine kinase 2 (PYK-2), and paxillin, involved in Ca2+ fluxes. Moreover, the nontargeted response induced by directing 5-[(125)I]iodo-2¢-deoxyuridine to the nucleus was comparable to that of125I-mAb against cell surface receptors. In vivo, we found that the statistical significance of tumor growth delay induced by125I-mAb was removed after MBCD treatment and observed oxidative DNA damage beyond the expected Auger electron range. These results suggest the involvement of nontargeted effects in vivo also. Innovation: Low-energy Auger electrons, such as those emitted by125I, have a short tissue range and are usually targeted to the nucleus to maximize their cytotoxicity. In this study, we show that targeting the cancer cell surface with125I-mAbs produces a lipid raft-mediated nontargeted response that compensates for the inferior efficacy of non-nuclear targeting. Conclusion: Our findings describe the mechanisms involved in the efficacy of125I-mAbs targeting the cancer cell surface. Antioxid. Redox Signal. 25, 467–484.

Introduction

The highly localized cytotoxicity of Auger electrons is particularly attractive for targeted radionuclide therapy of microscopic or metastatic disease because it allows the irradiation of tumor cells while sparing healthy tissues. Auger electrons are produced in atoms decaying by internal conversion or electron capture. These processes create a vacancy in the inner electron shell that is subsequently filled by electrons dropping in from higher energy levels. Thus, the vacancy moves to the outer shells, and the transition is accompanied by cascades of complex emissions of Auger, Coster–Kronig, and super Coster–Kronig electrons (collectively called Auger electrons) and soft X-rays [for

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Innovation

Because of their physical properties, Auger electron emitters, such as iodine 125 (125I), are usually targeted to the nucleus to maximize their cytotoxicity. In this study, we show that monoclonal antibodies labeled with 125I (125I-mAbs) and targeting the cell membrane are cytotoxic through oxidative stress-mediated nontargeted effects. As this nontargeted response is comparable to that observed with 125I-IdUrd, bystander effects induced by cell membrane irradiation could compensate for the anticipated inferior efficacy of the absence of nuclear targeting, particularly when vectors do not gain access to every tumor cell. Furthermore, Auger emitter-labeled mAbs bypass the disadvantages of using labeled deoxyribonucleotides.

results

125I-mAbs induce nontargeted effects

Clonogenic survival of p53+/− HCT116 cells targeted by 4 MBq/ml of noninternalizing anti-carcinoembryonic antigen (CEA) 125I-mAb (resulting in cell surface localization of 125I) or internalizing anti-Human Epidermal Receptor type 1 (HER1) 125I-mAb (resulting in cytoplasmic localization of 125I) (donor cells) was reduced to 58% ± 2.5% and 57.8% ± 7%, respectively. These values were significantly different (p < 0.001) from values measured using the nonspecific 125I-PX mAb (Fig. 1A, left panel). We also investigated the role of p53 by performing the same experiments in p53+/− HCT116 cells and showed a similar reduction in clonogenic survival to 54.6% ± 1.2% and 56.5% ± 1.9%, respectively (Fig 1B, left panel). Again survival was statistically significantly different from survival measured using non-specific 125I-PX mAb exposure (p < 0.001), where no toxicity was observed (Fig. 1B, left panel).

To test whether nontargeted effects were involved in the toxicity induced by 125I-mAb, nonirradiated p53+/− and p53−/− HCT116 cells (recipient cells) were incubated with medium in which donor cells had been cultured for 2 h following a 2-day exposure to 4 MBq/ml of anti-CEA or anti-HER1 125I-mAbs. Clonogenic survival of recipient cells was significantly reduced when compared to that of cells incubated with medium from cells exposed to non-specific 125I-PX mAb (p < 0.01). For example, in p53+/− HCT116 recipient cells, survival decreased to 71.2% ± 7.0% after incubation with medium from p53+/− HCT116 donor cells exposed to anti-CEA 125I-mAb and to 70.6% ± 8.4% after exposure to medium from cells exposed to anti-HER1 125I-mAb (Table 1 and Fig. 1A, right panel). Similarly, in p53−/− HCT116 recipient cells, survival decreased to...
73.3% ± 5.6% following incubation with medium from p53−/− HCT116 donor cells exposed to anti-CEA ¹²⁵I-mAb and to 74.5% ± 5.1% upon switch to medium from cells exposed to anti-HER1 ¹²⁵I-mAb (Fig. 1B, right panel). No cell killing was observed after incubation of recipient cells with medium from donor cells treated with the non-targeting ¹²⁵I-PX mAbs (Fig. 1B, right panel) or with unlabeled mAbs (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars). This suggests that the effect was specific and required ¹²⁵I-mAb binding to other cell surface. Similar results were obtained in A-431CEA cells treated with the same ¹²⁵I-mAbs. Gamma-H2AX foci (markers of DNA double strand breaks, DSBs) were detected in both p53−/− HCT116 donor (exposed to anti-CEA or anti-HER1 ¹²⁵I-mAbs) and recipient cells, again indicating the occurrence of non-targeted effects in recipient cells (Fig. 2A, B). It must be noted that cells were proliferating and that a larger number of gamma-H2AX foci are likely to be produced during the S phase. The proportion of donor cells showing foci was assessed to be about 10% in nonirradiated cells, while it increased up to 25% in cells exposed to the specific ¹²⁵I-PX mAb and 100% after exposure to the specific anti-HER1 and anti-CEA ¹²⁵I-mAbs. Corresponding values were 15% and 100% in recipient cells (data not shown).

Nontargeted effects do not require cellular internalization of ¹²⁵I

Localization of the anti-CEA and anti-HER1 mAbs at the cell membrane and cytoplasm, respectively, was confirmed by cell fractionation followed by Western blotting (Fig. 2C) and by immunofluorescence (Fig. 2D) in p53−/− HCT116 and A-431CEA cells. Coincubation of p53−/− HCT116 donor cells with anti-CEA or anti-HER1 ¹²⁵I-mAbs and sodium azide, a drug preventing antibody internalization after receptor binding, did not modify the nontargeted response in recipient cells (Fig. 2E). These results suggest that internalization of ¹²⁵I is not required for radiation-induced nontargeted effects.

The role of lipid rafts in ¹²⁵I-mAb cytotoxicity in vitro

Lipid rafts can be ceramide-enriched platforms in the cell membrane that are produced through sphingomyelin hydrolysis to ceramide and phosphorylcholine following acid sphingomyelinase (ASMase) activation by ROS (56). To investigate whether ¹²⁵I-mAbs affected the cell membrane
TABLE 1. 

<table>
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<th>p53+/+ HCT116 cells survival (%) at 4 MBq/ml</th>
<th>Targeted cytotoxicity</th>
<th>Nontargeted cytotoxicity</th>
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<td>Donor cells</td>
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| p53+/+ HCT116 donor cells exposed to 125I-anti-CEA, 125I-anti-HER1, 125I-PX mAbs, or 20 kBq/ml 125I-UdR and of the corresponding recipient cells. Cells were incubated with 125I-vectors for 2 days. Donor cells were in reality both donor and recipient cells. Filipin significantly reduced the formation of phosphorylated histone H2AX (γ-H2AX) foci in both donor and recipient cells (Fig. 4B). Finally, 125I-mAb cytotoxicity in both donor (Fig. 4C) and recipient cells (Fig. 4D) was significantly reduced when donor cells were incubated in the presence of filipin. Indeed, clonogenic survival increased from 58% ± 2.6% to 84.4% ± 2.4% from 57.8% ± 7.0% to 81.0% ± 1.6% in donor cells incubated with 4 MBq/ml anti-CEA or anti-HER1 125I-mAbs and filipin, respectively (Table 1). In recipient cells, survival increased from 71.2% ± 7.0% to 92.8% ± 2.4% to 94.2% ± 2.4%, respectively. Similar protection was afforded by methyl-β-cyclodextrin (MBCD), another lipid raft disruptor (Supplementary Fig. S2). These findings indicate that lipid raft formation is involved in both targeted and nontargeted cytotoxicity of 125I-mAbs.

Phosphorylated proteins associated with 125I-RIT

To identify the signaling pathways activated in cells exposed to anti-CEA or anti-HER1 125I-mAbs, the phosphorylation profiles of 46 kinases were assessed by array-based proteomics (Fig. 5 and Supplementary Fig. S3). Only extracellular signal-related kinase 1 (ERK1/2) and C-AMP Response Element-binding protein (CREB) were strongly activated after treatment with unlabeled anti-CEA mAb (Fig. 5A). Conversely, upon incubation with the unlabeled anti-HER1 mAb, several proteins were induced, including ERK1/2, CREB, p53, and AKT (Fig. 5B).

Thus, only the anti-CEA 125I-mAb was used to investigate the effect of targeting 125I to the cell surface. When cells were exposed to anti-CEA 125I-mAb (Fig. 5A), the ERK1/2, AKT, p38, and c-Jun N-terminal kinase (JNK) signaling pathways...
were induced. Activated downstream AKT proteins included p70S6 kinase (p70S6K), glycogen synthase kinase 3 (GSK3), and endothelial nitric oxide synthase (e-NOS) (61). Signal transducer and activator of transcription (STAT) 1 and 4, 90 kDa ribosomal S6 kinase (RSK), mitogen- and stress-activated protein kinase (MSK), and CREB proteins, which are downstream targets of ERK1/2 signaling, were also activated, as well as c-JUN, which is downstream of p38 and JNK (for review (61)). Phosphorylated proteins involved in Ca\(^2+\) fluxes, such as phospholipase C-\(\gamma\) (PLC-\(\gamma\)), proline-rich tyrosine kinase 2 (PYK-2), and paxillin, were strongly activated by exposure to anti-CEA \(^{125}\)I-mAb. Phosphorylation of AKT, p38, JNK, CREB, GSK3, STAT 1 and 4, e-NOS, MSK, PLC-\(\gamma\), PYK-2, and paxillin was abolished, whereas phosphorylation of ERK1/2, c-JUN, p53, RSK, SAT 2, and p70S6K was diminished in cells coincubated with filipin (Fig. 5), confirming that these proteins are downstream effectors of lipid raft formation and, thus, are likely to be involved in \(^{125}\)I-mAb nontargeted effects. Moreover, the use of different pharmacological inhibitors of MEK (U0126) and subsequently of downstream AKT signaling pathway, p38 (SB203580), and JNK (SP600125) indicated that, among the identified proteins activated, p38 and JNK were mainly involved in the response of cells to \(^{125}\)I-mAb exposure (Fig. 6). No effect of EGTA on clonogenic survival was observed (Supplementary Fig. S1C).

**ROS are involved in Auger electron-induced nontargeted response**

The involvement of ROS in the mediation of the bystander response was examined by culturing recipient cells in medium from \(^{125}\)I-mAb-exposed cells (Fig. 2). Clonogenic survival of recipient cells was assessed after incubation with conditioned medium from donor cells exposed to \(^{125}\)I-mAbs in the presence of sodium azide. Results are the mean ± SD of three experiments performed in triplicate. \(*p<0.05\), \(**p<0.01\), and \(***p<0.001\) compared to NT cells (B) or to the \(^{125}\)I-PX mAb-treated cells (E). Mb, membrane; Cyto, cytoplasm. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

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**FIG. 2.** Gamma-H2AX foci detection and optional role of \(^{125}\)I-mAb internalization for nontargeted cytotoxicity. (A) Detection of gamma-H2AX foci in p53\(^{++}\) HCT116 donor (top panels) and recipient cells (bottom panels) by immunofluorescence assay. (B) The number of foci per cell was counted in 100 cells. Antibody localization was assessed in p53\(^{++}\) HCT116 and A-431\_CEA cells by (C) subcellular fractionation and (D) immunofluorescence. F-actin was stained with conjugated phalloidin (Red) to visualize cytoplasm, nuclei with Hoechst (Blue), and mAbs with Alexa-488-conjugated anti-mouse secondary mAbs. (E) Clonogenic survival was assessed in p53\(^{++}\) HCT116 donor cells coincubated with 0.02% sodium azide (a drug blocking antigen internalization) and a range of activities of \(^{125}\)I-mAbs (non-internalizing anti-CEA, internalizing anti-HER1, or nontargeting PX mAbs). Clonogenic survival of recipient cells was assessed after incubation with conditioned medium from donor cells exposed to \(^{125}\)I-mAbs in the presence of sodium azide. Results are the mean ± SD of three experiments performed in triplicate. \(*p<0.05\), \(**p<0.01\), and \(***p<0.001\) compared to NT cells (B) or to the \(^{125}\)I-PX mAb-treated cells (E). Mb, membrane; Cyto, cytoplasm. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars
with $^{125}$I-mAbs in the presence or absence of the free radical scavengers dimethyl sulfoxide (DMSO) or N-acetylcysteine (NAC) (Fig. 7). We previously checked (using immunofluorescence detection of mAbs in the presence of cells grown on coverslips) that NAC did not prevent anti-HER1 or anti-CEA mAbs from binding to their receptors.

Survival of both donor and recipient cells was significantly increased compared with controls treated with $^{125}$I-mAbs in the absence of free radical scavengers ($p<0.001$ and $p<0.01$, respectively). This suggests that oxidative stress is involved in both targeted and nontargeted cytotoxic effects of $^{125}$I-mAbs.

**Nontargeted effects of $^{125}$I directed to the cell membrane or the nucleus are equipotent**

To compare the effect of targeting $^{125}$I to the cell membrane or the nucleus, $p53^{+/-}$ HCT116 donor cells were incubated with $^{125}$I-UdR (nuclear localization of $^{125}$I) or with anti-CEA or anti-HER1

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**FIG. 3.** Lipid rafts are formed upon exposure to $^{125}$I-mAbs via ASMase-induced ceramide formation. (A) Lipid rafts were detected by immunofluorescence analysis using Alexa-488-conjugated cholera toxin (a marker of lipid raft) in untreated (NT) and treated $p53^{+/-}$ HCT116 cells (noninternalizing $^{125}$I-anti-CEA, internalizing $^{125}$I-anti-HER1, nontargeting $^{125}$I-PX mAbs). (B) Ceramide was measured in $p53^{+/-}$ HCT116 cells by flow cytometry analysis after treatment with unlabeled mAbs (top panels), or with $^{125}$I-mAbs (bottom panels). Ctrl+, positive control (100 μl of 0.4 U/ml Bacillus cereus SMase and 10 μl sphingomyelin). (C) Colocalization of ceramide and Annexin V in apoptotic cells was determined by immunofluorescence, in untreated (NT) and $^{125}$I-anti-CEA-treated $p53^{+/-}$ HCT116 cells. (D) ASMase activity was determined in whole $p53^{+/-}$ HCT116 cell lysates using the Amplex Red Sphingomyelinase Fluorimetry Kit after incubation with unlabeled or $^{125}$I-mAbs. Ctrl+, positive control (100 μl of 0.4 U/ml B. cereus SMase and 10 μl sphingomyelin); Ctrl− (Amplex Red working solution without sphingomyelin). (E) S1P concentration was determined in whole $p53^{+/-}$ HCT116 cells lysates using the Sphingosine-1-Phosphate Assay Kit after incubation with unlabeled or $^{125}$I-mAbs or in nontreated cells (NT). Results are the mean ± SD of four experiments performed in triplicate. **$p<0.01$ and ***$p<0.001$ compared to cells treated with unlabeled mAbs. ASMase, acid sphingomyelinase; S1P, sphingosine-1-phosphate. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars
125I-mAbs. Subsequently, clonogenic survival was assessed in both donor and recipient cells. Results are summarized in Table 1. The uptake of 125I-UdR and 125I-mAbs was measured for every test activity for 6 days, and the total number of decays occurring in cells was calculated as described previously (46). The results of the clonogenic survival assay showed that, in donor cells, 125I-UdR (nuclear localization) was more cytotoxic than anti-CEA (cell surface localization) or anti-HER1 (cytoplasm localization) 125I-mAbs. Conversely, in recipient cells, cell survival was similar and independent of 125I localization, indicating that 125I at the cell membrane is as efficient as nuclear 125I in killing bystander cells.

The role of lipid rafts in 125I-mAb cytotoxicity in vivo

Mice bearing intraperitoneal tumor xenografts were treated with NaCl, anti-CEA mAb (100 μg/injection at day 8 and 11), 125I-anti-CEA mAb (37 MBq/injection at day 8 and 11), or MBCD (300 mg/kg; daily, from day 6 to 15), or with both 125I-anti-CEA mAb and MBCD. At day 30, tumor mass was significantly reduced in the group treated with 125I-anti-CEA mAb (RIT) compared with the NaCl group (p = 0.0227). No difference was observed between the groups treated with MBCD, NaCl (p = 0.9349), or anti-CEA mAb (p = 0.9563). The latter result is in agreement with a previously published study indicating that anti-CEA mAbs have no effect on mouse tumor growth (53). Treatment with MBCD removed the statistical significance of RIT-induced tumor growth delay such that no difference was observed between NaCl and RIT + MBCD groups (p = 0.2481) (Fig. 8A).

Oxidative DNA damage occurs beyond the range of Auger electrons in vivo

Digital autoradiographic analysis performed on sections of tumors collected in mice injected with 125I-anti-CEA mAb...
showed that radioactivity (and thus the absorbed dose) was mainly localized at the tumor periphery in A-431CEA tumor cell xenografts (Fig. 9A, inset in the bottom left corner). Conversely, DNA DSBs occurred homogeneously throughout the tumor, as indicated by immunofluorescence detection of p53-binding protein 1 (53BP1), a DNA damage sensor that is recruited to damaged chromatin (Fig. 9A). The average number of foci per cell was 4.4 at the center and 4.3 at the periphery of the tumor. The corresponding regional activities were 2.3 and 34.7 cpm per mm². Our observation of similar levels of DNA DSBs at the center of the tumor, independent of the absorbed dose and beyond the range of Auger electrons, supports the involvement of nontargeted effects also in vivo. 53BP1 expression was also investigated in A-431CEA tumor cell xenograft sections from mice injected with the irrelevant 125I-PX mAb (Fig. 9B). The numbers of foci per cell was 0.8 (tumor center) and 1.0 (tumor periphery). These values were lower than those observed following treatment with anti-CEA 125I-mAb, despite the higher uptake of 125I-PX mAb exemplified by activity per area values of 28 and 18.2 cpm/mm² at the tumor center and periphery, respectively. A similar level of 0.7 ± 0.1 53BP1 foci was found in tumors of NaCl or anti-CEA-treated mice (Fig. 9C).

This ruled out the possibility that the signal detected in tumors treated with anti-CEA 125I-mAb was caused by non-specific induction of 53BP1 due to cross-irradiation from the radioactivity present in nonmalignant regions of the animal.

Discussion

In the present study, we show that 125I translocation to the nucleus is not required for cell killing and that radiation-induced nontargeted effects contribute to 125I cytotoxicity when labeled with monoclonal antibodies (125I-mAbs) (Table 1). We also identify the signaling pathways involved in these nontargeted effects. In particular, we show using p53 and p53 CEAtreated HCT116 cells that the latter protein did not play a
A significant role in anti-CEA $^{125}$I-mAb cytotoxicity since no difference was observed between the two cell lines (Fig. 1A, B and Supplementary Fig. S4). We chose the nuclear p53 protein as it is one of the main proteins involved in signaling pathways activated by nuclear damage.

The potency of nontargeted cell kill, assessed using a medium transfer protocol, was independent of $^{125}$I localization (nucleus, cytoplasm, or cell surface) and of the magnitude of targeted cytotoxicity in donor cells (Table 1). In our in vitro experimental RIT model, donor cells were incubated with $^{125}$I-mAbs and were killed both by targeted and nontargeted effects. Therefore, it is possible to determine the relative contribution of targeted and nontargeted cytotoxicity to donor cells by comparing the survival values of donor and recipient cells reported in Table 1. As death of recipient cells (incubated in culture medium in which donor cells had been cultured for 2 h) ranged between 28.8% and 29.4% (Fig. 1B), it may be assumed that a similar (or even higher) magnitude of nontargeted effects also occurred in donor cells that were directly exposed to $^{125}$I-mAbs for 48 h. Accordingly, cell death by targeted effects in donor cells would be equal to or less than 13.2% (42.0% minus 28.8%) and 12.8% (42.2% minus 29.4%) for $^{125}$I localized at the cell surface or cytoplasm, respectively, and equal to or less than 50.7% (77.9% minus 27.2%) for $^{125}$I within the nucleus (Table 1). These data confirm the greater cytotoxicity of $^{125}$I when incorporated into DNA (22, 17, 64).

Our results indicate that the cell membrane plays a major role not only in the targeted cytotoxic effects of $^{125}$I-mAbs but also in nontargeted cytotoxic effects (Fig. 1). According to the fluid mosaic model (57), the membrane contains sphingolipids (predominantly sphingomyelin), cholesterol, and glycosphospholipids. Sphingolipids associate with each other and with cholesterol to form sphingolipid- and
cholesterol-enriched domains called lipid rafts that are stabilized in some cells by caveolin (32). It has been shown that ASMase activated by hydroxyl radicals (56) translocates from the cytoplasm to the outer layer of the cell membrane, where it hydrolyzes sphingomyelin into hydrophobic ceramide and hydrophilic phosphorylcholine (59, 68). Ceramide production is accompanied by the coalescence of lipid raft microdomains into ceramide-enriched large platforms that can participate in the spatial reorganization of membrane receptors and in the activation of multiple signaling pathways. Several mechanisms leading to protein activation through membrane reorganization have been identified in response to EBRT (8, 13, 47).

Our results confirm that similar mechanisms might be involved in 125I-mAb efficacy after binding to the cell surface because we show that the localized energy deposition associated with 125I decays was accompanied by ASMase activation (Fig. 3D), formation of ceramide-enriched large platforms (Fig. 3A), and subsequent activation of signaling pathways (Fig. 5). ROS scavenging or disruption of lipid rafts, using MBCD or filipin, reduced not only the targeted cytotoxic effect of 125I-mAbs but also the nontargeted cytotoxic effect (Figs. 4 and 7). The use of filipin, a cholesterol-depleting agent, reduced the cytotoxic effect of 4 MBq/ml anti-CEA or anti-HER1 125I-mAbs by about half in donor cells and by 75%–80% in recipient cells (Table 1), showing the involvement of lipid rafts in the cytotoxic effects of 125I-mAbs. These results are supported by in vivo data showing that MBCD treatment removes the statistical significance of the therapeutic efficacy of 125I-anti-CEA mAb (Fig. 8).

A schematic representation of the mechanisms that might be involved in 125I-mAbs targeted cytotoxicity is proposed in Figure 10. Formation of ceramide-enriched large platforms is induced following ASMase activation mediated by ROS (56) originating from water radiolysis (presumably in a dose-dependent manner) (Fig. 3D). ROS could also be generated...
A set of tumors in different mice were examined in 100 cells of a selected area and was correlated (daily from day 6 to 15 postgraft) (RIT with MBCD from day 6 to 15 postgraft (MBCD), or with group). Tumors were collected at days 30 postgraft, and the tumors of mice treated with 37 MBq of 125I-anti-CEA mAbs, in cells exposed to 125I-mAbs was not correlated with the abundance of micronuclei (36, 67). ROS contribute to “chronic” oxidative stress and following lipid raft-mediated activation of NAD(P)H oxidase (36, 67). ROS contribute to “chronic” oxidative stress and the subsequent DNA damage, including the formation of micronuclei in nontargeted cells (3, 44). This is in agreement with our previous finding that the abundance of micronuclei in cells exposed to 125I-mAbs was not correlated with the mean absorbed dose to the nucleus (48, 50). The involvement of oxidative stress in EBRT-induced nontargeted effects has been extensively reviewed by Havaki et al. (20). Irradiated cells produce ROS and RNS through radiolysis and cytokines. ROS and RNS are then directly transmitted to bystander cells through gap junctions or through release in the extracellular medium (20). In bystander cells, different signaling pathways participate in the upregulation of genes encoding cytokines, cyclooxygenase-2 (COX2), and inducible nitric oxide synthase (iNOS) that generate intracellular ROS and RNS (20). However, the involvement of such mechanisms following low dose rate and protracted exposure (as in RIT) needs to be confirmed. The present study focused on the mechanisms involved in irradiated donor cells, and we assume that the main source of oxidative stress was associated with radiation and hydroxyl radical formation. Elucidation of the precise mechanisms involved in recipient cells would require further work.

It is noteworthy that 125I decays must occur at the surface of targeted cells to initiate the nontargeted response. Indeed, our data show that low LET radiation (including electrons and soft X-rays) emitted by the irrelevant PX 125I-mAb, which does not interact with cells, did not produce a significant nontargeted response. This suggests that nontargeted effects are induced mainly by high LET Auger electrons (of very low range) generated at the cell surface when 125I-mAbs bind to CEA or HER1 receptors.

The relative contribution of the various signaling pathways activated by cell membrane reorganization following lipid raft formation is complex and remains to be elucidated. We show that the mitogen-activated protein kinases (MAPKs), ERK1/2, P38, JNK, and AKT, and their downstream proteins, are activated during exposure to 125I-mAbs. The role of these kinases in our model is still unclear because AKT and ERK1/2 promote cell survival, while ceramide, p38, JNK, and its downstream target c-JUN participate in cell death. However, the use of p38 or (to a lesser extent) of JNK inhibitors was accompanied by a stronger effect on clonogenic survival (increase) than the use of PI3K or MEK inhibitors, suggesting the cell death mechanisms predominate (Fig. 6). We also found that S1P, the hydrolysis product of sphingosine that is generated from ceramide by ceramidase (Figs. 3E and 10), was upregulated following exposure to anti-CEA 125I-mAb. Contrary to ceramide, S1P inhibits apoptosis and stimulates cell proliferation through activation of the AKT and e-NOS pathways (for review, Oskouian and Saba (45)). Paxillin also was activated by incubation with anti-CEA 125I-mAb (Fig. 5). Paxillin can be phosphorylated by focal adhesion kinase (FAK) or PYK-2, and phosphorylated paxillin serves as a scaffold protein to facilitate the functional integration of focal adhesion proteins. Paxillin is phosphorylated during EBRT as a downstream protein target (together with AKT and ERK1/2) of FAK (21).

Moreover, our data suggest a possible role of ion channels located in lipid rafts, such as Ca2+ channels. The role of Ca2+ in EBRT-associated nontargeted effects has clearly been demonstrated (38). The influx of extracellular Ca2+ can be regulated by ceramide (11), but also by PLC-γ that is activated upon exposure to anti-CEA 125I-mAb (Fig. 5A). During EBRT, PLC-γ localized at the plasma membrane produces inositol triphosphate (IP3) that consequently induces Ca2+ release from the endoplasmic reticulum through IP3 receptors (60). Such an increase in Ca2+ cytoplasmic concentration could also contribute to the phosphorylation of PYK-2 observed in cells exposed to anti-CEA 125I-mAb (Fig. 5A) (34). PYK-2, a member of the FAK family, is involved in cell proliferation and phosphorylates paxillin. However, incubation of donor cells with EGTA, a chelator of extracellular calcium, was not accompanied by modifications in clonogenic survival. In a previous study, Lyng et al. have used EGTA in combination with verapamil and nifedipine to block also voltage-dependent calcium channels (38) to investigate the role of Ca2+ ions. Such an approach merits further investigation in our models also.

Finally, we investigated in A-431CEA tumor xenografts from mice injected with anti-CEA 125I-mAb whether the induction of DNA DSBs could be correlated with the
distribution of radioactivity. We observed that radioactivity was mainly localized at the periphery of tumors, whereas DNA DSBs were homogenously distributed throughout the tumor (Figs. 8B and 9A).

A lower DSB induction was detected after $^{125}$I-PX mAb treatment, despite the higher nonspecific uptake of radioactivity, for some of the tumors, compared to anti-CEA $^{125}$I-mAb. These data indicate that the DSBs observed after treatment with anti-CEA $^{125}$I-mAb were not due to nonspecific irradiation. Indeed, we analyzed $^{125}$I-PX mAb-treated tumors showing high tumor uptake to be able to exclude the possibility that DNA DSBs measured in the center of the tumor were caused by nonspecific irradiation of $^{125}$I (mainly soft X-rays and electrons) from the tumor periphery to the center. We could also exclude any contribution from blood circulating $^{125}$I-mAbs in mouse. As the major contribution to the absorbed dose from $^{125}$I is due to Auger electrons, radioactivity distribution may be considered to be indicative of the distribution of the absorbed dose. The reason for the observed higher uptake in some areas of tumor of $^{125}$I-PX mAb-treated mice compared with those treated with $^{125}$I-anti-CEA mAb (especially the center) is unclear.

It could be that while the nonspecific antibody is able to freely diffuse to the center of the tumor, the CEA binding antibody binds to receptors, and the first of those encountered would be in the periphery. Unless these receptors in the periphery become saturated, substantial penetration of further unbound antibody molecules would be prevented. Notwithstanding this effect, whatever the distribution of radioactivity, we have previously shown that $^{125}$I-PX mAb does not have a therapeutic effect in vivo (53). Accordingly, our results indicate that DNA DSB formation did not correlate with

![FIG. 9. Radioactivity distribution and 53BP1 induction do not overlap in A-431_CEA tumor cell xenografts treated with $^{125}$I-anti-CEA mAbs. Digital autoradiography (inset on the bottom left side of each panel) and immunohistochemical detection of 53BP1 in tumor sections from mice xenografted intraperitoneally with A-431_CEA cells and treated 24 h before tissue collection with a single intraperitoneal injection of 37 MBq of (A) $^{125}$I-anti-CEA mAb, (B) $^{125}$I-anti-PX mAb, or (C) injected with NaCl. The distribution of 53BP1 foci was determined in 100 cells of relevant areas of tumors. Between 6 and 7 areas of tumors in different mice were selected. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars](https://liebertpub.com/ars)
the absorbed dose and that DNA damage occurred to a similar extent both within and beyond the range of the $^{125}$I Auger electrons. In a similar approach using $^{212}$Pb-mAbs for treating A-431CEA tumor xenografts, we also observed that distribution of DNA damage and induction of abnormal mitosis do not correlate with tumor absorbed dose, a phenomenon not observed with $^{212}$Pb-PX nonspecific mAb (data not shown). These data support the involvement, in vivo, of nontargeted effects during $^{125}$I-mAb RIT, but this needs to be confirmed in further experiments.

One of the issues raised by our study is related to radiation protection since nonirradiated tissues can show DNA damage and subsequent possible cell death or cell transformation. It was generally considered that Auger binding DNA, or localized in the cell nucleus, produces high LET radiation. However, there is no guidance on how to calculate the equivalent dose for these radionuclides. It has been proposed by the American Association of Physicists in Medicine that the component of dose from the Auger electrons for radionuclides bound to DNA should be given a preliminary radiation weighting factor of 10 for deterministic effects and 20 for stochastic effects for nuclear localization while ICRP has recommended considering each situation (isotope and localization) separately (23, 24).

In conclusion, we have shown that Auger electron emitters, such as $^{125}$I, conjugated to mAbs directed against the cancer cell surface can be cytotoxic by a cell oxidative, stress-
mediated nontargeted mechanism involving lipid rafts. This nontargeted effect is comparable to that induced by $^{125}$I-UdR concentrated in the nucleus of tumor cells. Compared with $^{125}$I-UdR, lower targeted cytotoxicity is expected when using $^{125}$I-mAbs, but the induction of nontargeted effects could compensate for the inferior efficacy of non-nuclear targeting. Moreover, $^{125}$I-mAbs should also be less toxic toward proliferating normal tissue, thus allowing an increase in the injected activities.

These findings are particularly relevant for targeted therapy in which vectors cannot gain access to every tumor cell. Their extrapolation to targeted alpha radiotherapy, now routinely used in clinical radionuclide therapy, should be evaluated.

Materials and Methods

Cell lines

A-431CEA and SK-OV-3 CEA cells were obtained by transfecting A-431 vulvar squamous and SKOV-3 ovarian carcinoma cells (from ATCC, Molsheim, France), respectively, using vectors encoding the CEA. $p^{53+/+}$ HCT116 colon adenocarcinoma cells were obtained from ATCC. The $p^{53+/+}$ HCT116 cell line was a kind gift from Professor Bert Vogelstein. HCT116 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM l-glutamine, and antibiotics (0.1 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere with 5% CO2. A-431 CEA and SKOV-3 CEA cells were grown as described (50). All cell culture reagents were from Life Technologies (Thermo Fisher Scientific, Waltham, MA).

Radiolabeled vectors for cell targeting

To target the cytoplasm, $^{125}$I was coupled to the internalizing mouse m225 mAb. $^{125}$I-labeled m225 mAb is internalized within the cytoplasm after binding to its receptor (HER1). For cell surface localization, $^{125}$I was coupled to the noninternalizing mouse IgG1k mAb 35A7. For the CEA Gold 2 epitope (50), 35A7 is specific. The nonspecific PX antibody was used as a control (irrelevant antibody). PX is an IgG1 mAb purified from mouse myeloma P3-X63 cells (30). The three mAbs were obtained from mouse hybridoma ascites fluids by ammonium sulfate precipitation followed by ion exchange chromatography on DE52 cellulose (Whatman, Balston, United Kingdom).

Antibody radiolabeling was performed according to the IODO-GEN (1,3,4,6-tetrachloro-3az, 6x-diphenylglycoluril) (Sigma-Aldrich, St. Louis, MO) method (50). $^{125}$I-mAb-specific activity was 370 MBq/mg of protein, and immunoreactivity was 60%–95%. All experiments used $^{125}$I-mAb activities ≤4 MBq/ml. $^{125}$I was targeted to the nucleus using the thymidine base analogue $^{125}$I-UdR at a specific activity of 81.4 TBq/mmol. $^{125}$I-UdR was purchased from PerkinElmer (Waltham, MA).

RIT, medium transfer, and clonogenic survival of irradiated and nonirradiated cells

$p^{53+/+}$ or $p^{53+/+}$ HCT116, SK-OV-3 CEA, and A-431_CEA cells were cultured in 6-cm Petri dishes (100–500 cells/dish) with 3 ml of medium. The following day, cells were incubated with $^{125}$I-mAbs at activities ≤4 MBq/ml for 48 h (donor cells). For targeting of the nucleus, due to the higher toxicity of $^{125}$I-UdR, between 100 and 1200 $p^{53+/+}$ HCT116 cells were seeded in 6-cm Petri dishes containing 3 ml of medium and exposed, the following day, to $^{125}$I-UdR activities of ≤20 kBq/ml for 48 h. Culture medium was then removed, and cells were washed twice with 4 ml phosphate buffered saline (PBS). For clonogenic assays, performed as described in (50), fresh medium (3 ml) was added and donor cells were cultured for 12 days. For investigating the nontargeted response, fresh medium (3 ml) was added to donor cells for 2 h and then transferred to dishes in which cells had been seeded the day before (recipient cells: 100–500 cells/6 cm dish). The transferred medium was checked for radioactivity (values were generally very low, comprising between 0.08% and 0.10% of the activity incubated with donor cells). Donor and recipient cells were then grown for 12 days and colonies stained with crystal violet (2.5 g/l in 45:5 30% [v/v] methanol/paraformaldehyde). Colonies containing 50 or more cells were scored, and the surviving fraction was calculated. The toxicity of unlabeled mAbs was assessed using the same experimental conditions.

Drug treatment

To determine the role of mAb internalization in the bystander response, $p^{53+/+}$ HCT116 cells were exposed for 2 days to $^{125}$I-mAbs of activity concentration ≤4 MBq/ml in the presence of 0.02% (w/v) sodium azide, a drug that blocks mAb transport into the cytoplasm.

The role of cell membrane lipid raft formation in $^{125}$I-mAb-induced bystander effects was investigated by coincubating $p^{53+/+}$ HCT116 donor cells with $^{125}$I-mAbs and 4 mM MBCD (Sigma-Aldrich) for 6 h or with 2.5 μg/ml filipin (Sigma-Aldrich) overnight.

The potential role of oxidative stress in the induction of bystander effects was assessed by coincubating $p^{53+/+}$ HCT116 donor cells with $^{125}$I-mAbs and 50 μM NAC (Sigma-Aldrich) (for 2 days) or 0.5% DMSO (Sigma-Aldrich) (overnight).

Immunofluorescence analysis of mAb localization, lipid rafts, and γH2AX foci

For immunofluorescence studies, $p^{53+/+}$ HCT116 cells were plated on 12-mm glass coverslips in culture dishes. They were fixed in 3.7% (v/v) formaldehyde and then permeabilized in 0.1% (w/v) Triton X-100 for 15 min followed by incubation with 1% (v/v) PBS/bovine serum albumin (BSA) for 1 h. To confirm the mAb localization, an Alexa-488-conjugated anti-mouse antibody (1:200; Jackson Immuno Research) was used as a control (irrelevant antibody). PX is an IgG1 mAb purified from mouse myeloma P3-X63 cells (30). The three mAbs were obtained from mouse hybridoma ascites fluids by ammonium sulfate precipitation followed by ion exchange chromatography on DE52 cellulose (Whatman, Balston, United Kingdom).

Antibody radiolabeling was performed according to the IODO-GEN (1,3,4,6-tetrachloro-3az, 6x-diphenylglycoluril) (Sigma-Aldrich, St. Louis, MO) method (50). $^{125}$I-mAb-specific activity was 370 MBq/mg of protein, and immunoreactivity was 60%–95%. All experiments used $^{125}$I-mAb activities ≤4 MBq/ml. $^{125}$I was targeted to the nucleus using the thymidine base analogue $^{125}$I-UdR at a specific activity of 81.4 TBq/mmol. $^{125}$I-UdR was purchased from PerkinElmer (Waltham, MA).
Ceramide levels present in the cell membrane of $p53^{+/+}$ HCT116 cells were assessed by flow cytometry. Cells were treated with unlabeled mAbs (20 $\mu$g/ml) or $^{125}$I-mAbs (4 MBq/ml) at 37°C for 1 h. After three washes, cells were fixed in 3.7% (v/v) parafomaldehyde for 15 min. Then, cells were washed three times in PBS buffer and incubated with the anti-ceramide 15B4 mAb at a dilution of 1:50 (Alexis Biochemicals) at 37°C for 1 h. After three washes in PBS—2% FCS, cells were incubated with PE-conjugated goat anti-mouse IgM at a dilution of 1:100 (Jackson Immunoresearch) in the dark for 1 h. Cells were then washed three times and suspended in PBS for analysis using a Muse flow cytometer (Merck Millipore, Fullerton, CA). The y axis corresponds to the total number of analyzed cells (arbitrary units), while the x axis represents the fluorescence signal intensity (PE-conjugated goat anti-ceramide 15B4 mAb). The numbers provided in the red boxes correspond to the proportion of ceramide-positive cells within the total population of cells compared with the nontreated control.

Inhibitors of MAPK pathways and chelation of extracellular Ca$^{2+}$

To confirm the involvement of MAPK proteins, including AKT, ERK1/2, p38, and JNK on final survival outcome, HCT116 donor cells were incubated for 48 h with $^{125}$I-anti-CEA mAb in the presence of 1 $\mu$M of PI3K inhibitor (ZSTK474), and indirectly of downstream AKT signaling pathway, 1 $\mu$M of MEK1/2 inhibitor (U0126), and indirectly of downstream ERK1/2 signaling pathway, 10 $\mu$M of SP600125 (JNK inhibitor), 10 $\mu$M of SB203580 (p38 inhibitor), or 5 mM EGTA (to chelate extracellular Ca$^{2+}$ ions). Donor cells were preincubated with inhibitors 1 h before addition of $^{125}$I-mAbs. After 48 h, medium was removed and standard medium transfer protocol was followed as previously described. Clonogenic survival of both donor and recipient cells was measured. The efficacy of ZSTK474 and of U0126 in inhibiting AKT and ERK1/2 was confirmed by Western blotting (Supplementary Fig. S3B, right panel).

Measurement of ASMase activity

ASMase activity in whole cell lysates of $p53^{+/+}$ HCT116 cells exposed to $^{125}$I-mAbs (0–4 MBq/ml) for 2 days was measured using the Amplex Red Sphingomyelinase Assay Kit (Molecular Probes, Saint Aubin, France). The ASMase activity of experimental samples and positive control was calculated as a percentage of the ASMase activity of the negative control.

Measurement of S1P concentration

The level of S1P was determined in $p53^{+/+}$ HCT116 cells exposed to $^{125}$I-mAbs (4 MBq/ml) for 2 days using the Sphingosine 1 Phosphate Assay Kit according to the manufacturer’s protocol (Echelon Biosciences, Salt Lake City, UT).

Animal model

Swiss nude mice (7-week-old females) from Charles River were acclimated for 1 week before experimental use. They were housed at 22°C and 55% humidity with a light–dark cycle of 12 h. Food and water were available ad libitum. Body weight was determined weekly, and the mice were clinically examined throughout the study. All animal experiments were performed in compliance with the guidelines of the French government and the INSERM standards for experimental animal studies (agreement B34-172–27).

In vivo experiments

Swiss nude mice (7-week-old females) from Charles River were xenografted intraperitoneally (i.p.) with 1.5 x 10$^6$ A-431 CEA cells suspended in 0.3 ml PBS. The A-431 CEA cell line was chosen for in vivo $^{125}$I experiments because it expresses high levels of CEA receptors (compared with HCT116 cells), which enables treatment with a tumoricidal dose.

RIT experiments with or without MBCD. For these experiments, 32 mice were divided in four groups (n = 8 each) that were treated as follows: (i) two i.p. injections of NaCl at day 8 and 11 postgraft, (ii) two i.p. injections of anti-CEA mAb (100 $\mu$g) at day 8 and 11, (iii) two i.p. injections of 37 MBq of $^{125}$I-anti-CEA mAb at day 8 and 11, (iv) daily i.p. injections of 300 mg/kg MBCD from day 6 to 15 postgraft, and (v) daily i.p. injections of MBCD from day 6 to 15 and two injections of 37 MBq of $^{125}$I-anti-CEA mAb at day 8 and 11 postgraft. At day 30 postgraft, mice were sacrificed, tumors collected, and the total tumor mass was determined.
For this purpose, length, width, and depth of each tumor nodule was measured and used for calculating the nodule volume and a density of 1.05 g/cm³ was used to determine each nodule mass. The average total tumor mass for each mouse was calculated by summing up the mass value of each nodule.

Digital autoradiography and immunohistochemical detection of 53BP1. Ten days postgraft, eight mice were injected i.p. with 37 MBq of 125I-PX or of 125I-anti-CEA mAbs. Four and 24 h later, mice were anesthetized, bled, and dissected. Tumors were collected and frozen after inclusion in optimal cutting temperature embedding matrix. Two consecutive 10 µm-thick frozen sections were analyzed by digital autoradiography (DAR) or by immunohistochemical analysis. DAR acquisition was performed using a Beta Imager 2000 instrument (Biospace, Paris, France) over 24 h and analyzed using M3vision software to determine the radioactivity distribution. Immunofluorescence analysis of 53BP1 expression was performed to assess DNA DSB formation. Frozen sections were rehydrated in PBS for 10 min, fixed in 3.7% (v/v) formaldehyde, and then permeabilized in 0.5% (v/v) Triton X-100 for 15 min followed by incubation with 10% (v/v) PBS/BSA for 1 h. Sections were incubated at 37°C with an anti-53BP1 mAb (1:400) for 1 h followed by incubation with an Alexa-594-conjugated anti-rabbit secondary antibody (1:500; Invitrogen). Nuclei were stained with 0.1 µg/ml Hoechst. The distribution of 53BP1 foci was determined in 100 cells in two relevant areas of representative tumors. All animal experiments were performed in compliance with the guidelines of the French government and the INSERM standards for experimental animal studies (agreement B34-100). They were approved by the local ethic committee of Languedoc-Roussillon (CEEA LR France No. 36) for animal experiments. All in vitro data were obtained from four independent experiments in triplicate. Data were described using mean ± standard deviation. Analysis of variance was used for multigroup comparisons (significance level set at $p < 0.05$). Pairwise comparisons were performed using the Student’s $t$ test, and to control for multiple testing, the significance threshold was set at 0.013 in these comparisons.

Data were analyzed using Stata software, version 13 (StataCorp, College Station, TX).

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Author Disclosure Statement

No competing financial interests exist.

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### Abbreviations Used

- 53BP1 = p53-binding protein 1
- ASMase = acid sphingomyelinase
- CREB = C-AMP Response Element-binding protein
- DAR = digital autoradiography
- DMSO = dimethyl sulfoxide
- DSB = double-strand break
- ECL = enhanced chemiluminescence
- e-NOS = endothelial nitric oxide synthase
- ERK = extracellular signal-related kinase
- FAK = focal adhesion kinase
- FCS = fetal calf serum
- GSK3 = glycogen synthase kinase 3
- HER1 = Human Epidermal Receptor type 1
- $^{125}$I = iodine 125
- $^{123}$I = iodine 125
- $^{111}$In = indium 111
- i.p. = intraperitoneally
- IP3 = inositol triphosphate
- $^{125}$I-UdR = 5-[(125)I]iodo-2'-deoxyuridine
- JNK = c-Jun N-terminal kinase
- LET = linear energy transfer
- MAPK = mitogen-activated protein kinases
- MBCD = methyl-β-cyclodextrin
- MSK = mitogen- and stress-activated protein kinase
- NAC = N-acetyl cysteine
- p70S6K = p70S6 kinase
- PBS = phosphate buffered saline
- PLC-γ = phospholipase C-γ
- PYK-2 = proline-rich tyrosine kinase 2
- RIT = radioimmunotherapy
- RNS = reactive nitrogen species
- ROS = reactive oxygen species
- RSK = 90kDa ribosomal S6 kinase
- S1P = sphingosine-1-phosphate
- STAT1/2/3/4 = signal transducer and activator of transcription 1/2/3/4

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