Epithelial Junction Opener JO-1 Improves Monoclonal Antibody Therapy of Cancer

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Abstract

The efficacy of monoclonal antibodies (mAb) used to treat solid tumors is limited by intercellular junctions which tightly link epithelial tumor cells to each another. In this study, we define a small, recombinant adenovirus serotype 3-derived protein, termed junction opener 1 (JO-1), which binds to the epithelial junction protein desmoglein 2 (DSG2). In mouse xenograft models employing Her2/neu- and EGFR-positive human cancer cell lines, JO-1 mediated cleavage of DSG2 dimers and activated intracellular signaling pathways which reduced E-cadherin expression in tight junctions. Notably, JO-1-triggered changes allowed for increased intratumoral penetration of the anti-Her2/neu mAb trastuzumab (Herceptin) and improved access to its target receptor, Her2/neu, which is partly trapped in tight junctions. This effect translated directly into increased therapeutic efficacy of trastuzumab in mouse xenograft models using breast, gastric, and ovarian cancer cells that were Her2/neu-positive. Furthermore, combining JO-1 with the EGFR-targeting mAb cetuximab (Erbitux) greatly improved therapeutic outcomes in a metastatic model of EGFR-positive lung cancer. A combination of JO-1 with an approach that triggered transient degradation of tumor stroma proteins elicited eradication of tumors. Taken together, our findings offer preclinical proof of concept to employ JO-1 in combination with mAb therapy. Cancer Res; 71(22): 11. ©2011 AACR.

Introduction

Trastuzumab (Herceptin) and cetuximab (Erbitux) are humanized monoclonal antibodies (mAb) used for the therapy of Her2/neu- and EGFR-positive cancers, respectively. The mechanisms of trastuzumab and cetuximab action include the activation of antibody-dependent or complement-dependent cytotoxicity, and interference with tyrosine kinase receptor signaling (1). A unifying aspect among these mechanisms is that tumor cell growth inhibition is dependent on the binding of these mAbs to their corresponding receptors, that is, Her2/neu and EGFR. Therefore, molecules that prevent access and binding to the receptor, either by physically inhibiting intratumoral transport from blood vessels to malignant cells or masking of receptors, are predicted to block trastuzumab and cetuximab activity (2). Several studies showed that the expression or upregulation of epithelial proteins correlated with increased resistance to trastuzumab (3) and cetuximab (4) therapy. Epithelial cells maintain several intercellular junctions (tight junctions, adherens junctions, gap junctions, and desmosomes), a feature which is often conserved in epithelial cancers in situ and in cancer cell lines (5). Epithelial junctions are composed of adhesive dimers consisting of cadherin molecules derived from 2 neighboring cells (6). Desmoglein 2 (DSG2), an epithelial caderhin, is overexpressed in a series of epithelial malignancies, including breast cancer (ref. 7; Supplementary Fig. S1), ovarian cancer (ref. 7; Supplementary Fig. S1), lung cancer (7), gastric cancer (8), squamous cell carcinomas (9), melanoma (10), metastatic prostate cancer (11), and bladder cancer (12).

Recently, we showed that a group of human adenoviruses (Ads; Ad serotype 3, 7, 11, and 14) use DSG2 as a primary attachment receptor for the infection of cells (7). Importantly, in epithelial cells, Ad3 binding to DSG2 triggered activation of signaling pathways resulting in the transient opening of epithelial junctions (7). The opening of the epithelial junctions was also achieved with recombinant survilval particles, such as Ad3 penton-dodecahedra (PtDd; Fig. 1A). We subsequently generated a minimal Ad3-derived DSG2 ligand formed by 2 fiber knob domains (13). This protein, with a molecular weight of approximately 50 kDa, is produced in E. coli and can be easily purified. In a series of functional studies, we showed that this protein efficiently triggers the opening of junction. In the following study, we therefore refer to this protein as junction opener-1 (JO-1).
Figure 1. Transient opening of epithelial junctions by JO-1. A, structure of Ad3 viral particles. Left: complete, infectious Ad3 particle. The capsid proteins fiber and penton base are shown in green and blue, respectively. The trimeric fiber knob is shown in red. Middle: Ad3 PtDd formed by spontaneous assembly of 12 recombinant pentons (fiber + penton base). Right: dimeric Ad3 fiber (JO-1). B, schematic structure of JO-1 containing an N-terminal His-tag, a dimerization domain (K-coil ref. 32), a flexible linker, one fiber shaft motif, and the homotrimeric Ad3 fiber knob domain. C, left: simplified structure of epithelial junctions with tight junctions, desmosomes, and adherens junctions. Confocal immunofluorescence microscopy of T84 cells. Shown are stacked XZ images. Cells were treated with JO-1 (5 μg/mL) for 1 hour on ice. After removal of JO-1, cells were incubated at 37°C and analyzed 0, 30, and 60 minutes later. Top: DSG2 (green) seems at the apical site of baso-lateral junctions marked by claudin 7 (red). Middle: within 30 minutes after adding JO-1, claudin 7 staining increases and DSG2 staining becomes visible along the upper part of the lateral membrane (yellow signals). Bottom: by 60 minutes, lateral junctions resemble those of time point "0 minute." The scale bar is 40 μm. D, transmission electron microscopy of junctional areas of polarized colon cancer T84 cells. Cells were either treated with PBS (left) or JO-1 (right) for 1 hour on ice, washed, and then incubated for 1 hour at 37°C. At this time, the electron-dense dye ruthenium red (33) was added together with the fixative. The scale bar is 1 μm. E, 14C-PEG-4,000 diffusion through monolayers of T84 cells at different time points after adding JO-1 or anti-DSG2 antibody 6D8 (directed against ECD3/4). F, effect of various DSG2 ligands on the TEER of polarized T84 epithelial cells. Cells were treated as described in (E).
In this study, we have partially delineated the in vivo mechanism of JO-1–mediated junction opening. Furthermore, we show that JO-1 treatment greatly increases the permeation of mAbs in tumors and significantly enhances the efficacy of trastuzumab and cetuximab therapy in a series of xenograft tumor models.

Material and Methods

Proteins

JO-1 (also known as Ad3-K/S/Kn) is produced in E. coli as described previously (13). Recombinant Ad3 PtDd protein complexes were produced in insect cells and purified as described elsewhere (14).

Cell lines

BT474-M1 is a tumorigenic subclone of BT474 (ATTC, HTB-20) that was generously provided by Mien-Chie Hung (Department of Molecular and Cellular Oncology, University of Texas MD Anderson Cancer Center, Houston) in 2009 (15). BT474-M1 and HCC1954 cells (ATTC, CRL-2338) were cultured in RPMI-1640 with 10% FBS, 1% Pen/Strep and L-Glutamine. A549 (ATCC, CCL-185) and T84 (ATCC, CCL-248) were cultured in DMEM/F-12 with 10% FBS, 1% Pen/Strep and L-Glutamine. To achieve cell polarization, 1.4×10^5 A549 (ATCC, CCL-185) and T84 (ATCC, CCL-248) were cultured in DMEM/F-12 with 10% FBS, 1% Pen/Strep and L-Glutamine. To achieve cell polarization, 1.4×10^5 T84 cells were cultured in collagen-coated 6.5 mm Transwell inserts (0.4 μm pore size; Costar Transwell Cclears) for a period of 14 to 20 days until transepithelial resistance was stable (7). Cell lines from the ATCC were obtained in December 2010. All cell lines have been passaged vigorously (19). Mice were sacrificed when the tumor volume reached 1,000 mm^3 or ulcerated. Lung cancer xenografts were established by injecting 4×10^6 cancer cells into the mammary fat pad of CB17 SCID-beige mice. Trastuzumab was injected intraperitoneally (i.p.) at a dose of 10 mg/kg, PtDd or JO-1 was given i.v. at a dose of 2 mg/kg. Tumor volumes were measured as described previously (19). Mice were sacrificed when the tumor volume reached 1,000 mm^3 or ulcerated. Lung cancer xenografts were established by injecting 4×10^6 A549 s.c. into the right flank of CB17 SCID-beige mice. Cetuximab was injected at 10 mg/kg i.p. For the disseminated lung tumor model, mice were intravenously injected with 2×10^5 A549 cells. Animals were sacrificed when the first mouse of the control group was moribund. India ink (15% in PBS) was injected intratracheally prior to the removal of the lungs.

Transmural electrical resistance and PEG permeability assays

A total of 5×10^5 T84 cells were seeded on 12 mm transwell inserts (PET membrane, with 0.4 μm pore size) and cultured for 20 days. Culture medium was changed every 2 to 3 days. The cells were exposed to DSG2 ligands (20 μg/mL) in adhesion medium (DMEM, 1% FBS, 2 mmol/L MgCl₂, 20 mmol/L HEPES) for 15 minutes at room temperature and transepithelial electrical resistance (TEER) was measured and calculated as described elsewhere (16). For permeability assays 15 minutes after adding the DSG2 ligands, 1 μCi of [³⁴C] polyethylene glycol-4000 (PEG-4000; Perkin Elmer) was added to the inner chamber. Medium aliquots were harvested from the inner and outer chambers and measured by a scintillation counter. Permeability was calculated as described elsewhere (17).

Transmission electron microscopy

Transmission electron microscopy (TEM) was done as described previously (13).

Hematopoietic stem cell–based relaxin expression

The protocol has been described elsewhere (18). Briefly, transplant recipients were 6- to 10 weeks old, female CB17 severe combined immunodeficient (SCID)-beige mice, subcutaneously irradiated with 350 cGy immediately before tail vein injection with 6×10^⁶ lentivirus vector—transduced bone marrow cells from 5-FU—treated mice. After engraftment of cells in the recipients' bone marrow was confirmed, a total of 4×10⁶ HCC1954 were injected into the mammary fat pad. The lentivirus vector expressing relaxin under the control of doxycycline (Dox) has been described previously (18).

Human IgG (Herceptin) ELISA

A polyclonal goat anti-human IgG antibody (G-101-C-ABS, R&D Systems) was used as a capture antibody. Tissues were lysed as for Western blots. Purified human IgG served as a standard. Binding was detected with a mouse monoclonal anti-human IgG1 Fc antibody (MAB 110, R&D Systems), followed by an anti-mouse IgG-HRP conjugate.

Animal studies

Breast cancer xenografts were established by injecting 4×10⁶ cancer cells into the mammary fat pad of CB17 SCID-beige mice. Trastuzumab was injected intraperitoneally (i.p.) at a dose of 10 mg/kg. PtDd or JO-1 was given i.v. at a dose of 2 mg/kg. Tumor volumes were measured as described previously (19). Mice were sacrificed when the tumor volume reached 1,000 mm^3 or ulcerated. Lung cancer xenografts were established by injecting 4×10⁶ A549 s.c. into the right flank of CB17 SCID-beige mice. Cetuximab was injected at 10 mg/kg i.p. For the disseminated lung tumor model, mice were intravenously injected with 2×10⁵ A549 cells. Animals were sacrificed when the first mouse of the control group was moribund. India ink (15% in PBS) was injected intratracheally prior to the removal of the lungs.

Statistical analysis

All results are expressed as mean ± SD. Student t test or 2-way ANOVA for multiple testing, were applied when applicable. A value P < 0.05 was considered significant.

Results

JO-1 triggers opening of epithelial junctions

As the large size of Ad3 or PtDd particles can affect their egress from blood vessels and tissue penetration, we attempted to generate smaller Ad3-derived DSG2 ligands that are functionally active as epithelial junction openers. We therefore designed JO-1 (aka Ad3-K/S/Kn; ref. 13), a small, self-dimerizing Ad3 fiber derivative (Figs. 1A and B; ref. 13). JO-1 has a molecular weight of approximately 50 kDa and is produced in E. coli prior to purification by affinity chromatography. In contrast, PtDd have to be produced in insect cells and have a molecular weight of 4,860 kDa and a diameter of approximately 50 nm.

The functional activity of JO-1 was tested on polarized colon cancer T84 cells. Incubation of T84 cells with JO-1 triggered remodeling of epithelial junctions, as shown by confocal...
microscopy for claudin 7 and DSG2 (Fig. 1C). Opening of the tight junctions, which are localized apical to the desmosomal and adherence junctions, is illustrated by electron microscopy (Fig. 1D). Microphotographs of untreated epithelial cells show intact tight junctions as judged by the exclusion of the apically applied electron-dense dye ruthenium red from basolateral space. Incubation of epithelial cells with JO-1 for 1 hour resulted in the disassembly of tight junctions and leakage of ruthenium red into the basolateral space (Fig. 1D, right panel). Exposure of polarized epithelial cells to JO-1 also increased the transepithelial permeability, as shown by transflux of 14C-PEG-4000 with a molecular weight of 4,000 Da (Fig. 1E). Importantly, monoclonal antibodies against different regions of the extracellular domain of DSG2 did not significantly increase transepithelial permeability. We speculate that the ligation of several DSG2 molecules is required to trigger the opening of the junctions. Finally, transient opening of junction was confirmed by measuring the TEER in polarized epithelial cells (Fig. 1F). Notably, JO-1 had no significant effect on the TEER when studies were done in subconfluent cell cultures where mature junction had not yet formed (i.e., when TEER was not constant).

JO-1 triggers intracellular signaling and increases penetration of mAb in epithelial tumors in vivo

An orthotopic breast cancer xenograft model (HCC1954) was used to study the effect of JO-1 on epithelial junctions in vivo. HCC1954 xenograft tumors resembled the histology of breast cancer in humans (20), that is, tumors were vascularized and contained nests of epithelial cells glued together by epithelial junctions and surrounded by extracellular matrixes (Supplementary Fig. S2). JO-1 was injected intravenously into

Figure 2. Analysis of mechanism of JO-1 action in tumors in an orthotopic (HCC1954) breast cancer model. When tumors reached a volume of approximately 200 mm³, JO-1 (2 mg/kg in 200 µL PBS) was injected intravenously. Tumors were harvested either 1 or 12 hours after JO-1 injection. Control mice received 200 µL PBS and tumors were collected 1 hour later. A, kinetics of JO-1 accumulation in tumors. Left: immunofluorescence analysis of tumor sections using anti-His tag antibodies (for visualization of JO-1). The scale bar is 20 µm. Right: Western blot analysis of tumor tissue using Ad3-fiber knob specific antibodies (7). B, analysis of DSG2 in tumors. Left: immunofluorescence analysis of tumor sections using DSG2 antibodies (mAb 6D8 against extracellular domain 3/4 of DSG2). The inserts show a higher magnification. Right: the same anti-DSG2 antibody was used for Western blot analysis of tumor tissue. C, intracellular signaling in vivo. Left: Western blot analysis of tumor tissue for E-cadherin and phosphorylated E-cadherin, Erk 1/2, phosphorylated Erk1/2, claudin 7, and vimentin. Antibodies against γ-tubulin were used to assess sample loading ("loading control"). Right: immunofluorescence analysis using antibodies against E-cadherin and phosphorylated Erk1/2.
mice with preestablished tumors, JO-1 could be detected in the tumors by immunofluorescence microscopy as early as 1 hour postinjection. JO-1 accumulated in the tumors as is indicated by the increased immunofluorescence at 12 hours postinjection (Fig. 2A, left 3 panels). This is also confirmed by Western blot analysis of tumor lysates (Fig. 2A, right panel). Analysis of DSG2 on tumor sections by immunofluorescence microscopy in PBS-treated animals showed membrane localized signals (Fig. 2B, left panel). One hour subsequent to JO-1 injection, DSG2 molecules were mostly found in the cytoplasm of the tumor cells (second panel). By 12 hours membrane localization of DSG2 seemed to be partly restored (third panel). Western blot analysis using anti-DSG2 antibodies against the extracellular domain of DSG2 revealed smaller fragments of the DSG2 (80 and 45 kDa) at the 1 hour time point (Fig. 2B, right panel). These fragments represent the extracellular domains (ECD) and proteolytic cleavage products of the ECD. Proteolytic cleavage of DSG2 to stable fragments in normal epithelial tissue and cancer has been reported before (21).

Recently, it was found in in vitro studies that Ad3 binding to DSG2 of epithelial cells triggered intracellular signaling including pathways that are involved in epithelial-to-mesenchymal transition (EMT; 7). Among the features that characterize EMT are decreased expression of epithelial markers and activation of Erk1/2 (MAPK; 5). In our studies with xenograft tumors, we found less nonphosphorylated and phosphorylated forms of Erk1/2 (MAPK; 5). In our studies with xenograft tumors, we found less nonphosphorylated and phosphorylated forms of Erk1/2 (MAPK; 5). In our studies with xenograft tumors, we found less nonphosphorylated and phosphorylated forms of Erk1/2 (MAPK; 5).

In breast cancer xenograft sections and in cultured breast cancer cells, we found costaining of Her2/neu and the adherens junction protein claudin 7 (Fig. 4A, Supplementary Fig. S3). Confocal microscopy of breast cancer BT474 cells confirmed the trapping of Her2/neu in lateral junctions. Incubation of the Her2/neu positive breast cancer cell lines BT474 (Fig. 4) or compare pErk1/2 PBS vs. JO-1 (1 hour). The decrease in E-cadherin and an increase in signals for phosphorylated Erk1/2 upon JO-1 injection were also observed by immunofluorescence microscopy (Fig. 2C, right panels). These studies indicate that JO-1 triggers transient activation of Erk1/2 pathways in vivo.

Next, we tested whether JO-1-triggered opening of epithelial junctions in tumors would increase the penetration of mAbs in xenograft tumors. Trastuzumab, a humanized IgG1 mAb, was injected intraperitoneally at a dose of 10 mg/kg into HCC1954 tumor-bearing mice (24). In tumor sections and Western blot analyses, trastuzumab was detectable 1 hour postinjection and at higher levels 12 hours after injection (Fig. 3A and B). Quantitative analysis of human IgG1 in tumor lysates by ELISA showed approximately 6-fold higher levels in mice that received JO-1 injection + trastuzumab (12 hours time point) compared with mice that received trastuzumab alone (Fig. 3C).

In conclusion, intravenous injection of JO-1 1 hour prior to the administration of trastuzumab, significantly increased the amount of trastuzumab in the tumors, indicating either better egress from blood vessels, better intratumoral penetration, and/or longer intratumoral half-life.

mAb targets are trapped in epithelial junctions

In breast cancer xenograft sections and in cultured breast cancer cells, we found costaining of Her2/neu and the adherens junction protein claudin 7 (Fig. 4A, Supplementary Fig. S3).
imab. enhanced killing of cancer cells by trastuzumab and cetuximab resulted in a translocation of EGFR to the cell surface. We observed for Her2/neu, which confers resistance to cetuximab (25). Similar to what we observed for Her2/neu in the corresponding cell lines, although the effect of JO-1 was relatively modest.

In vitro killing of BT-474 breast cancer and A549 lung cancer cells. C, JO-1 enhances killing of Her2/neu-positive breast cancer cells by trastuzumab. BT-474 cells were incubated with JO-1 (5 μg/mL) or PBS. Trastuzumab (15 μg/mL) was added 1 hour later. Cells viability was measured after 3 hours by WST-1 assays as described earlier (34). Viability of PBS-treated cells was taken as 100%. n = 5. D, JO-1 enhances cetuximab killing of EGFR-positive A549 cells. n = 5; *, P < 0.05.

HCC1954s (Supplementary Fig. S3) with JO-1 changed the composition of the lateral epithelial junctions within 1 hour. As a result of this, Her2/neu staining at the cell surface became more intense, while it faded in areas distal of the cell surface. This suggests that JO-1 mediated junction opening triggered a translocation of Her2/neu from lateral membranes to the cell surface. Being trapped in epithelial junctions also seems to be a problem for EGFR as costaining for EGFR and the tight junction protein E-cadherin suggests (Fig. 4B, Supplementary Fig. S3B). In our studies with cetuximab, we focused on a lung cancer model (A549 cells), as most colon cancer cell lines have mutations in K-ras, which confers resistance to cetuximab (25). Similar to what we observed for Her2/neu, incubation of A549 cells with JO-1 resulted in a translocation of EGFR to the cell surface.

Release of mAb receptors from trapping is supported by the enhanced killing of cancer cells by trastuzumab and cetuximab. In vitro killing of BT-474 breast cancer and A549 lung cancer cells by trastuzumab and cetuximab, respectively, was inefficient (Fig. 4C and D). Pretreatment of these cells with JO-1 significantly increased in vitro cytotoxicity of both antibodies in the corresponding cell lines, although the effect of JO-1 was relatively modest.

JO-1 improves trastuzumab therapy in vivo

JO-1’s potential enhancement of trastuzumab therapy was first tested in an orthotopic breast cancer model based on Her2/neu positive BT-474-M1 cells. JO-1 injection alone had no significant effect on tumor growth (Fig. 5A). BT-474-M1 tumors initially responded well to trastuzumab, however, preinjection of JO-1 significantly enhanced the therapeutic efficacy of trastuzumab (Fig. 5A). The enhancing effect of JO-1 pretreatment becomes more apparent when treated mice were followed long term, that, for 136 days. Although 60% of the animals that received trastuzumab monotherapy relapsed around day 100, none of the animals treated with JO-1+trastuzumab showed tumor regrowth (data not shown).

A second breast cancer model involved HCC1954 cells. Tumors derived from these cells are more resistant to trastuzumab (Fig. 5B). As seen in the BT-474-M1 model, JO-1 pretreatment significantly improved trastuzumab therapy and stalled tumor growth. On the basis of our previous study with PtDd (7), we chose a time interval of 10 hours between JO-1 and trastuzumab injections. This regimen is supported by the kinetics of JO-1 accumulation in tumors and the kinetics of E-cadherin decrease (see Fig. 2A and C). On the other hand,
events that seem to be linked to junction opening, that is, DSG2 cleavage or Erk1/2 activation, occur already within 1 hour after JO-1 injections. We therefore investigated how simultaneous JO-1/trastuzumab injection and injection of trastuzumab 1 hour after JO-1 application influenced the therapeutic outcome (Fig. 5C). In this study, no significant difference was found when compared with the treatment approach used initially (trastuzumab 10 hours after JO-1). We speculate that this is due to the relative slow accumulation of the protein in the tumors. To further consolidate the clinical relevance of JO-1 as a cotherapeutic for trastuzumab, we conducted efficacy studies in Her2/neu-positive gastric cancer (NCI-N87) and ovarian cancer (SKOV3-1ip) models (Supplementary Fig. S4).

Similar to the breast cancer model, we found costaining of Her2/neu and claudin 7 in NCI-N87 cultures and xenografted tumors, suggesting trapping of Her2/neu in epithelial junctions. Pretreatment of NCI-N87 tumor-bearing mice with JO-1 significantly improved trastuzumab therapy as revealed by delayed tumor growth (Supplementary Fig. S4A). To establish the ovarian cancer model, SKOV3-1ip, cells were injected intraperitoneally and survival was monitored after treatment (Supplementary Fig. S4B). Although all mice treated with trastuzumab alone had reached the endpoint by day 80, 80% of the animals that received the combination treatment JO-1 plus trastuzumab were still alive at this time.

**JO-1 improves cetuximab therapy in vivo**

Cetuximab treatment of mice with preestablished subcutaneous A549 tumors did not result in a significant delay of tumor growth when compared with treatment with PBS (Fig. 6A). JO-1 was injected intravenously or intraperitoneally followed by cetuximab 12 hours later. Both treatment approaches had a significant therapeutic effect and resulted in a decrease of tumor volumes. An additional combination of intravenously injected JO-1 with an intratumoral application of the junction opener did not further increase the therapeutic efficacy. As seen in the breast cancer model, JO-1 treatment alone did not exert a significant antitumor effect. JO-1 pretreatment enhanced cetuximab therapy to a similar degree as seen with PTDd (Fig. 6B).

Next, the cotherapy approach was tested in a metastatic lung cancer model. In this model, mice became morbid within 37 days of tumor cell transplantation with predominant tumor localization to the lung (Fig. 6C, "PBS" group). Treatment of mice was started at day 10. All animals were sacrificed at day 40. Although lung metastases were clearly visible in the control group, JO-1 group, and the cetuximab-treated animals, 80% of the lungs in the JO-1 + cetuximab-treated animals were free of tumor when inspected macroscopically. Microscopy of lung sections showed that in PBS-treated animals, tumor cells almost completely replaced normal lung tissue and also filled...
the bronchioli (Fig. 6C, right panels). Although cetuximab-treated animals had considerable, infiltrating tumor growth, the majority of JO-1+ cetuximab-injected animals showed only micrometastases.

**Combined tumor stroma protein degradation and junction opening**

Extracellular matrix proteins forming the tumor stroma tightly surround nests of malignant breast and colon cancer cells (26). We have recently shown that transient degradation of tumor stroma proteins by intratumoral expression of the peptide hormone relaxin significantly enhanced trastuzumab therapy (24). Here, we utilized the HCC1954 model to test whether additional transient tumor stroma protein degradation, would further increase the effect of JO-1 on trastuzumab therapy (Fig. 7A). To deliver the relaxin gene to the tumor, we employed an approach based on hematopoietic stem cells (HSC; ref. 26). The approach involved the ex vivo transduction of bone marrow derived HSCs with lentivirus vectors expressing relaxin under the control of a.
Figure 7. Combination therapy of JO-1 and relaxin in the HCC1854 breast cancer model. A, schematic illustration of the experiment. Lethally irradiated mice received either mock transduced or LV-EF1α/Rlx transduced Lin− bone marrow cells. Six weeks later, after engraftment of HSCs, mice were injected into the mammary fat pad with 4 x 10⁶ HCC1854 cells. Relaxin (Rlx) expression was activated by Dox 7 days later. Mice were then given weekly treatment of PBS, PBS/trastuzumab or JO-1/trastuzumab and tumor volumes were measured. B, tumor volumes of individual mice. n = 5. (trastuzumab vs. JO-1 + trastuzumab, P < 0.001; Rlx + JO-1 + trastuzumab vs. Rlx + trastuzumab, P < 0.001; Rlx + PBS vs. PBS, P < 0.001; Rlx + PBS vs. Rlx + JO-1 + PBS, P < 0.001; Rlx + PBS vs. Rlx + JO-1 + trastuzumab, P < 0.001).

Mechanisms of action

Our data suggest that JO-1 triggers junction opening in epithelial tumors through several, potentially connected, mechanisms: (i) cleavage of the DSG2 ECD, and disruption of DSG2 dimers between neighboring cells; (ii) intracellular signaling that leads to a transient decrease of E-cadherin and potentially other junction proteins; and (iii) changes in the membrane distribution of Her2/neu. JO-1 treatment resulted in transient phosphorylation of Her2/neu in tumors (Supplementary Fig. S5). However, trastuzumab treatment alone also triggered Her2/neu phosphorylation, a phenomenon that has been observed before (27), and JO-1 plus trastuzumab cotherapy did not further increase the levels of phosphorylated Her2/neu. This makes it unlikely that JO-1 enhances trastuzumab therapy through its effect on the biology of Her2/neu.

Discussion

JO-1 as new cotherapeutic

The epithelial phenotype of cancer, that is, intercellular junctions, creates obstacles to mAb therapy. The small recombinant protein JO-1 increased the penetration of trastuzumab in the tumor and allowed for better access to mAb target receptors, which, in turn, facilitated mAb therapy in a series of xenograft models involving human epithelial tumor cells. Potentially, the combination of JO-1 with trastuzumab and cetuximab might allow for the reduction of the effective dose of these mAbs, thereby reducing critical side effects, that is, trastuzumab-associated cardiotoxicity and acne-like rashes that often occur during cetuximab therapy.
Side effects on normal epithelial tissues

Because the mouse orthologue of DSG2 is not recognized by Ad3 or JO-1 (7), we generated transgenic mice containing the human DSG2 locus. The expression pattern and level of human DSG2 in these animals were similar to those found in humans. Furthermore, we showed that JO-1 binding to human DSG2 in transgenic mouse epithelial cells triggered junction opening to a degree similar to data observed in human cells. In preliminary studies with DSG2-transgenic mice we did not find critical side effects of intravenous JO-1 injection (2 mg/kg; 28). We speculate that DSG2 in normal epithelial cells is not readily accessible to intravenously applied JO-1. On the other hand, greater leakage of tumor-associated blood vessels and the lack of strict cell polarization might make epithelial tumors more responsive to JO-1. Lack of toxicity after intravenous injection of JO-1 ligands is also underscored by studies with adenoviruses containing Ad3 fibers (29).

JO-1 immunogenicity

As JO-1 is a viral protein, adaptive immune responses might develop in humans, particularly after repeated injection. This might, however, not be a problem clinically because both trastuzumab and cetuximab are used in combination with immunosuppressive chemotherapeutic drugs.

Potential risk to enhance tumor invasion and metastasis

In agreement with other studies (8, 9), we found a higher DSG2 expression in malignant tissues than in the surrounding normal epithelial tissue. There are, however, also studies reporting a reduction in the amounts of DSG2 in invasive pancreatic or gastric cancer (23, 30). The latter, and the finding that JO-1 triggers EMT-like signaling, raises the question of whether JO-1 would facilitate metastasis. Notably, in all models used in this study, we did not see stimulation of tumor growth or macroscopic/microscopic signs of metastasis in animals treated with JO-1 alone. Tumor invasion and metastasis requires more than transient activation of EMT pathways. Detachment from epithelial cancers and migration of tumor cells is only possible after long-term crosstalk between malignant cells and the tumor microenvironment, resulting in changes in the tumor stroma and phenotypic reprogramming of epithelial cells into mesenchymal cells (31).

In summary, the epithelial junction opener JO-1 has the potential to improve mAb therapies of cancer both in terms of efficacy and safety, that is, by allowing lower therapeutic mAbs doses. This study also sheds light on the mechanisms of Ad3 infection of epithelial cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

The work was supported by NIH grants R01 CA080192, R01 HL078836 (A. Lieber), the Pacific Ovarian Cancer Research Consortium/Specialized Program of Research Excellence in Ovarian Cancer Grant P50 CA03636, FHCR Breast Cancer Research Program Pilot Project Fund, the Danish Cancer Society, the Danish National Research Foundation (R. Strauss and J. Bartek), and the European Commission (grant CZ.1.05/21.0008; J. Bartek). I. Beyer is a recipient of a postdoctoral fellowship award from 'Deutsche Krebshilfe' (108968).

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Received June 16, 2011; revised September 13, 2011; accepted September 28, 2011; published OnlineFirst October 11, 2011.

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Cancer Res  Published OnlineFirst October 11, 2011.