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RON Receptor Tyrosine Kinase as a Target for Delivery of Chemodrugs by Antibody Directed Pathway for Cancer Cell Cytotoxicity

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Abstract: Overexpression of the RON receptor tyrosine kinase exists in various cancers and contributes to malignant progression. To validate RON as a targeting moiety for delivery of chemoagents for enhanced tumor cytotoxicity, immunoliposomes (IL) loaded with doxorubicin (Dox) were formulated followed by postinsertion of monoclonal antibodies Zt/g4, Zt/c1, or their Fab fragments specific to the RON extracellular domains. Flow cytometry analysis showed that Zt/g4 or Zt/c1-IL binds to cancer cells and causes RON internalization as evident in confocal analysis of intracellular fluorescence intensity. The antibody-directed IL uptake by cancer cells is in both dose and time-dependent manners. Studies of cytotoxicity of individual IL *in vitro* against colon or breast cancer cell lines revealed that Zt/g4 directed Dox-IL displayed increased cytotoxic activities with a significant reduction of IC_{50} values. An average of 8-fold increases in cytotoxic efficiency was achieved among four cell lines tested. Moreover, Zt/g4 directed Dox-IL also displayed the effective killing of cancer cells that are insensitive to pegylated liposomal doxorubicin. The effect of Zt/c1-Dox-IL was not as strong as Zt/g4-Dox-IL, and only moderate activities were observed. IL coupled with the Fab fragments of Zt/g4 or Zt/c1 show moderate activities against cancer cells. The ineffectiveness seemed to be related to the weak activities of the Fab fragments in the induction of RON internalization, which resulted in reduced drug uptakes. We conclude that anti-RON antibody-directed drug delivery is effective for increased uptake of cytotoxic drugs. Antibody-based RON targeting could be developed into a potential therapeutic for treatment of malignant cancers.

Keywords: Receptor tyrosine kinase; monoclonal antibody; immunoliposomes; drug delivery; targeted therapy

Introduction

Targeted cancer therapy with monoclonal antibodies (mAb) specific to proteins on the surface of tumor cells has achieved clinical significance in treatment of several malignancies.[1,2] Such approaches also open a new avenue for the development of novel therapeutics.[3,4] Cell surface proteins such as receptor tyrosine kinases are often overexpressed in cancerous cells and contribute to the pathogenesis of tumors. Because of their pathogenic features, these molecules have been recognized as either the direct drug target or a suitable moiety for delivery of cytotoxic drugs. Development of antibody-directed delivery of chemoagents

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in the form of immunoliposomes (IL) to cancer cells overexpressing epidermal growth factor receptor (EGFR) is a good example.[5−7] This process significantly increases the internalization of chemoagents in cancer cells and enhances the efficacy of multiple anticancer drugs *in vivo*.[5−7] Thus, antibody-directed drug delivery targeting receptor tyrosine kinases overexpressed in solid tumors is a strategy to achieve maximal therapeutic activities.

The RON receptor tyrosine kinase is a member of the Met proto-oncogene family.[8] The protein is mainly expressed at relatively low levels in various epithelial cells.[9] Upon interaction with specific ligand macrophage-stimulating protein (MSP),[10] also known as hepatocyte growth factor-like protein,[11] RON undergoes dimerization leading to protein phosphorylation and activation of downstream signaling cascades such as the MAP kinase pathway.[12,13] Activation also results in RON internalization into the cytoplasm, which is a mechanism that regulates a delicate signaling balance in response to the extracellular stimulation.[14] Altered RON expression, characterized by overexpression, is often observed in colon and breast cancer samples.[9,15] As shown by immunohistochemical analysis, RON is minimally expressed in normal or benign cells but overexpressed at significant numbers in primary colon and breast cancers.[9,15] Overexpression is often associated with disease progression and worsened prognosis.[9,15] Considering these facts, RON is regarded as a potential drug target for therapeutic interventions. Development of RON-specific small chemical inhibitors or antibodies that block oncogenic RON signaling is currently under intensive investigation.[15−17] Results from published studies indicate that the application of specific mAb or small molecule inhibitors attenuates RON-mediated signaling in various cancer cells and reduces tumor growth in animal models.[15−17] These findings suggest that targeting RON has therapeutic significance.

The present study is to validate if RON is a useful molecule for delivery of chemodrugs into cancerous cells. We hypothesized that antibody-induced internalization of RON is an effective pathway for delivery of cytotoxic drugs into the cancerous cells leading to increased cytotoxicity. To test this hypothesis, two mAb that bind to human RON extracellular domains[18] were selected to deliver doxorubicin (Dox) into colon and breast cancer cells. By formulation of antibody-directed IL loaded with Dox, we demonstrated that anti-RON antibodies effectively induce internalization of the receptor, which facilitates the uptake of drug-loaded liposomes (LS) by cancerous cells. The increased drug uptake resulted in effective killing of tumor cells with significant reduction of IC_{50} values required for therapeutic activities. Thus, anti-RON antibody directed delivery of chemoagent is the effective pathway for increased uptake of cytotoxic drug. Antibody-based RON targeting could be a novel

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approach for delivery of chemoagents for treatment of malignant cancers.

Materials and Methods

Chemicals and Reagents. Various chemicals and phospholipids (PL), including cholesterol, hydrogenated soya phosphatidylcholine (HSPC), mPEG-DSPE, Mal-PEG-DSPE and rhodamine phosphatidylethanolamine, were from Avanti Polar Lipid (Birmingham, AL). Doxorubicin (Dox) was from Alexis Biochemicals (San Diego, CA). Pegylated-liposomal doxorubicin (PLD) was from Ortho Biotech Products LP (Horsham, PA).

Cell Lines and Reagents. Tumor cell lines T-47D, HCC1937, MCF-7, SW620, and SW837 were from ATCC (Manassas, VA). Mouse anti-RON mAb Zt/g4, Zt/c1, and 2F2 were produced by us and purified by a protein G-Sepharose column as previously described.[18] Fab fragments of Zt/g4 or Zt/c1were prepared according to the manufacturer's instruction (Pierce). Normal mouse IgG and goat anti-mouse IgG labeled with FITC were from Jackson Immunoresearch Lab (West Grove, PA). Human recombinant MSP was provided by Dr. E. J. Leonard (National Cancer Institute, Bethesda, MD). Rabbit IgG antibody to RON C-terminal peptides was used as previously described.[9] Mouse IgG antibody specific to phosphotyrosine (clone PT100) was from Cell Signaling Technology Inc. (Danvers, MA). Enhanced chemiluminescent reagents were from Thermo Scientific.

Formulation of Dox-Loaded LS and IgG-Conjugated IL. LS were prepared by hydration of thin lipid film and adjusted to the size of 110 ± 10 nm by sonication as previously described.[19] They contained HSPC:Chol:$MPEG_{2000}$-DSPE at a 2:1:0.08 molar ratio. Dox was loaded using the ammonium sulfate exchange method.[19] Incorporation of fluorescent dye rhodamine (RD) into LS (RD-LS) was performed by using RD-PE as previously described and used for uptake experiments.[19,20]

Incorporation of antibodies into LS was carried out by the postinsertion technique as previously described.[20] Zt/g4, Zt/c1, or control IgG were first thiolated using Traut's reagents and then conjugated with micelles prepared from $mPEG_{2000}$DSPE:Mal-PEG_{2000}DSPE (4:1 molar ratio). Micelles and thiolated antibody were reacted at a 10:1 molar ratio of Mal-PEG_{2000}DSPE:antibody under oxygen free conditions, which were achieved by purging nitrogen gas according to a previously described method.[19,20] Fab fragments of Zt/g4 and Zt/c1 were prepared by ficin digestion followed by treatment with 2-mercaptoethanolamine as previously described.[5] The thiolated Fab fragments were then conjugated to Mal-PEG2000-DSPE at a ratio of 4:1 (Mal-PEG200-DSPE: protein) according to a previously published method.[5,19,20] Conjugation efficacy of Zt/g4 Fab and Zt/c1 Fab ranged at 40−50% or 65−70%, respectively. The whole antibody or Fab containing micelles were then incorporated into LS by incubation at 55 °C for 30 min. The ratio of micelles to LS was 75 μg of Fab fragments per μM of PL or 120 μg of IgG per μM of PL for postinsertion of antibody micelles in preformed LS. As a result, the micelles were attached to the outer lipid layer of the LS via hydrophobic DSPE domains. The efficacy of IgG or Fab fragment incorporation on LS was determined by 10% SDS−PAGE analysis using IgG standards followed by densitometry analysis.

Western Blot, Immunoprecipitation, and Protein Phosphorylation Assays. Western blot analysis was performed as previously described.[9] Rabbit anti-RON IgG antibody was used to detect RON followed by reaction with chemiluminescent reagents. To measure RON phosphorylation, cells were stimulated with MSP, Zt/g4, or Zt/c1 for 15 min at 37 °C, lysed in lysis buffer, and then immunoprecipitated with mAb 2F2.[9] The phosphorylated RON was detected by Western blotting using PY100 specific to phosphotyrosine.[9]

Fluorescent Analysis of Antibody Binding and Antibody-Induced RON Internalization in Cancer Cells. Cell surface-binding activities of Zt/g4, Zt/c1, or their Fab fragments were determined as previously described.[21] Briefly, cells at 1×10^6 cells/sample were treated with individual mAb or Fab fragments at 4 °C for 45 min followed by incubation with anti-mouse IgG coupled with FITC. Normal mouse IgG was used as the control. To determine mAb-induced RON internalization, cells (1×10^6 cells/sample) were first incubated at 37 °C with 2 μg of FITC-labeled Zt/g4 or Zt/c1 for various times and then washed with an acidic buffer (150 mM NaCl, pH 2.5) to eliminate antibodies bound on the cell surface. Cells without acid wash served as the control. In certain experiments, cytochalasin B (CC-B), an endocytic inhibitor,[22] was used to verify receptor internalization. Cellular fluorescent intensities were measured by BD FACScan.

Methods for Measuring Cellular Uptakes of Zt/g4-IL or Zt/c1-IL. Two methods were used to determine cellular uptake of antibody-coupled IL. The first is a confocal microscope-based method, in which RD is used as the indicator of cellular uptake.[22] Briefly, T47-D or other cells were cultured overnight in a slide and then treated with antibody- or Fab-RD-IL for 60 min. Cells treated with NIg-RD-IL served as the control. After incubation, cells were washed with PBS, fixed with 4% paraformaldehyde solution, and then observed under the Zeiss confocal microscope. The second method is to use the uptake of PL by cells as the

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indicator.[23] In this assay, the fluorescent intensities of RD were quantitatively measured and then converted into the amounts of PL incorporated into IL.[23] Cells were incubated with various amounts of Zt/g4-, Zt/c1-, or NIg-RD-IL at 4 or 37 °C for 60 min and then washed with PBS. The fluorescent intensities from cell lysates were measured by a microplate fluorescent reader. The uptake was calculated and converted to the amounts of PL in the cells.[23]

Fluorescent Assay for Measuring Cellular Uptake Dox. The assay was performed as previously described.[5] Briefly, cells (1×10^6 cells/sample) were treated at 37 °C for 60 min with Zt/g4-Dox-IL at a PL concentration of 2.4 μM as described in the above method for the uptake of PL by cells. NIg-Dox-IL was used as control. After incubation, cells were washed and lysed. Fluorescent emission of Dox in cell lysates was measured at 592 nm using a microplate fluorescence reader (Bio-Tek, Winooski, VT).[5]

Uptake of Zt/g4-RD-IL in the Presence of Phosphorylation and Endocytic Inhibitors. T-47D cells were first treated with tyrosine phosphorylation inhibitor herbimycin A (HB-A, 5 μg/mL)[24] and then treated with antibody or MSP for 15 min. Cellular proteins were immunoprecipitated with mAb 2F2 followed by Western blotting to determine RON phosphorylation. For uptake experiments, T-47D cells (1×10^6 cells/sample) were pretreated with HB-A and then treated with Zt/g4-RD-IL at 37 or 4 °C for 60 min. Fluorescence intensities from cell lysates were measured as previously described.[23] To determine the effect of endocytic inhibitor CC-B[22,25] on IL uptake, T-47D cells were first incubated with 10 μg/mL of CC-B and then treated with Zt/g4-RD-IL at 37 °C for 60 min. Fluorescence intensities in the cell lysates were measured as described above. Mouse IgG-RD-IL was used as the control.

Cell Growth/Cytotoxicity Assays. The MTT assay was used to monitor cell growth and viability.[24] Briefly, cells (1×10^4 cells per well) in a 96-well plate were cultured in triplicate in DMEM with 10% FBS and treated with various amounts of Zt/g4 or Zt/c1 for 3 days. For cytotoxicity, cells were treated with various amounts of Dox, PLD, or IL for 60 min and then washed. After incubation for 3 days, cells were washed with PBS and then MTT was added to each well. The MTT assay was performed as previously described.[24]

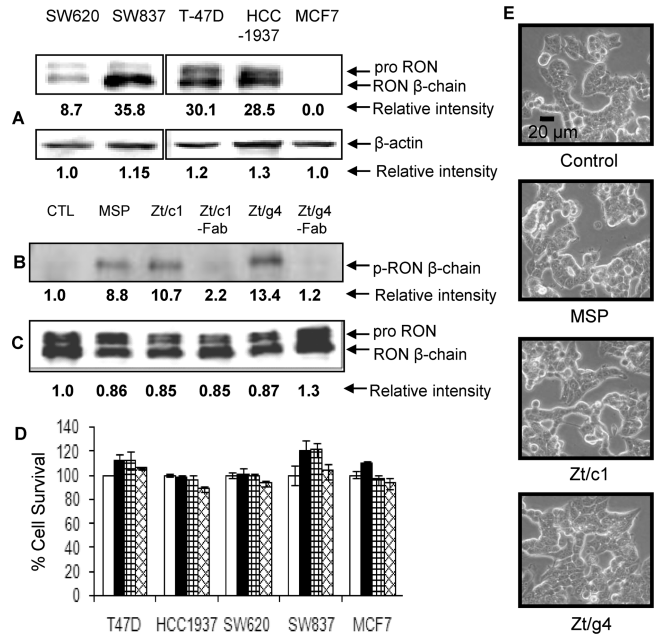

Figure 1. Functional analysis of anti-RON mAbs Zt/g4 or Zt/c1 on cancer cells: (A) Expression of RON in a panel of human cancer cell lines. Proteins (50 μg/sample) were subjected to Western blot analysis using rabbit IgG antibody to RON. β-Actin served as the loading control. (B) Effect of anti-RON mAb on RON phosphorylation. T-47D cells were serum-starved overnight and then treated with 10 μg/mL of Zt/g4, Zt/c1, Zt/g4Fab, or Zt/c1 Fab fragments for 15 min at 37 °C. Cells treated with or without 2 nM MSP served as the controls. Phosphorylated RON was detected in Western blotting using PT100. Intensities of individual bands were determined by densitometry analysis. (C) The loading control of B. The same membrane was stripped and reprobed with rabbit anti-RON IgG antibody. (D) Effect of anti-RON mAb on cell growth. Cells (8×10^3 cells/well) were cultured in DMEM + 10% FBS in a 96-well plate and then treated with 0, 2, 10, and 30 μg/mL of Zt/g4 for 5 days. Proliferation was determined by MTT assay. (E) Morphological effect of anti-RON mAbs. T-47D cells were treated with 10 μg/mL of Zt/g4 or Zt/c1 for 48 h. Cells treated with or without MSP (2 nM) were used as the control. Cell morphologies were photographed by CCD camera. Intensities of individual proteins in Western blotting were determined by densitometry analysis.

Results

Biological Effects of Zt/g4 and Zt/c1 on Colon and Breast Cancer Cells. Zt/g4 and Zg/c1 have been previously studied in RON-transfected NIH3T3 cells.[28] To determine their activities in human cancer cells, we first determined RON expression in five cell lines by Western blot analysis. T-47D, SW837, and HCC1937 cells expressed relatively high levels of RON. Moderate expression was seen in SW620 cells and MCF-7 cell do not express RON (Figure 1A). Zt/g4 or Zt/c1 stimulated RON phosphorylation in T-47D cells

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(Figure 1B), which is consistent with results reported previously.[18] Similar results were also seen in SW620, SW837, and HCC1937 cells (data not shown). Fab fragments from Zt/g4 or Zt/c1 had no effect on RON phosphorylation (Figure 1B,C). Moreover, they failed to induce RON phosphorylation when RON-transfected cells were used (data not shown). The effect of Zt/g4 or Zt/c1 on cell growth as measured by the MTT assay is shown in Figure 1D. The proliferation rates of T-47D, SW620, HCC1937 and others were not affected by Zt/g4 when even higher concentrations of IgG were used. These results were also confirmed by kinetic analysis of cell growth (data not shown). Moreover, the effect of Zt/g4 or Zt/c1 on T-47D cell morphological changes was not observed (Figure 1E). Similar results were also seen in other cancer cell lines. Observations from the migration study indicated that Zt/g4 and Zt/c1 failed to enhance or reduce the spontaneous cell migration by T-47D, HCC1937, SW620 and other cell lines (data not shown). These results demonstrated that Zt/g4 and Zt/c1 were able to increase certain degrees of RON phosphorylation, but were unable to modulate biological behaviors of cancer cells.

Induction of RON Internalization by Zt/g4 and Zt/c1. To serve as a drug carrier, a selected antibody must induce receptor internalization as a critical step for drug uptake.[22,26] We tested if Zt/g4 or Zt/c1 induces internalization of RON into the cytoplasm by monitoring intracellular fluorescence intensity of internalized FITC-labeled antibody. Cells were incubated with FITC-labeled Zt/g4 or Zt/c1 at 37 °C for 30 or 60 min to aid antibody binding and RON internalization. Antibodies bound on the cell surface were then eliminated by washing with acidic buffer.[7] As shown in Figure 2A,B, FITC-labeled Zt/g4 produced high levels of fluorescence in T-47D and SW837 cells. After washing with acidic buffer, a significant amount of fluorescence was still detected although the intensity was reduced. The effect of Zt/c1 on RON internalization was relatively weak in comparison with Zt/g4 (Figure 2C,D). Zt/c1 caused only moderate amounts of internalization. Similar results were also obtained by using other cancer cell lines (data not shown). Thus, Zt/g4 was a highly specific antibody that binds to RON and induces a significant amount of receptor internalization into the cytoplasm of cancer cells.

To verify the above results, a control experiment with endocytic inhibitor CC-B was performed. As shown in Figure 2E, pretreatment of T-47D cells with CC-B did not interfere the binding of Zt/c1 to RON on the cell surface. The obtained fluorescence intensity was e similar to those from the positive control. However, CC-B pretreatment completely blocked the Zt/c1-induced internalization. In this case, the fluorescence intensity was at the background levels of mouse IgG control. Clearly, the observed internalization is not the residual anti-RON IgG bound on the cell surface.

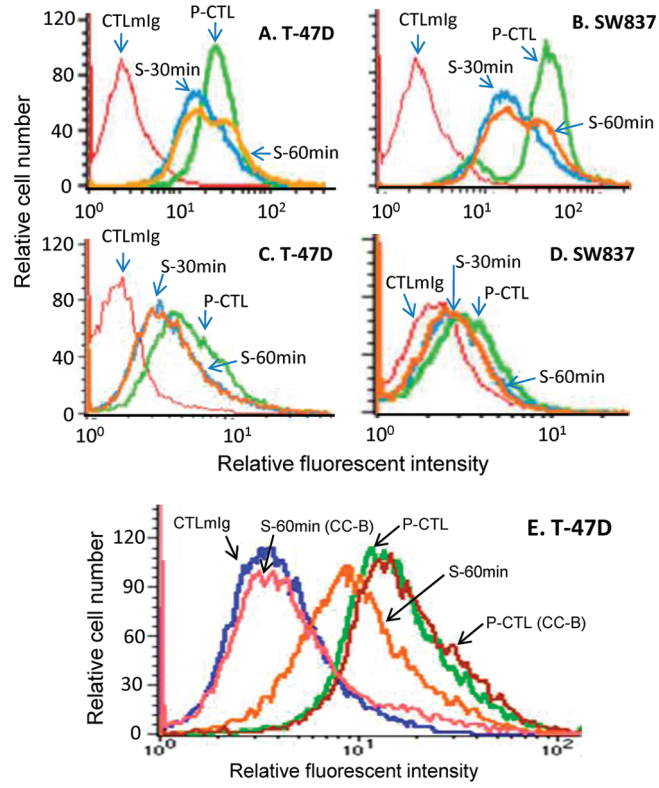

Figure 2. Effect of anti-RON mAbs on RON internalization in cancer cells: T-47D and SW837 cells (1×10^5 cells/sample) were incubated at 37 °C for 30 or 60 min with 2 μg of FITC-labeled Zt/g4 (A and B) or Zt/c1 (C and D) followed by acid wash. Cells treated with FITC-labeled Zt/g4 or Zt/c1 without acid wash were used as the positive control (P-CTL). Normal mouse IgG was used as the negative control (CTLmIg). Antibodies bound to cell surface were eliminated by washing with acidic buffer. The internalized FITC-mAbs were determined by flow cytometric analysis. (E) T-47D cells (1×10^5 cells/sample) were pretreated at 37 °C with or without CC-B (10 μg/mL) for 30 min and then incubated with FITC-Zt/c1 or normal mouse IgG as described above. After wash with or without acidic buffer, cellular fluorescence was determined by flow cytometric analysis. CTLmIg, mouse IgG control without acid wash; P-CTL, positive Zt/c1 control without acid wash; P-CTL (CC-B), positive Zt/c1 control pretreated with CC-B, without acid wash; S-60 min, treated with Zt/c1 and acid wash; S-60 min (CC-B), pretreated with CC-B followed by Zt/c1 and acid wash.

Biochemical Properties of Zt/g4 or Zt/c1-Conjugated IL Loaded with Dox. The biological properties of Zt/g4 and Zt/c1 described above prompted us to test if Zt/g4 and Zt/c1 are suitable for delivery of cytotoxic drugs for cancer cell killing. LS were prepared, underwent a standardized sonication procedure to generate IL with a size of 100 ± 20 nm as previously described,[22] and then were loaded with Dox (Dox-LS). Dox-LS were inserted with Zt/g4 or Zt/c1 to form IL (Zt/g4-Dox-IL or Zt/c1-Dox-IL). Fab fragments were also generated from Zt/g4

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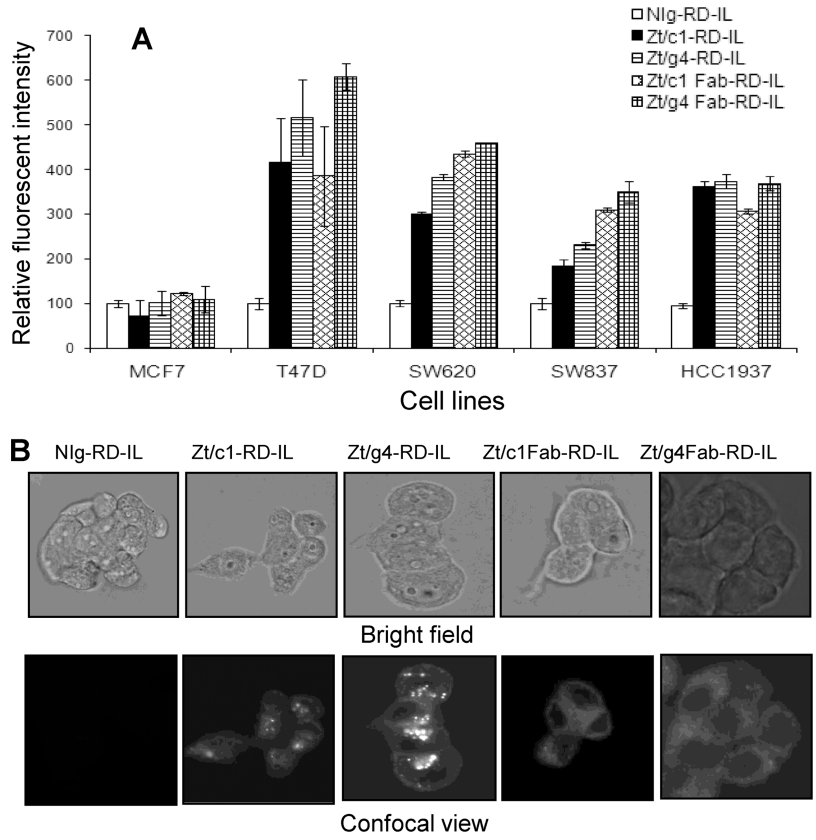

Figure 3. Effect of anti-RON mAb in induction of RD-IL binding and internalization by cancer cells: (A) Binding of Zt/g4-, Zt/c1, or their Fab-RD-IL to cancer cells. Cells (1×10^6 cells/sample) were incubated with individual IL (equivalent to 2 μM PL) at 4 °C for 45 min followed by measurement of fluorescent intensities using flow cytometry analysis. MCF-7 cells (RON negative) were used as the control. (B) Confocal analysis of anti-RON mAb-induced IL internalization: T-47D cells (1×10^4 cells/slide) were treated with individual IL containing Zt/g4, Zt/c1, or their Fab fragments (equivalent to 1 μM PL) at 37 °C for 60 min. Cells treated with NIg-RD-IL served as the control. RD was used as the fluorescent indicator. After treatment, cells were washed and fixed with 4% formaldehyde solution. Cell morphologies were observed and photographed under bright field. Intracellular fluorescence was determined by using the Olympus DSU confocal microscope.

or Zt/c1 and used to make Zt/g4Fab-Dox-IL and Zt/c1Fab-Dox-IL. ILs conjugated with normal mouse IgG (NIg-Dox-IL) and its Fab fragment (NIgFab-Dox-IL) were used as controls. The amounts of IgG inserted on IL were usually at 65 ± 10 μg of proteins per mg of PL per 200 ± 30 Dox. Incorporation of Fab fragments on IL was usually low (25 μg of proteins per 1 mg of PL per 200 ± 30 μg/mL Dox) when compared with whole IgG molecules. From available information, our IL formulation was comparable in various aspects with those published.[5,7,19,20]

Binding and Internalization of Antibody-Directed IL by Cancer Cells. To determine if Zt/g4, Zt/c1, or their Fab fragments inserted on IL still bind to cancer cells, RD was incorporated into IL to form Zt/g4-RD-IL, Zt/c1-RD-IL, Zt/g4Fab-RD-IL, and Zt/c1Fab-RD-IL. Cells were incubated with individual RD-labeled IL for 45 min at 4 °C followed by flow cytometry analysis. Compared with control MCF-7 cells that showed the baseline of fluorescence, increased amounts of fluorescence were observed in RON positive cell lines incubated with Zt/g4- or Zt/c1-RD-IL (Figure 3A). Similar fluorescent intensities were also observed in cells treated with Fab fragment-conjugated IL. These results demonstrated that Zt/c1, Zt/g4, and their Fab fragments in IL retained their binding capability.

We then determined Zt/g4 or Zt/c1-RD-IL induction of RON internalization by confocal analysis. Incubation of T-47D cells with NIg-RD-IL did not show visible fluorescence either on the cell surface or in the cytoplasm. In contrast, cells treated with Zt/g4-RD-IL displayed high levels of intracellular RD fluorescence (Figure 3B). Cytoplasmic fluorescence was also seen in cells treated with Zt/c1-RD-IL although the levels were relatively low. From analysis of confocal images, the IgG-RON complex seems to be in endosome-like small vesicles in the cytoplasm. Similar results were also observed in SW620 cells (data not shown). Incubation of cells with Zt/g4Fab- or Zt/c1Fab-RD-IL for 60 min did not reveal significant amounts of intracellular fluorescence. However, fluorescence was visible after a prolonged incubation (data not shown). Thus, Zt/g4 and Zt/

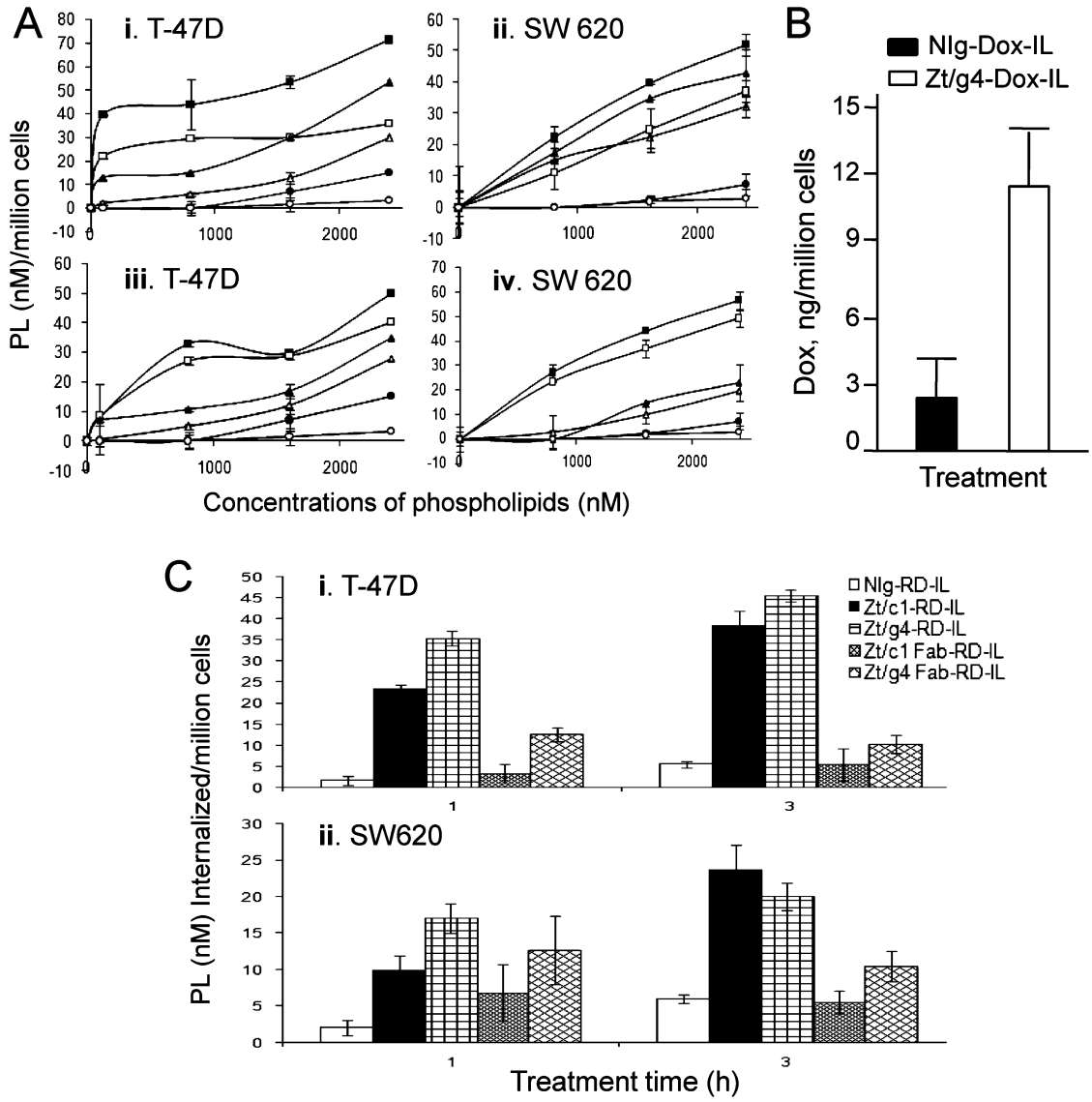

Figure 4. Analysis of IL uptakes induced by anti-RON mAb or their Fab fragments in cancer cells: (A) Effect of whole IgG molecule or Fab fragment on RD-IL uptake. T-47D or SW620 cells (1×10^6 cells/sample) were incubated with different amounts of IL at 4 or 37 °C for 60 min. After washing, RD fluorescence was determined. The amount of IL bound (in nmol of PL) was calculated from the initial specific activity of RD-IL. Cells treated with whole antibody-conjugated IL are shown in (i) and (ii), and cells treated with Fab-conjugated IL are shown in (iii) and (iv): ○, ●, NIg-RD-IL; □, ■, Zt/g4- or Zt/g4Fab-RD-IL; and ▲, Δ, Zt/c1- or Zt/c1Fab-RD-IL. Open symbols were carried out at 4 °C. The filled symbols were treated at 37 °C. (B) The amount of Dox was measured by its fluorescence emission at 592 nm.[5] SW620 cells were treated for 60 min at 37 °C with NIg-Dox-IL or Zt/g4-Dox-IL as described above. Fluorescent measurement was performed as described in Materials and Methods. (C) Kinetic effect of whole IgG molecule or Fab fragment in RD-IL uptake. T-47D (i) or SW620 (ii) cells (1×10^6 cells/sample) were incubated with individual RD-IL (adjusted to 2.4 μM of PL/sample) for various times. After incubation, cells were washed and subjected to fluorescent analysis.

c1 incorporated into IL was capable of inducing RON internalization. The effect of Fab fragments was relatively low.

Uptakes of Zt/g4 or Zt/c1-RD-IL by Cancer Cells. To study IL internalization in more detail, we performed a series of experiments in which PL in IL was used as the indicator. T-47D and SW620 cells were treated with different amounts of IL (adjusted to the levels of PL) for 60 min at 4 or 37 °C. Results in Figure 4Ai,Aii showed that in cells treated with Zt/g4-RD-IL there is a marked increase in the amount of PL associated with cells at 37 °C compared to 4 °C. This suggested that IL uptake is increased through a Zt/g4-mediated mechanism. The uptake of Zt/c1-RD-IL is relatively low compared to Zt/g4-RD-IL in SW620 and T-47D cells (Figure 4Aiii,Aiv). Consistent with those shown in Figure 4, the uptake of IL mediated by Fab fragments of Zt/g4 or Zt/c1 was at low levels at 37 °C compared to those at 4 °C. Only a moderate increase was observed. These results

Table 1. Kinetic Analysis of IL Internalization Induced Anti-RON mAb or Its Fab Fragment in Cancer Cells

times of cell incubation (min)	PL internalization (nM)/1 × 10^6 cells[a]						
	Nig-RD- IL	Zt/g4-RD-IL	Zt/g4Fab-RD-IL	Zt/g4-RD-IL:Zt/g4Fab-RD-IL	Zt/c1-RD-IL	Zt/c1Fab-RD-IL	Zt/c1-RD-IL:Zt/c1Fab-RD-IL
				T-47D			
60	1.8 ± 1.0	35.6 ± 1.2	12.7 ± 4.7	2.8:1	23.4 ± 1.1	6.9 ± 3.9	3.4:1
180	5.5 ± 0.7	45.5 ± 3.5	10.5 ± 2.1	4.3:1	38.4 ± 0.7	5.6 ± 1.6	6.9:1
				SW620			
60	2.1 ± 1.1	17.1 ± 2.0	12.6 ± 1.7	1.4:1	9.9 ± 2.1	3.4 ± 2.2	2.9:1
180	6 ± 0.6	20.1 ± 1.9	10.4 ± 2.1	1.9:1	23.7 ± 3.4	5.6 ± 3.8	4.2:1

[a] Experimental conditions were similar to those described in Figure 4.

Table 2. Effectiveness of Zt/g4- or Zt/c1-Dox-IL in Comparison with PLD *in Vitro* in Killing of Various Cancer Cells

cell lines	IC_{50} values (μg/mL)[a]					IC_{50} ratio	
	Dox	PLD	NIg-Dox-IL	Zt/g4-Dox-IL	Zt/c1-Dox-IL	PLD:Zt/g4-Dox-IL	PLD:Zt/c1-Dox-IL
MCF-7	4.6 ± 0.3	201 ± 38.7	267 ± 39	329 ± 7	277 ± 28	0.61:1	0.72:1
T-47D	1.9 ± 0.8	39.0 ± 1.4	48.1 ± 2.4	3.9 ± 0.9	6.7 ± 1.2	10.0:1	5.8:1
SW 620	0.7 ± 0.1	34.1 ± 4.2	42.3 ± 4.1	8.9 ± 1.3	8.0 ± 1.6	3.8:1	4.3:1
HCC1937	3.3 ± 0.3	156 ± 21	199.8 ± 30.1	8.7 ± 1.6	20.6 ± 3.5	17.9:1	7.6:1
SW 837	3.4 ± 1.1	1200 ± 105	1273 ± 84	74.4 ± 16.7	68.6 ± 18.7	16.1:1	17.8:1

[a] Cells were cultured overnight and then treated at 37 °C with various amounts of individual drugs (from 0 to 2000 μg/mL of Dox) for 60 min. After washing, cells were incubated for an additional 72 h. The MTT assay was used to determine % of cytotoxicity. The IC_{50} values from individual drugs were calculated from data of three experiments using statistical software.

demonstrated that whole IgG molecules are highly effective in the induction of IL uptake by cancer cells.

To verify the above results, levels of Dox associated with cells between control and Zt/g4-Dox-IL treated cells were directly measured by fluorescence emission of Dox at 592 nm.[5] Results in Figure 4B show that the amount of Dox in Zt/g4-IL treated cells was significantly higher than that of control IL-treated cells. By comparison upon conversion, the amounts of cell-associated Dox in Zt/g4-Dox-IL-treated cells (Figure 4B) is equivalent to the amounts of PL shown in SW620 cells treated at 37 °C with Zt/g4-RD-IL (Figure 4Aii).

We further studied the kinetics of anti-RON mAb-induced IL uptake in T-47D and SW620 cells (Figure 4Ci,Cii). The uptake of IL was time-dependent in both cell lines tested. Both Zt/g4 and Zt/c1 induced significant amounts of IL uptake. The peak of the uptake was reached at 3 h after cells were exposed to Zt/g4- or Zt/c1-RD-IL. Further incubation had no significant effect on IL uptake. We also tested the effect of Fab fragments in IL uptake. Fab fragments only caused a slight increase compared to NIg-RD-IL, which was significantly lower than Zt/g4- or Zt/c1-induced IL uptake. Table 1 summarizes the results from these experiments and demonstrates that whole antibody is more effective than Fab fragments in the induction of IL uptake by cancer cells.

Cytotoxic Effect of Zt/g4-Dox-IL and Zt/c1-Dox-IL on Cancer Cells. T-47D and SW620 cells were selected for their high sensitivity (IC_{50} values) toward Dox and PLD (Table 2). Cells were treated with various amounts of drugs to determine cytotoxic efficacy of Zt/g4-Dox-IL and Zt/c1-Dox-IL. Dox, PLD, and NIg-Dox-IL were used as controls. Results in Figure 5A show the dose-dependent cytotoxicity of Zt/g4-Dox-IL or Zt/c1-Dox-IL in comparison with PLD and NIg-Dox-IL. Although the responsiveness to the treatment varied between the two cell lines, enhanced cytotoxicity was observed after cells were treated with Zt/g4-Dox-IL or Zt/c1-Dox-IL, which resulted in a significant reduction of IC_{50} values. As shown in Table 2, IC_{50} values obtained from T-47D cells were reduced to 3.9 μg/mL (Zt/g4-Dox-IL) or 6.7 μg/mL (Zt/c1-Dox-IL) compared to 39 μg/mL (NIg-Dox-Il). Similar results were also seen in SW620 cells, in which the IC_{50} of PLD (34.1 μg/mL) was reduced to 8.9 μg/mL (Zt/g4-Dox-IL) or 8.0 μg/mL (Zt/c1-Dox-IL). These results demonstrated that the anti-RON directed approach is highly effective in the delivery of Dox which leads to increased cytotoxicity.

Increased Cytotoxicity of Anti-RON-Directed IL in Cancer Cells Less Sensitive to PLD. Sensitivity of cancer cells toward LS coated drugs such as PLD differs significantly from Dox.[27] As shown in Figure 5B and Table 2, HCC1937 and SW837 cells were highly sensitive to Dox with IC_{50} values at 3.3 and 3.4 μg/mL, respectively. However, these cells were less sensitive toward PLD with IC_{50} values increased up to 156 and 1200 μg/mL, respectively. To determine if RON targeted IL restores the IC_{50} values for effective killing, HCC1937 or SW837 cells were

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(28) Kesarwala, A. H.; Samrakandi, M. M.; Piwnica-Worms, D. Proteasome inhibition blocks ligand-induced dynamic processing and internalization of epidermal growth factor receptor via altered receptor ubiquitination and phosphorylation. *Cancer Res.* **2009**, *69*, 976–983.

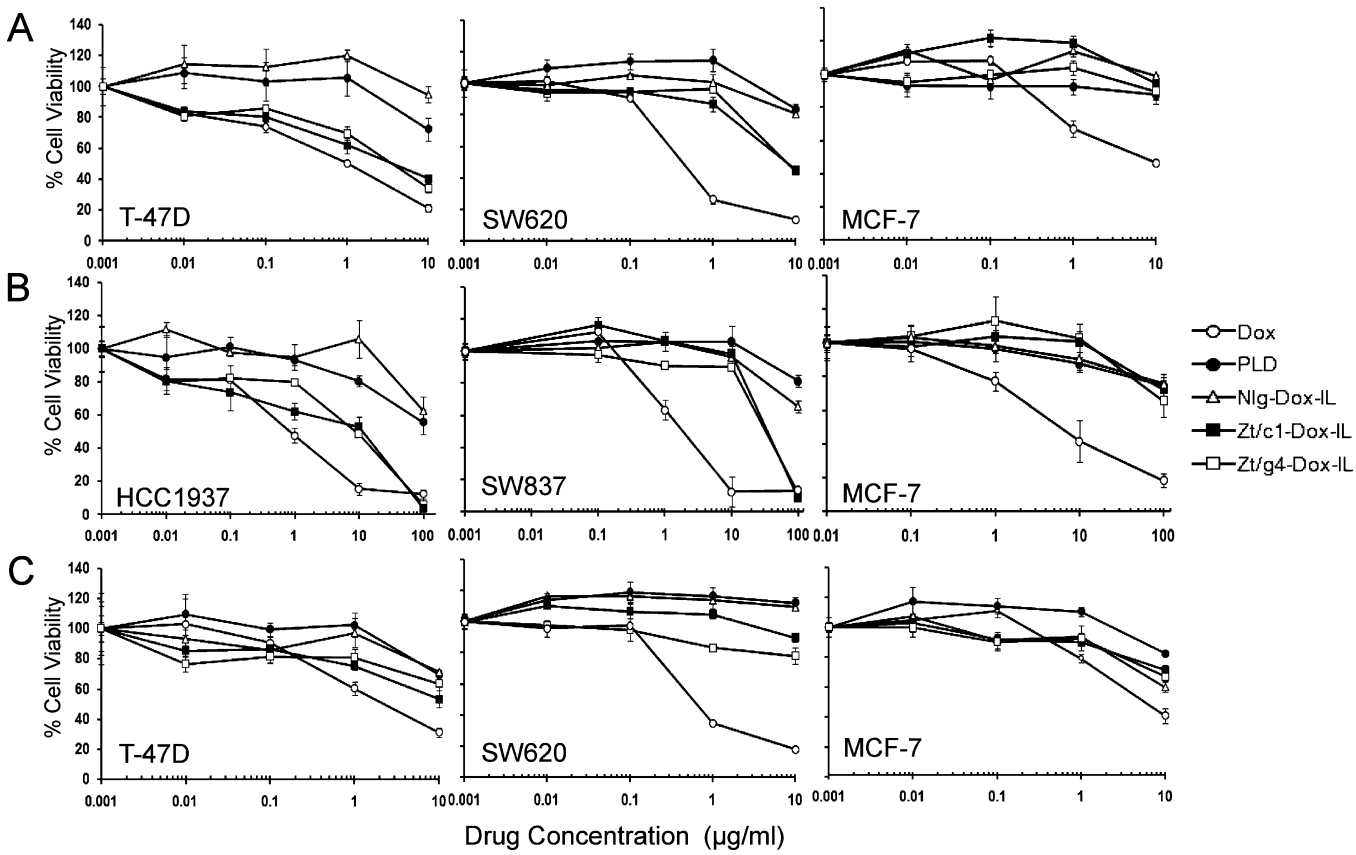

Figure 5. Enhanced cytotoxic activities of Zt/g4 or Zt/c1-Dox-IL in cancer cells: Cells (1×10^4 cells/well in a 96-well plate) in triplicate were treated with various amounts of individual IL for 60 min at 37 °C. Dox, PLD, and NIg-Dox-IL were used as the controls. After changing medium, cells were cultured for an additional 72 h and cell survivals were determined by MTT assay. (A) Cytotoxic effect of antibody-directed Dox-IL on cancer cells sensitive to PLD. T-47D and SW620 cells were used. (B) Effect of Zt/g4- or Zt/c1-Dox-IL on cancer cells insensitive to PLD: HCC1937 and SW837 cells were used. (C) Effect of Fab fragment-directed IL in killing cancer cells. T-47D and SW620 cells were used. In all cases, MCF-7 (RON negative) cells were used as the control. (D) Effect of Fab fragment-directed IL in PDL insensitive cells. HCC1937 and SW837 cells were used as the target cells and incubated with individual Fab-fragment-Dox-ILs as described above. One of three experiments with similar results.

treated with various amounts of Zt/g4-Dox-IL or Zt/c1-Dox-IL. PLD and NIg-Dox-IL were used as the controls. Results in Figure 5B and Table 2 showed that cellular sensitivities to IL in both cell lines were dramatically increased with significant reduction of IC_{50} values (8.7 μg/mL for Zt/g4-Dox-IL and 20.6 μg/mL for Zt/c1-Dox-IL in HCC1937 cells; 74.4 μg/mL for Zt/g4-Dox-IL and 68.6 μg/mL for Zt/c1-Dox-IL in SW837 cells). These results demonstrated that by targeting RON, Zt/g4-Dox-IL or Zt/c1-Dox-IL is highly effective in killing cancer cells which are insensitive to PLD.

Effect of Anti-RON Fab Fragment in Mediation of IL in Cancer Cell Cytotoxicity. We evaluated the effectiveness of Fab fragment-conjugated Dox-IL in killing several cancer cells under conditions described above. As shown in Figure 5C,D, Zt/g4Fab-Dox-IL displayed dose-dependent cytotoxic activities toward cancer cells when compared to PLD or NIg-Dox-IL. A 3- to 5-fold reduction in IC_{50} values was observed in T-47D, SW620, HCC1937, and SW837 cell lines treated with Zt/g4Fab-Dox-IL, respectively (Table 3). The effect of Zt/c1Fab-Dox-IL was relatively weak when compared with Zt/g4Fab-Dox-IL. A 2-fold reduction in IC_{50} values were only seen in 2 out of 4 cell lines tested. These results suggested that the efficiency of the Fab fragments for targeted delivery of chemoagent is relatively low in comparison with whole antibody directed Dox-IL.

Requirement of RON Phosphorylation in Endocytosis of Anti-RON Antibody-IL by Cancer Cells. To study Zt/g4 or Zt/c1 directed delivery of Dox in more detail, we first studied if tyrosine phosphorylation is required for anti-RON-IL-induced RON endocytosis.[13,24,28] Tyrosine kinase inhibitor HB-A was used to block RON phosphorylation followed by analysis of Zt/g4 or Zt/c1-induced IL uptake. Results in Figure 6A showed that HB-A inhibits not only spontaneous but also Zt/g4- or Zt/c1-enhanced RON phosphorylation. Such inhibition resulted in a 50% of reduction in uptake of Zt/g4-RD-IL in T-47D cells at 37 °C compared to cells at 4 °C (Figure 6B). Similar results were also seen in SW620 cells (data not shown). These results indicated that blocking RON phosphorylation by HB-A affects anti-RON mAb-induced receptor endocytosis.

Table 3. Efficiency of Zt/g4Fab- or Zt/c1Fab-Dox-IL in Comparison with PLD in Cytotoxicity against Different Cancer Cells

	IC_{50} values (μg/mL)[a]					IC_{50} ratio	
cell lines	Dox	PLD	NIg-Dox-IL	Zt/g4Fab-Dox-IL	Zt/c1Fab-Dox-IL	PLD:Zt/g4Fab-Dox-IL	PLD:Zt/c1Fab- Dox-IL
MCF-7	4.6 ± 0.3	201 ± 38.7	267 ± 38.7	292 ± 11	279 ± 32	0.68:1	0.72:1
T-47D	1.9 ± 0.8	39.0 ± 1.4	48.1 ± 2.4	44.3 ± 8.4	18.6 ± 4.5	0.9:1	2.1:1
SW 620	0.7 ± 0.1	34.1 ± 4.2	42.3 ± 4.1	11.2 ± 2.3	89.3 ± 21.2	3.0:1	0.4:1
HCC1937	3.3 ± 0.3	156 ± 21	199.8 ± 30.1	30.6 ± 3.3	63.4 ± 15	5.1:1	2.4:1
SW 837	3.4 ± 1.1	1200 ± 105	1273 ± 84	111 ± 18.5	466 ± 31	10.8:1	2.6:1

[a] Cells (1×10^4 cells/well) were cultured overnight and then treated at 37 °C with various amounts of individual drugs (from 0 to 2000 μg/mL of Dox) for 60 min. After washing, cells were incubated for an additional 72 h. The MTT assay was used to determine % of cytotoxicity. The IC_{50} values from individual drugs were calculated from data of three experiments using statistical software.

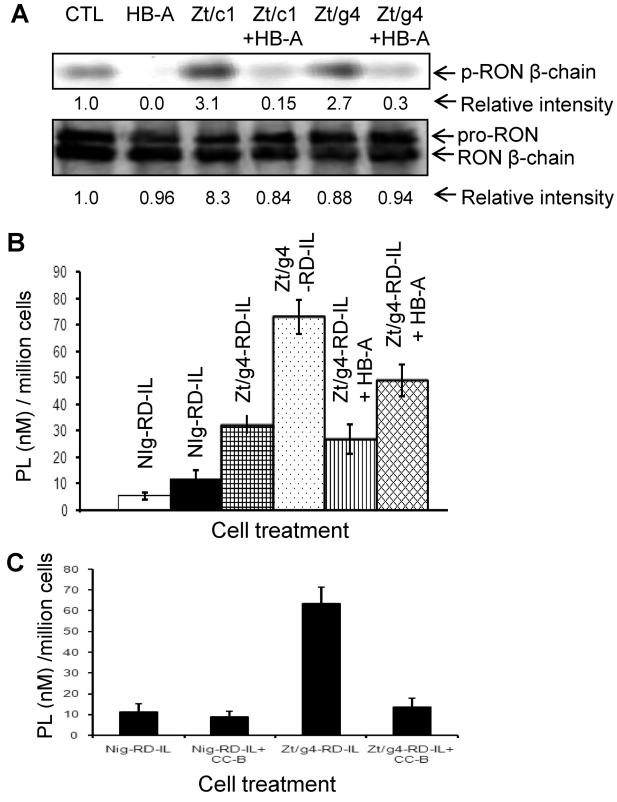

Figure 6. Potential mechanisms of anti-RON-mAb directed delivery of Dox for cytotoxicity against T-47D cells: (A) Effect of tyrosine kinase inhibitor HB-A on antibody-induced RON phosphorylation. Cells (3×10^6 cells/sample) were pretreated at 37 °C with 5 μg/mL of HB-A for 60 min and then treated with 10 μg/mL of Zt/g4, Zt/c1 for 15 min. Phosphorylated RON was detected by PT100 in Western blotting. The same membrane was also stripped and reprobed with rabbit IgG to RON for the loading control. (B) Effect of HB-A on Zt/g4-induced IL uptake. Cells (1×10^6 cells/sample) were pretreated with 5 μg/mL of HB-A for 60 min and then incubated with Zt/g4-RD-IL (equivalent to 2.4 μM LP) at 4 or 37 °C for 60 min. The IL uptake was determined as detailed in Materials and Methods. (C) Effect of endocytic inhibitor CC-B on Zt/g4-induced IL uptake. Cells (1×10^6 cells/sample) were pretreated with 10 μg/mL of CC-B for 60 min and then incubated at 37 °C with individual IL (equivalent to 2.4 μM PL) for 60 min. The IL uptake was determined as described above. One of two experiments with similar results.

We next studied if blocking RON internalization by endocytic inhibitor CC-B affects IL uptake. Results in Figure 6C showed that pretreatment of cells with CC-B (10 μg/mL) almost completely inhibits the uptake of Zt/g4-RD-IL by T-47D cells. CC-B had no effect on the binding of Zt/g4-RD-IL on cells (data not shown). These results, suggested that the uptake of anti-RON-Dox-IL is via an endocytic pathway.

Discussion

The findings of this study demonstrate that antibody-directed RON targeting is a rational approach for delivery of chemoagents such as Dox for tumor cell cytotoxicity. Using Zt/g4 and Zt/c1, IL loaded with Dox were formulated and tested in colon and breast cancer cell lines. By focusing on antibody-induced RON internalization, cellular IL uptakes, and *in vitro* cytotoxicity, we showed that Zt/g4 and Zt/c1 induce RON internalization, resulting in significant amounts of IL uptake by cancer cells. Both Zt/g4- and Zt/c1-directed Dox-IL displayed increased cytotoxic activities with significant reduction of IC_{50} values compared to control NIg-Dox-IL. We also found that tumor cells that are relatively insensitive to PLD showed increased sensitivity toward Zt/g4- or Zt/c1-Dox-IL. However, the Fab fragments derived from Zt/g4 and Zt/c1 were not as effective as the whole antibody in directing Dox-IL cytotoxicity. The weak action seems to be related to their low capabilities in induction of IL uptake. We further showed that receptor phosphorylation is partially involved in the endocytosis of RON and uptake of IL. We conclude that anti-RON antibody-directed delivery of chemodrugs has potential to be developed into a novel therapeutic with implications in targeted cancer therapy.

Targeting cell surface proteins for intracellular delivery of cytotoxic drugs is successful in treatment of leukemia and lymphoma.[1,29] The effectiveness of this approach for solid epithelial tumors has also improved significantly. The clinical use of therapeutic antibodies specific to EGFR or HER2 has provided a platform for further development of novel therapeutics.[5−7] One approach is to combine antibody specificity with liposomes to improve the therapeutic efficacies of chemoagents. The development of IL conjugated with

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anti-EGFR antibody such as cetuximab is one example.[5−7] Cetuximab is an interesting antibody because it induces EGFR phosphorylation but inhibits cancer cell growth.[30] Studies have shown that by targeting cancer cells overpressing EGFR or HER2, cetuximab or trastuzumab-directed drug delivery significantly enhances the efficacies of multiple chemoagents against cancer cells.[31,32] We have used Zt/g4 and Zt/c1 to formulate Dox-loaded IL for *in vitro* cytotoxic studies. Zt/g4 and Zt/c1 both bind to the RON extracellular domain, but their binding regions are different.[18] Zt/g4 recognizes a region containing the second and fourth IPT domains in the RON β-chain extracellular sequences.[18] In contrast, Zt/c1 interacts with the RON sema domain in both RON α- and β-chain.[18] Although they had no biological effects as shown in Figure 1, both mAbs were able to slightly enhance spontaneous RON phosphorylation in colon or breast cancer cells (Figure 1). Available evidence suggests that internalization of RON requires its phosphorylation.[14] Considering these facts, we determined to use them as the directing molecules for delivery of chemoagents for cytotoxic analysis.

Internalization of RON upon activation by ligand-dependent or independent pathways is a mechanism that controls the levels of cellular responsiveness to stimulation from the extracellular environment.[14] This property is also the basis for selecting RON as a pathway to deliver chemoagents for therapeutic purposes. Previous studies have shown that MSP induces RON phosphorylation leading to increased receptor endocytosis.[14] This process is mediated by recruiting c-Cbl ubiquitin ligase to phosphorylated sites in the RON intracellular sequences.[14] Our results demonstrated that binding of Zt/g4 or Zt/c1 to RON results in its internalization, which facilitates the intracellular uptake of Dox-IL. This conclusion was supported by the following evidence. First, Zt/g4- or Zt/c1-induced RON internalization in colon SW620 and breast T-47D cancer cells as evident by detection of intracellular fluorescence. Zt/g4 seemed to induce more RON internalization than Zt/c1 as shown in Figures 2 and 3. Second, the RON internalization resulted in uptake of Dox-IL by cancer cells (Figure 4 and Table 1). Kinetic studies further demonstrated that significant uptakes were seen at 1 h, peaking at 3 h after cells were incubated with IL (Figure 4). Again, cellular uptake was relatively higher in cells treated with Zt/g4-IL than in cells treated with Zt/c1-IL. This suggested that Zt/g4 is more potent in the induction of RON internalization and subsequent IL uptake. Currently, we do not know the underlying mechanism(s) differing Zt/g4 from Zt/c1. Since the binding region of Zt/g4 is different from Zt/c1, it is possible that binding to the IPT units by Zt/g4 triggers an event favorable for increased RON endocytosis. Third, the RON-mediated IL uptake required, to a certain degree, receptor phosphorylation. Zt/g4 or Zt/c1 was capable of induction RON phosphorylation,[18] which provided a mechanism for efficient RON internalization.[14] As shown in Figure 6, inhibition of RON phosphorylation by herbimycin A significantly prevented Zt/g4- or Zt/c1-directed IL uptake, although it was not complete. Clearly, other mechanisms were also involved in RON-mediated IL uptake. Finally, by using Fab fragments, we showed that the whole antibody molecule is more effective in induction of RON-mediated IL uptake. The Fab fragments bound to RON with similar affinity but were not as effective as the whole antibody in induction of RON internalization. A possible explanation might be the inability of Fab fragments to induce RON phosphorylation in cancer cells. Therefore, the observed uptake occurred in tumor cells treated with Fab fragment-Dox-IL was mediated through the baseline levels of RON phosphorylation.

Effectiveness of Zt/g4- or Zt/c1-Dox-IL *in vitro* was reflected in their reduced IC_{50} values required for cytotoxic activities against different cancer cells (Figure 5). T-47D, SW620, HCC1937 and SW837 cells were highly sensitive to Dox with IC_{50} values ranging from 0.7 to 4.6 μg/mL. The sensitivities of these cells toward PLD were significantly different. As shown in Table 2, a 20- or 50-fold increase in the amount of PLD was required to reach IC_{50} values. These features provided a foundation for us to evaluate the effectiveness of Zt/g4 or Zt/c1 in directing Dox-IL cytotoxicity over PLD. As shown in Figure 5, Zt/g4- or Zt/c1-directed Dox-IL cytotoxicity was indeed more effective in killing T-47D and SW620 cells. Although cellular responses to the treatment of Zt/g4-Dox-IL or Zt/c1-Dox-IL differed between the two cell lines, an average of 6-fold reduction in the IC_{50} values was achieved in comparison to IC_{50} values from cells treated with PLD. These results suggested that Zt/g4- or Zt/c1-directed delivery of Dox-IL overcame the insensitivity of cancer cells toward PLD. Experiments are currently underway to study the *in vivo* distribution, tissue localization, tumoral cell uptake, and therapeutic activities of Zt/g4- or Zt/c1-Dox-IL.

Previous studies have shown that IL formulated by using Fab fragments derived from trastuzumab were effective in the induction of Her2-mediated uptake of chemoagents, which led to increased cytotoxicity.[5−7,22] Although not directly compared, Fab-ILs targeting EGFR are highly specific and effective in induction of receptor internalization and drug uptake.[7,33] Results from our study of using Zt/g4Fab or Zt/c1Fab fragment-directed cytotoxicity were different from these data. We showed that the cytotoxic effect of Zt/g4Fab or Zt/c1Fab-Dox-IL is not as effective as the whole antibody. The IC_{50} values obtained from using Zt/g4Fab- or

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(31) Ciardiello, F.; Tortora, G. EGFR Antagonists in Cancer Treatment. *N. Engl. J. Med.* **2008**, *358*, 1160–1174.

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Zt/c1Fab-Dox-IL in both T-47D and SW620 cells were generally higher than those from the whole antibody-conjugated IL (Table 3). The ratio of the IC_{50} values of PLD to Zt/g4Fab-Dox-IL or to Zt/c1Fab-Dox-IL was quite low, ranging only from 0.4 to 3.0, respectively. Considering these facts, we reasoned that low levels of cytotoxicity mediated by Zt/g4Fab- or Zt/c1Fab-Dox-IL is mainly a result of their inability to induce high levels of RON internalization and subsequent IL uptakes. The possible explanation might be their inability to induce RON phosphorylation.

The finding that antibody-directed IL induced the significant killing of PLD-insensitive tumor cells as shown in HCC1937 and SW837 cells was quite interesting. Both cell lines were highly susceptible *in vitro* to Dox-induced cytotoxicity as evident by their IC_{50} values. In contrast, their sensitivities toward PLD-induced cytotoxic activities were significantly reduced with IC_{50} values ranging from 1400 and >2000 μg/mL, respectively. Clearly, such changes were caused mainly by the biophysical nature of lipids coated on Dox that formed a physical barrier preventing the direct interaction of Dox with cancer cells. The lipid coating in the form of PLD has clinical advantage for evading detection and destruction by the immune system, therefore increasing its capability for long-term circulation in the body.[34] However, the coating affected the interaction of tumor cells with Dox, which reduced the PLD cytotoxic efficiency with increased IC_{50} values. Considering these facts, the antibody targeted approach had advantages in overcoming these shortfalls. As demonstrated in Table 3, Zt/g4- or Zt/c1-Dox-IL killed HCC1937 and SW837 cells with significantly reduced IC_{50} values. The use of targeted antibodies promoted the interaction of IL with cancer cells, caused IL uptake and facilitated the release of Dox for intracellular cytotoxicity.

Abbreviations Used

CC-B, cytochalasin-B; Chol, cholesterol; Dox, doxorubicin; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HB-A, herbimycin A; HSPC, hydrogenated soy phosphatidylcholine; IL, immunoliposome; LS, liposome; mAb, monoclonal antibody; MAP, mitogen-activated protein; MDCK, Madin−Darby canine kidney; MSP, macrophage-stimulating protein; $mPEG_{2000}$-DSPE, polyethylene glycol conjugated to dihexadecylphosphatidylglycerol; Mal, maleimide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PLD, pegylated-liposomal doxorubicin; PE, dihexadecanoylphosphatidylethanolamine; PL, phospholipid; RD, rhodamine; RON, recepteur, d'origine nantais.

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