213Bi-induced death of HSC45-M2 gastric cancer cells is characterized by G2 arrest and up-regulation of genes known to prevent apoptosis but induce necrosis and mitotic catastrophe

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Abstract

Tumor cells are efficiently killed after incubation with α-emitter immunoconjugates targeting tumor-specific antigens. Therefore, application of α-emitter immunoconjugates is a promising therapeutic option for treatment of carcinomas that are characterized by dissemination of single tumor cells in the peritoneum like ovarian cancer or gastric cancer. In diffuse-type gastric cancer, 10% of patients express mutant d9-E-cadherin on the surface of tumor cells that is targeted by the monoclonal antibody d9Mab. Coupling of the α-emitter 213Bi to d9Mab provides an efficient tool to eliminate HSC45-M2 gastric cancer cells expressing d9-E-cadherin in vitro and in vivo. Elucidation of the molecular mechanisms triggered by α-emitters in tumor cells could help to improve strategies of α-emitter radioimmunotherapy. For that purpose, gene expression of 213Bi-treated tumor cells was quantified using a real time quantitative-PCR low-density array covering 380 genes in combination with analysis of cell proliferation and the mode of cell death. We could show that 213Bi-induced cell death was initiated by G2 arrest; up-regulation of tumor necrosis factor (TNF), SPHK1, STAT5A, p21, MYT1, and SSTR3; and down-regulation of SPP1, CDC25 phosphatases, and of genes involved in chromosome segregation. Together with morphologic changes, these results suggest that 213Bi activates death cascades different from apoptosis. Furthermore, 213Bi-triggered up-regulation of SSTR3 could be exploited for improvement of the therapeutic regimen. [Mol Cancer Ther 2007;6(8):2346–59]

Introduction

Selective targeting of tumor cells with radionuclides is achieved via antibodies recognizing cell surface proteins that are overexpressed or exclusively expressed by tumor cells (1). α-Emitters, such as 177Lu, 131I, 188Re, and 90Y, with mean ranges in tissue between 0.7 and 3.9 mm, are used for therapy of solid tumors. Currently, 90Y- and 131I-labeled antibodies that are directed to CD20 are approved for the treatment of non–Hodgkin’s lymphoma. In contrast, α-emitters, such as 225Ac, 211At, and 213Bi, with ranges in tissue between 50 and 100 μm, show a very high relative biological effectiveness due to a high linear energy transfer of ~100 keV/μm. As few as four α-particles traversing the nucleus are sufficient to irreversibly damage a cell (2). The use of α-emitter immunoconjugates is promising in the therapy of minimal residual disease as well as in the elimination of disseminated single tumor cells (3). Successful clinical trials have been done with 211Bi-HuM195 conjugates targeting CD33 in the treatment of myeloid leukemia as well as with 211At-antitenascin in malignant glioma with intrasional application after resection of the primary tumor (4, 5).

In diffuse-type gastric cancer, ~10% of patients express mutant d9-E-cadherin that is selectively targeted by the monoclonal antibody d9Mab (6). 213Bi-d9Mab conjugates have been used to selectively target and to eliminate tumor cells expressing d9-E-cadherin both in vitro and in vivo (7–10). However, the molecular mechanisms that are induced by α-particle–emitting nuclides are poorly understood. Insight into these mechanisms could lead to improved strategies for radioimmunotherapy with α-emitters. Incubation of human prostate cancer cells and lymphocytes from leukemia patients with 213Bi-immunoconjugates has been reported to activate apoptotic pathways (11, 12), whereas treatment of gastric cancer cells expressing d9-E-cadherin with 213Bi-d9Mab triggered nonapoptotic pathways (10).

The aim of this study was to investigate key biological processes such as the mode of cell death, cell survival, and proliferation with regard to gene expression after incubation

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of HSC45-M2 gastric cancer cells with 213Bi-d9MAb conjugates. Gene expression was quantified using a newly developed real time quantitative-PCR (RTQ-PCR) low-density array: The CLARCC (cell cycle regulation, lipid metabolism, apoptosis, repair, cytokinesis, and chromosome segregation) array covers 380 genes measured simultaneously coding for cell cycle regulation, lipid metabolism, apoptosis, repair, cytokinesis, and chromosome segregation. We could show that cell death of HSC45-M2 gastric cancer cells induced by 213Bi-d9MAb is preceded by G2 arrest, as determined via bromodeoxyuridine (BrdUrd) double labeling and flow cytometry. G2 arrest was accompanied by up-regulation of SPHK1, STAT5A, p21, and MYT1 together with down-regulation of SPP1 and CDC25 phosphatases as well as genes involved in chromosome segregation. Because up-regulation of SPHK1, STAT5A, and p21 as well as down-regulation of SPP1 have been described to prevent apoptosis, 213Bi-induced cell death of HSC45-M2 cells as initiated by up-regulation of tumor necrosis factor (TNF) should be different from apoptosis.

Materials and Methods

213Bi Labeling of d9MAb

The rat antibody d9MAb targeting mutant E-cadherin lacking exon 9 (d9-E-cad; ref. 6) specifically binds to HSC45-M2 gastric cancer cells. For labeling with the α-emitter 213Bi (t1/2 = 46 min), d9MAb conjugated to the chelate 2-(4-isothiocyanatoethyl)-1-cyclohexyl-diethylenetriaminepentaacetic acid (SCN-CHX-A2'-DTPA) was incubated with Bi52/Bi522 eluted from a 225Ac/213Bi generator system (Institute for Transuranium Elements, Karlsruhe, Germany; ref. 13) for 7 min in 0.4 mol/L ammonium acetate (pH 5.3). The resulting 213Bi immunoconjugates were purified from unbound 213Bi by size exclusion chromatography using PD-10 columns (GE Healthcare). Binding of 213Bi-d9MAb to HSC45-M2 cells was assayed as described previously (8).

Incubation of Gastric Cancer Cells with 213Bi-d9MAb

The human stomach cancer cell line HSC45-M2 established from a patient suffering from diffuse-type gastric carcinoma was reported to express d9-E-cad (14). The cells were grown in DMEM supplemented with 10% FCS at 37°C in a humidified atmosphere with 5% CO2.

Aliquots of 5 × 106 cells were transferred into 175-cm2 tissue culture flasks 24 h before radiation exposure. Cells were incubated with 213Bi-d9MAb (1.5 MBq/mL) for 3 h in a total volume of 10 mL culture medium. Subsequently, medium was exchanged and the cells were allowed to grow for definite time intervals depending on the end points examined.

Fluorescence In situ Hybridization Analysis of HSC45-M2 Metaphase Spreads with a Chromosome 1 Probe

For preparation of metaphase spreads, HSC45-M2 cells (1 × 106/mL), cultured for 24 h in 25-cm2 flasks, were incubated with colcemid (0.15 μg/mL) overnight. Subsequently, cells were detached with 1 mmol/L EDTA in PBS, washed twice in Hanks medium at 37°C, incubated in 0.56% KCl for 7 min, fixed in acetic acid/methanol (1:3), dropped on ethanol-cleaned slides on a thermoplate (35°C), and allowed to dry for 20 min. Fluorescence in situ hybridization using a FITC-conjugated whole painting probe for chromosome 1 was done according to the manufacturer’s descriptions (Cytocell Technologies). Finally, slides were immersed in 4,6-diamidino-2-phenylinodole (DAPI) antifade (Cytocell Technologies), overslipped, and analyzed using confocal laser scanning microscopy (TCS NT, Leica). The confocal laser scanning microscopy was run in horizontal scanning mode using two channels for sequential FITC (excitation 488 nm, emission 530 nm) and DAPI (excitation 280 nm, emission 420 nm) excitation. Images of 512 × 512 pixels in size were scanned manually. Detection of chromosome 1 in the metaphase spreads was done via merge of corresponding DAPI and FITC images.

RNA Isolation

213Bi-irradiated and control cells were detached using 1 mmol/L EDTA in PBS at 6, 24, and 48 h after start of exposure. Total RNA was isolated with the Prep Station 6100 (Applied Biosystems) according to the manufacturer’s instructions. DNA was digested with RNase-free DNase set (Qiagen). Purified RNA was stored at −80°C until use.

To control quality and purity of isolated total RNA, spectrophotometry, agarose gel electrophoresis, and PCR (using β-actin primers for detection of DNA contamination) were done. Only total RNA samples with A260/A280 ratios >1.9, with 28S rRNA present at approximately twice the amount of the 18S RNA and without detectable contamination of DNA, were used for RTQ-PCR.

RTQ-PCR: CLARCC Array (Low-Density Array)

All materials and instruments used for RTQ-PCR were purchased from Applied Biosystems. Aliquots of total RNA (5 μg) were reversely transcribed for 2 h using a two-step PCR protocol (High Capacity Kit). A volume of 50 μL of the resulting cDNA typically contained 1 μg RNA equivalents. To this volume, an aliquot of 50 μL 2×RT-PCR master mix was added, mixed, and pipetted into one fill port of a low-density array. The remaining seven fill ports of the array were filled identically. Cards were centrifuged twice and sealed. After removal of the emptied fill ports, cards were transferred into the 7900 RTQ-PCR instrument equipped with a specially designed thermal cycler to run the RTQ-PCR with the 384-well low-density array format for 2 h.

Every well contained a unique lyophilized primer probe designed for detection of one specific human gene. This chemistry became solubilized after transfer of aliquots of the template/PCR master mix solution from the fill ports into the wells via centrifugation resulting in a total volume of 1 μL per well. This chemistry uses the 5’ nuclease activity of Taq DNA polymerase to cleave a minor groove binding probe during PCR. The minor groove binding probe contains a reporter dye at the 5’ end, the fluorescence of which is quenched by a minor groove binder located at the 3’ end. The cleavage separates the reporter dye from the 3’ end, resulting in a fluorescence signal increasing with every cycle of the PCR reaction. Three hundred eighty genes involved in cell cycle regulation, lipid metabolism, apoptosis, repair,
cytokinesis, and chromosome segregation (CLARCC) array were selected. The respective probes were synthesized by Applied Biosystems as a customer-specific fluidic card design. The residual four wells of the card were used for 18S rRNA detection to normalize the results. Differential gene expression was calculated by a software using the ΔΔCT quantitative approach.

Quantification of $^{213}$Bi-Induced Cell Death

Cell death was measured using the MAA assay [i.e., by quantification of micronucleated (M), apoptotic (A), and abnormal (A) cells at different time points after radiation exposure; ref. 15]. For that purpose, $^{213}$Bi-d9MAb exposed cells (1.5 MBq/mL; 3 h) and untreated controls were harvested at 24 and 48 h after start of incubation. Cells were rinsed twice in PBS, fixed in methanol, gently cytopun (50 g, 5 min), air dried (10 min), stained with DAPI (1 µg/mL in PBS; Serva) for 5 min, washed in distilled water (5 min), air dried again, and stored at 4°C. Analysis of cells was done using an epifluorescence microscope (DM RBE, Leica) equipped with an UV filter (excitation 270–380 nm; emission 410–580 nm). At least 1,500 cells were scored for each time point.

Criteria for scoring micronucleated cells included sharply bordered nuclei and a spatial separation between the micronuclei and the cell nucleus (16). Micronuclei had to be less than or equal to one fifth of the nucleus diameter. Additionally, the fluorescence of micronuclei and the nucleus should have roughly the same intensity and color. Micronuclei difficult to distinguish from cell protrusions were not counted. Apoptotic cells show a characteristic condensation of the DNA. All other categories of damaged cells (e.g., binucleated cells, tetranucleated cells, and necrotic cells) were comprised under abnormal cells.

Cell survival after incubation of HSC45-M2 cells with $^{213}$Bi-d9MAb (1.5 MBq/mL; 3 h) was measured by standard clonogenic cell survival assay.

Analysis of Cell Proliferation after $^{213}$Bi-d9MAb Incubation

Twenty-four hours after seeding, HSC45-M2 cells (5 × 10^4/75-cm² flask) were incubated with $^{213}$Bi-d9MAb (1.5 MBq/mL) for 3 h. Incubation was stopped by change of culture medium. Pulse labeling with 10 µmol/L BrdUrd (Serva) for 20 min was done 20 min before start of incubation with $^{213}$Bi-d9MAb. BrdUrd was removed completely by repeated washings with BrdUrd-free culture medium. At defined time points after start of irradiation (4, 6, 12, and 24 h), cells were detached with 1 mmol/L EDTA in PBS and fixed in 80% ethanol. For preparation of nuclei, cells were treated with RNase A (Sigma) and subsequently incubated with pepsin (Merck; 0.7 FIP-U/mg; 5% in 0.05 N HCl) for 10 min at 37°C. DNA was denatured with 2 N HCl (10 min, room temperature). After three washes in PBS-albumin (1%), cell nuclei were incubated with anti-BrdUrd mouse IgG (1:10 in PBS-albumin; Becton Dickinson) and FITC-conjugated rabbit anti-mouse IgG (1:50 in PBS-albumin; Dako) for 30 min each (room temperature). DNA was counterstained with propidium iodide (50 µg/mL). DNA content and BrdUrd incorporation were analyzed by dual-parameter flow cytometry (FACS caliber, Becton Dickinson). Histograms were evaluated as described (17).

Statistics

At least three independent experiments were done for each of the end points described. The means, geometric means, and SE were calculated with the aid of statistical software (Sigma Plot 2000, Jandel).

Results

Specific Binding of $^{213}$Bi-d9MAb to HSC45-M2 Gastric Cancer Cells

Labeling of d9MAb with $^{213}$Bi resulted in maximal specific activities of 1.5 GBq/mg d9MAb. Following size exclusion chromatography to remove unbound $^{213}$Bi, the radiochemical purity of the labeled antibody fractions varied between 95% and 99%. The in vitro stability of $^{213}$Bi-d9MAb at room temperature exceeded four half-lives (3 h) of $^{213}$Bi. The gastric cancer cell line HSC45-M2 targeted by $^{213}$Bi-d9MAb due to expression of ~3 × 10^5 molecules of d9-E-cadherin per cell (10) proved to be highly aneuploid, comprising ~80 to 88 chromosomes per nucleus. Typically five to six copies of chromosome 1 or fragments of chromosome 1 could be detected using fluorescence in situ hybridization (Fig. 1A). Binding of $^{213}$Bi-d9MAb to HSC45-M2 turned out to be highly specific (25% binding) compared with an unspecific $^{213}$Bi-immunoconjugate of the same species and subclass targeting mutant d8-E-cadherin not expressed on HSC45-M2 cells (2% binding; data not shown).

$^{213}$Bi-d9MAb Triggered Killing of HSC45-M2 Gastric Cancer Cells Is Associated with Up-regulation of TNF

As revealed via the clonogenic assay (plating efficiency 61%), almost 99% of HSC45-M2 cells were killed after incubation with $^{213}$Bi-d9MAb (1.5 MBq/mL). However, cells died relatively slowly. Dying was initiated by massive swelling of the cells ~24 to 48 h after radiation exposure. Forty-eight to 72 h postincubation with $^{213}$Bi-d9MAb, large cells characterized by enlarged nuclei with prominent nucleoli and vacuoles in the cytoplasm as well as multinucleated cells predominated (Fig. 1B). Cells started to disintegrate at 72 h after radiation exposure and cell death usually was completed at 96 h. In contrast to the results of the clonogenic assay, only 11.4% of cells investigated using the MAA assay showed microscopic lesions in the nucleus 48 h after radiation exposure: micronucleated cells, 3.2%; apoptotic cells, 1.2%; abnormal cells, 7%. These values were not significantly different from untreated controls (micronucleated, 1–2%; apoptotic, 0–0.3%; abnormal, 0.5–3%; data not shown).

Of a total of 79 genes examined, all of them involved in regulation of apoptotic processes, at 6 h after irradiation with $^{213}$Bi-d9MAb a ≥4-fold difference in gene expression relative to control was found only for six genes being upregulated and one gene being down-regulated (Fig. 2A). A similar number of genes appeared differentially expressed at 24 h (five genes up-regulated and one gene
down-regulated) and at 48 h (eight genes up-regulated and two genes down-regulated) after radiation exposure. From these, cell death–inducing genes were associated with the TNF family (TNF ligand was 4- and 6-fold up-regulated and TNFRSF9 11- and 2-fold up-regulated at 6 and 48 h after irradiation). Furthermore, SSTR3 appeared up-regulated 4-fold and 10-fold 24 and 48 h after incubation with $^{213}$Bi-d9MAb, respectively. No changes in Fas-related receptors were found. Genes related to the integration phase of apoptosis in general showed no changes greater than 2-fold over control, except for BAG5 and the BCL-2 family member BCL2L14, which both revealed a 2-fold increase in gene expression: BCL2L14 at 6 and 48 h; BAG5 at 48 h after irradiation. From the numerous genes of the caspase family examined, only CASP1 (2- to 3-fold at 6, 24, and 48 h) and CASP14 (2-fold at 24 h and 8-fold at 48 h) showed up-regulation. Among various DNases, only DNase1L3 was up-regulated 11-fold at 48 h after radiation exposure.

Likewise, expression of 16 genes associated with the sphingomyelin fatty acid metabolism related to apoptotic processes was measured (Fig. 2B). At 24 and 48 h, only SPHK1 (2-fold) and at 48 h after radiation exposure the genes A4GALT (4.5-fold) and CD36 (2.5-fold) appeared up-regulated, whereas the remaining genes revealed control levels.

Additionally, 46 genes that have shown differential expression in various in vitro models characterized by radiation-induced micronucleation were examined (Fig. 2C). Four genes were up-regulated either at all three time points (COL6A1, 3- to 10-fold), at 24 and 48 h (SDC3, 2- to 4-fold and GNAS, 3.5- to 9-fold), or at 6 and 48 h (GSTP1, 2.5- to 6.5-fold) after radiation exposure. The gene coding for SPP1 became down-regulated 0.2- and 0.4-fold at 24 and 48 h, respectively. The remainder appeared at control levels.

Taken together, necrotic forms of cell death and micronucleation were associated with an up-regulation of (a) genes related to induction (TNF family), integration (BCL2L14), and execution (caspase-1 and caspase-14, DNase) of cell death; (b) genes of the sphingomyelin metabolism (e.g., SPHK1 and A4GALT); and (c) genes found to play a role in the process of micronucleation (e.g., COL6A1, SDC3, GNAS, and GSTP1).

$^{213}$Bi-d9MAb Continuously Down-regulates Genes Involved in Chromosome Segregation in HSC45-M2 Cells

Gene expression related to cytokinesis and chromosome segregation was examined in 65 genes (Fig. 3). Genes of this group showed the highest number of differentially expressed genes (30) with most of them being down-regulated (28). Moreover, several of the genes showed a constant down-regulation at all time points investigated. Among them were genes coding for central spindle formation (KIF20A, 0.2-fold down-regulated) and other processes of chromosome segregation (STK6, CENP-E, PLK1, all of them 0.2- to 0.5-fold down-regulated). Other genes being down-regulated distinctly at 6 and 24 h after incubation with $^{213}$Bi-d9MAb included BUB1 and KPNA2 (0.4-fold, respectively). Only two genes showed significant up-regulation: MYL9 at 48 h (4-fold) and ACTA2 at 24 and 48 h (2- to 3-fold).
Figure 2. Gene expression related to regulation of cell death using a customer-designed low-density array (CLARCC). $^{212}$Bi-irradiated HSC45-M2 cells were examined at 6, 24, and 48 h after irradiation. Gene expression related to apoptotic processes (A), sphingomyelin fatty acid metabolism (B), and radiation-induced micronucleation (C). Points, geometric mean of three independent experiments; bars, SE. Points with missing bars represent measurements of single experiments, because the other repeats showed values that were outside the linear dynamic range of the method. If data points are missing at a time point, the values of all three independent experiments were outside the linear dynamic range.
Selected genes related to signal transduction pathways showed altered gene expression only in case of the PKC pathway (10-fold up-regulation of PRKCB1 at 24 and 48 h), the serine-threonine kinase pathway (0.2–0.5 down-regulation of STK6 at all time points examined), and the TP53 pathway; although MDM2 showed a 0.1- to 0.4-fold down-regulation, expression of TP73 was up-regulated increasingly to a maximum of 5-fold at 48 h after radiation exposure (Fig. 4).

$^{213}$Bi-d9MAb Induces S-Phase Delay and G2 Arrest in HSC45-M2 Cells Accompanied by Up-regulation of STAT5A, p21, and MYT1

Pulse-labeled, BrdUrd-positive HSC45-M2 cells that had been incubated with $^{213}$Bi-d9MAb showed a delayed S-phase progression compared with untreated controls and were finally arrested in G2 phase as shown by flow cytometry (Fig. 5A). Up to 9 h after radiation exposure, the number of S-phase cells remained in the range of 43%, whereas in the controls it continuously decreased from 43% to 3% (Fig. 5Bb). As a consequence, irradiated BrdUrd-positive cells showed a delayed entry into G2 phase and accumulated in G2 phase: Starting with 3% at 0 h, at 24 h after irradiation 48% of cells were in G2 phase. In untreated controls, only 21% were found in G2 phase (Fig. 5Bc). As a result, none of the $^{213}$Bi-irradiated BrdUrd-positive cells entered G1 phase (Fig. 5Ba). A G2 arrest after $^{213}$Bi-d9MAb incubation could also be shown for BrdUrd-negative cells (Fig. 5Bf). Results were almost...
identical if BrdUrd pulse labeling was done 1 h after stop of incubation with $^{213}$Bi-d9MAb, indicating that BrdUrd incorporation has no effect on $^{213}$Bi-induced changes of cell cycle progression (data not shown).

Most of the differentially expressed genes involved in cell cycle control were found among genes controlling G$_1$-S and G$_2$-M transition: Cyclins E1 and E2 (CCNE1, CCNE2) showed a 2-fold up-regulation at all time points (6, 24, and 48 h) and cyclin D2 (CCND2) a 6- to 12-fold up-regulation at 24 and 48 h after radiation exposure. Although certain cyclin B (CCNB) and CDC25 transcripts revealed up to 0.2-fold down-regulation throughout the measurements, MYT1 was up-regulated drastically (up to 20-fold) at 6 and 24 h (Fig. 6). Among other genes regulating cell cycle progression, cyclin A1 (CCNA1; 9- to 12-fold up-regulation at 24 and 48 h); the Cdk inhibitors p21 (CDKN1A; 2-fold increase at all time points), CDKN1C (0.5-fold down-regulation at 24 and 48 h), and CDKN2C (0.6-fold down-regulation throughout); the FGF1 receptor (2- to 4-fold up-regulation at 24 and 48 h); and STAT5A (2- to 5-fold up-regulation throughout) appeared differentially expressed (Fig. 6). Taken together, $^{213}$Bi-d9MAb–induced S-phase delay and G$_2$ arrest measured by flow cytometry can be associated with drastic up-regulation of genes regulating phase transitions and slight down-regulation of cyclin B and CDC25 phosphatases.

$^{213}$Bi-d9MAb Treatment Induced Only Little Changes in Expression of DNA Repair Genes

Gene expression analysis of 40 genes related to DNA repair nearly showed no changes except a 2-fold up-regulation of RAD51 at 48 h after incubation of HSC45-M2 cells with $^{213}$Bi-d9MAb (Fig. 7).

Discussion

The reduction of clonogenicity over two log scales after a single treatment of HSC45-M2 cells with $^{213}$Bi-d9MAb reflects the cytotoxic potency of the $\alpha$-emitter $^{213}$Bi. Light microscopic examinations suggest a predominant mode of cell death characterized by necrosis and multinucleation (Fig. 1B). In contrast to the clonogenic data, only discrete changes in the number of micronucleated, apoptotic, and abnormal cells (primarily multinucleated cells) were counted with the MAA assay, suggesting that the altered cells might be destroyed during preparation in the cytoplasm, which is in line with own experiences with irradiated lymphocytes. The results further suggest that the $^{213}$Bi-induced cellular damages prevent cell division and thus formation of micronuclei and of other abnormalities that are detected using the MAA assay. This is in accordance with the finding that $^{213}$Bi-d9MAb treatment induced G$_2$ arrest in HSC45-M2 cells.

Analysis of gene expression via RTQ-PCR suggests a TNF pathway–mediated cell death because increased transcripts of TNF ligand and TNF receptor as well as up-regulated caspases were found in HSC45-M2 cells after $^{213}$Bi-d9MAb incubation (Fig. 2A). The induction of either necrosis or apoptosis via TNF pathways as outlined above has been described for IEC-6 cells (18). Complete inhibition of the caspase cascade with zVAD-fmk resulted in TNF$\alpha$-induced necrosis rather than apoptosis. However, after exclusive inhibition of activation of caspase-3 via the specific inhibitor zDEVD-fmk, TNF$\alpha$ could induce apoptosis. Accordingly, caspase-3 is not activated in the course of TNF$\alpha$-induced apoptosis. As we have shown previously, $^{213}$Bi-d9MAb treatment of HSC45-M2 cells also did not induce significant activation of caspase-3. Nevertheless, cell death induced by $\alpha$-particle–emitting $^{213}$Bi-d9MAb showed unequivocal signs of necrosis in both the presence and the absence of zVAD-fmk (10).

Up-regulated somatostatin receptor 3 (SSTR3) found in our experiments could lead to induction of apoptosis as shown in colorectal cancer cells (19). Thus far, only a TP53- and Bax-mediated mechanism was described for SSTR3 (20); however, both transcripts remained at control in our experiments. Instead, the TP53 family member TP73 (Fig. 4) and the BCL2 member BCL2L14 (Fig. 2A) were elevated. The $^{213}$Bi-triggered, massive DNA damages are thought to activate DNA repair processes through up-regulation of TP53. However, activation of TP53 could not be observed at any time point after $^{213}$Bi treatment (Fig. 4). Therefore, the function of TP53 in HSC45-M2 gastric cancer cells might have been lost, as is the case in >50% of all cancers analyzed. However, HSC45-M2 cells have been shown to undergo a caspase-3–mediated mode of cell death, most likely apoptosis, after treatment with sorbitol (10).

Down-regulation of MDM2 as observed in our study after treatment of HSC45-M2 cells with $^{213}$Bi-d9MAb (Fig. 4) has also been described for primary rat/mouse cells after high-dose UV irradiation (20 J/m$^2$; ref. 21). Because binding of MDM2 to TP53 leads to an inhibition of the ability of TP53 to function as a tumor suppressor (22), reduced expression of MDM2 should promote processes to overcome radiation damage as triggered by TP53.

It has been reported that the expression of SSTR3 protein was significantly lower in gastric cancer cells compared with normal mucosa (23). Nevertheless, treatment of gastrointestinal carcinoids with somatostatin analogues targeting SSTR3, like octreotide or octreotides labeled with radionuclides ($^{111}$In, $^{90}$Y, $^{177}$Lu), have proved to efficiently inhibit cell growth and induce apoptosis (24). Therefore, after $^{213}$Bi-d9MAb therapy of diffuse-type gastric cancer in the nude mouse model (8), up-regulation of SSTR3 expression could be exploited to enhance therapeutic efficacy via additional administration of radiolabeled octreotide.

Changes observed in the sphingomyelin fatty acid metabolism after incubation of HSC45-M2 cells with $^{213}$Bi-d9MAb included up-regulation of sphingosine kinase 1 (SPHK1) and the thrombospondin 1 receptor CD36 (Fig. 2B). With respect to regulation of proliferation and apoptosis, the CD36 receptor ligand thrombospondin 1 presents both stimulatory and inhibitory effects (25). As observed in PC12 cells, increased expression of SPHK1 resulted in increases of sphingosine-1-phosphate levels and marked suppression of apoptosis (26). This again suggests
that $^{213}$Bi-induced cell death is different from apoptosis as already postulated (10).

In previous studies, certain genes could be defined that were associated with radiation-induced micronucleated cells as well as apoptotic cells (Fig. 2C). Among these genes, four appeared up-regulated after $^{213}$Bi-treatment: COL6A1, SDC3, GNAS, and GSTP1. Collagen IV (COL6A1) and syndecan-3 (SDC3) function in muscle metabolism and in cell adhesion. The complex GNAS locus, giving rise to several different transcripts, is involved in signal transduction, whereas glutathione S-transferase π (GSTP1) plays a key role in detoxification.

Figure 3. Gene expression related to cytokinesis and chromosome segregation using a customer-designed low-density array (CLARCC). $^{213}$Bi-irradiated HSC45-M2 cells were examined at 6, 24, and 48 h after irradiation. Points, geometric mean of three independent experiments; bars, SE. Points with missing bars represent measurements of single experiments, because the other repeats showed values that were outside the linear dynamic range of the method.

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Up-regulation of these four genes after $^{213}$Bi treatment is in accordance with the idea that independent of the mode of cell death, there exist certain similarities within the different activated signal transduction pathways (27). Because cell death in osteopontin (SPP1)–deficient cardiac fibroblasts occurs independently of caspase-3 (28), $^{213}$Bi-d9MAb–induced down-regulation of SPP1 strongly suggests induction of necrotic cell death.

Following treatment of HSC45-M2 cells with $^{213}$Bi-d9MAb, chromosome segregation was blocked due to down-regulation of the key regulatory serine/threonine kinases aurora-A kinase (STK6), BUB1 (budding uninhibited by benzamidazoles 1 homologue), and polo-like kinase 1 (PLK1), all of them implicated in the assembly of the mitotic spindle and in the spindle checkpoint (Fig. 3; refs. 29–31). PLK1 is overexpressed in many human tumors, including gastric
carcinoma, and therefore PLK1 inhibitors are of interest in terms of suppression of tumor growth in vivo (32). Other proteins involved in chromosome/spindle stability during mitosis being down-regulated after 213Bi treatment of HSC45-M2 cells were centromere protein E (CENP-E; ref. 33), the kinesin family member 20A (KIF20A; ref. 34), and importin a1 (KPNA2; ref. 35; Fig. 3). Down-regulation of these genes is in accordance with the occurrence of multinucleated/mitotic catastrophe cells. KPNA2 also mediates nuclear localization of the MRN complex in response to radiation (36). This complex consisting of MRE11, RAD50, and NBS1 forms nuclear foci that are required for DNA double strand break repair. Irradiation of HeLa cells (15 Gy) resulted in increased nuclear activity of MRN and DNA repair (36). Interestingly, incubation of HSC45-M2 cells with the α-emitter 213Bi decreased KPNA2 expression, implying a reduction of MRN-mediated double strand break repair. Nevertheless, drastic DNA damage induced by 213Bi-d9MAb is repaired efficiently, as has been shown using the COMET assay.6 Thus, high linear energy transfer α-radiation and low linear energy transfer γ-radiation might induce different signal cascades triggering DNA double strand break repair. The only genes involved in cytokinesis and chromosome segregation being up-regulated after 213Bi-d9MAb incubation of HSC45-M2 cells—MYL9 (myosin light chain polypeptide 9) and ACTA2 (actin, α2, smooth muscle, aorta)—might be involved in generation of stress fibers (37), which has been described to increase in human lung cancer cells after irradiation (38).

Up-regulation of FGF1 receptor 24 and 48 h after incubation with 213Bi-d9MAb should stimulate cell proliferation through a number of different pathways (39). Accordingly, STAT5A activation as observed at all time points after incubation of HSC45-M2 cells with 213Bi-d9MAb signals proliferation and thus should prevent cells from undergoing apoptosis (Fig. 6). In murine T cells, STAT5A deficiency causes a proliferative deficiency and enhanced apoptotic rates (40). STAT5A has also been described to positively regulate transcription of cyclin D (41). This finding is in accordance with the observed up-regulation of cyclin D2 after 213Bi-d9MAb incubation. Because D-type cyclins are essential for the progression through the G1-phase of the cell cycle, G1 progression should not be inhibited by 213Bi-d9MAb as observed in

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6 U. Roessler, personal communication.
Gene expression related to cell cycle control using a customer-designed low-density array (CLARCC). $^{213}$Bi-irradiated HSC45-M2 cells were examined at 6, 24, and 48 h after irradiation. Points, geometric mean of three independent experiments; bars, SE. Points with missing error bars represent measurements of single experiments, because the other repeats showed values that were outside the linear dynamic range of the method. If data points are missing at a time point, the values of all three independent experiments were outside the linear dynamic range.
BrdUrd-labeling experiments. Moreover, STAT5A has been referred to induce expression of p21 (42). Accordingly, we could observe up-regulation of p21 at all time points after incubation of HSC45-M2 cells with 213Bi-d9MAb (Fig. 6). Up-regulation of p21 after irradiation with 20 Gy of human MCF-7 and HCT116 carcinoma cells has been associated with G2 arrest and prevention of apoptosis (43). Likewise, cells homozygously deleted for p21 displayed no irradiation-induced G2 arrest but enhanced apoptosis via a caspase-3-dependent mechanism. G2 arrest as a consequence of incubation of HSC45-M2 gastric carcinoma cells with 213Bi-d9MAb has been shown after flow cytometry analysis of BrdUrd-labeled cells (Fig. 5).

Strong up-regulation of MYT1, particularly at 6 and 24 h after incubation of HSC45-M2 cells with 213Bi-d9MAb, is also in accordance with the observed G2 arrest (Fig. 6). MYT1 negatively regulates CDC2/cyclin B1 by phosphorylation of Thr14 and Tyr15 and thus overexpression of MYT1 inhibits G2-M phase progression (44). RNA interference–triggered down-regulation of MYT1 could abrogate DNA damage–induced G2 arrest (45). Such a correlation remains to be shown in our setting.

The inhibitory phosphates at Thr14 and Tyr15 of CDC2 are removed at the onset of mitosis by the CDC25 dual-specificity phosphatases. All three CDC25 isoforms (i.e., CDC25A, CDC25B, and CDC25C) cooperate to activate CDC2/cyclin B1 complexes during the G2-M transition (46). Thus, the observed down-regulation of CDC25B/C after 213Bi-d9MAb incubation is also in accordance with G2 arrest as determined via flow cytometry analysis (Figs. 5 and 6).

Surprisingly, mRNA levels of cyclins A and B behaved contrarily after incubation of HSC45-M2 with 213Bi-d9MAb. Although cyclin A1 was significantly up-regulated, all isoforms of cyclin B (cyclin B1, cyclin B2, and cyclin B3) were down-regulated (Fig. 6). Because synthesis of cyclins drives cells into mitosis and their destruction allows cells to finish the cell cycle (47), one would expect that in case of radiation-induced G2 arrest, synthesis and/or degradation

Figure 7. Gene expression related to different DNA repair mechanisms using a customer-designed low-density array (CLARCC). 213Bi-irradiated HSC45-M2 cells were examined at 6, 24, and 48 h after irradiation. Points, geometric mean of three independent experiments; bars, SE. Points with missing bars represent measurements of single experiments, because the other repeats showed values that were outside the linear dynamic range of the method.
of cyclins are inhibited. Thus, due to incubation with $^{213}$Bi-d9MAb, degradation should be disrupted in case of cyclin A1 and synthesis in case of cyclins B.

Only little changes in expression of genes associated with DNA repair could be detected after incubation of HSC45-M2 cells with $^{213}$Bi-d9MAb (Fig. 7). For example, the mRNA coding for the DNA-dependent ATPase RAD51 was up-regulated 2-fold 48 h after irradiation. These results are in accordance with observations generally made on eukaryotic cells after irradiation: At most, there is only a 2-fold up-regulation of proteins involved in DNA repair (48). Instead, in response to DNA damage, repair proteins that are found diffused in the nucleus are rapidly concentrated into subcellular complexes that can be visualized as foci containing RAD51 and RAD52. This relocation of repair proteins can occur within a few minutes to ~1 h after irradiation. In fact, repair of double-strand breaks after irradiation of HSC45-M2 cells with $^{213}$Bi-d9MAb seems to be completed within 1 h, as derived from COMET assay experiments. Up-regulation of RAD51 at a late time point (48 h) after $^{213}$Bi-d9MAb treatment also might support a role of RAD51 in recombination repair processes associated with postreplicative chromatin, as has been described for a fibroblast cell line (49).

Taken together, the mode of cell death found in $^{213}$Bi-treated HSC45-M2 cells obviously involves the TNF and SSTR3 pathways as well as an inhibition of cytokinesis and chromosome segregation. This was accompanied by a G2 arrest most likely induced via up-regulated STAT5A, p21, and MYT1 as well as down-regulation of CDC25 phosphatases. Future experiments will focus on targeting the SSTR3 pathway via octreotide to further improve the therapeutic outcome by reducing clonogenicity. Furthermore, RNA interference experiments are needed to elucidate the causal relationship between application of $^{213}$Bi-d9MAb and the TNF pathway induction. Finally, further studies on other gastric cancer cells will be done to clarify the molecular mechanisms induced by $\alpha$-emitter immunonjugates.

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References


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213Bi-induced death of HSC45-M2 gastric cancer cells is characterized by G₂ arrest and up-regulation of genes known to prevent apoptosis but induce necrosis and mitotic catastrophe

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