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Semi-quantitative Assessment of Microdistribution of Fluorescence-labeled Monoclonal Antibody in Small Peritoneal Disseminations of Ovarian Cancer

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Summary

Uniform antibody microdistribution throughout tumor nodules is crucial for antibody-targeted therapy, because nonuniform microdistribution leads to suboptimal therapeutic effect, a commonly observed limitation of therapeutic antibodies. Herein, we evaluated the microdistribution of different doses of intraperitoneally-injected^{R2-2} fluorescence-labeled full-antibody, trastuzumab (15, 50, and 150 μ g) and its Fab fragment (trastuzumab-Fab: 15 and 50 μ g) in a mouse model of ovarian cancer with peritoneal disseminated tumor. A semiquantitative approach (central/peripheral accumulation ratio; C/P ratio) was developed using *in situ* fluorescence microscopy. Furthermore, we compared the microdistribution of intact trastuzumab with a mixed-injection of trastuzumab and trastuzumab-Fab or serial injections of trastuzumab using *in situ* multicolor fluorescence microscopy. Fluorescence images after the administration of 15- μ g or 50- μ g trastuzumab and 15- μ g trastuzumab-Fab demonstrated antibody accumulation in the tumor periphery, whereas administration of 150- μ g trastuzumab and 50- μ g trastuzumab-Fab showed relatively uniform accumulation throughout the tumor nodule. Serial injections (19-h interval) of trastuzumab-rhodamine green and TAMRA study, the 2nd injection of trastuzumab distributed more centrally than the first, while no difference was observed in control mixed-injection group. Moreover, the mixed injection of trastuzumab and trastuzumab-Fab showed that trastuzumab-Fab distributed more centrally than the same amount of co-injected trastuzumab. Our results suggest that the strategies of increasing dose and using Fab fragments can be used to achieve a uniform antibody distribution within peritoneal disseminated nodules after intraperitoneal injection. Furthermore, serial-injection and mixed-injection strategy can modify antibody microdistribution within tumors and has the potential for preferential delivery of anti-cancer drugs to either the tumor periphery or its center.

Introduction

Monoclonal antibody-targeted cancer therapy is an important, growing class of targeted anti-cancer therapies. Monoclonal antibodies can target specific cell-surface markers and enable delivery of anti-cancer agents, such as radionuclides, cytotoxic drugs, and photo-sensitizers in addition to inducing antibody-dependent cellular cytotoxicity (ADCC) and complement-

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dependent cytotoxicity (1). Currently, 9 cancer-specific monoclonal antibodies and antibody-conjugates have been approved for the treatment of human cancers by the US Food and Drug Administration (FDA) and many more are currently undergoing clinical trials (1–5). For instance, anti-CD20 monoclonal antibody (rituximab), first approved by the FDA in 1997 as an anti-cancer monoclonal antibody, demonstrated excellent therapeutic outcomes in patients with follicular lymphoma and diffuse large B cell lymphoma. The combination of rituximab and chemotherapy is now the standard of care in clinical oncology (2). In spite of considerable success in the field of hematological cancers, the anti-cancer responses have been more modest in solid tumors (5,6). With hematological cancers, the desired serum antibody concentration can be readily achieved, while mechanical and physiological barriers exist within solid tumors which effectively inhibit the penetration of the antibody, leading to a nonuniform distribution of antibody (5,7–13). Such non-uniformity leads to suboptimal therapeutic effects (9,14), thus explaining, in part, the differences in response rates of hematologic and solid malignancies to monoclonal antibody therapies.

The factors underlying the failure of antibodies to distribute uniformly through a solid tumor include large tumor size, increased central interstitial pressure, and mechanical barriers (e.g. collagen and tight-junction) (7,8,10,11,14–16). However, the majority of this work has been conducted in subcutaneous xenografts (except for a few studies involving lung (13) or liver metastasis models (10)), which may not adequately simulate the microenvironmental conditions found in human solid metastases. Small peritoneal tumors are a realistic model with which to study the microdistribution of antibody-targeted therapy (17–19). Such a small peritoneal implant can be a target of antibody therapy after cytoreductive surgery (18).^{R2–3} Since the surface of the tumor is exposed to the peritoneal cavity except for its narrow attachment to the peritoneal membrane, antibodies can interact with tumor nodules at high concentrations after intraperitoneal injections. Although the intraperitoneal injection of antibodies with and without therapeutic radionuclides, has been extensively used for preclinical and clinical studies of cancer treatment (17–19), microdistribution studies within tumors has not been extensively evaluated. In this study, therefore, we evaluated the microdistribution of different doses of intraperitoneally-injected^{R2–2} fluorescence-labeled IgG and Fab fragments in a peritoneal dissemination model of ovarian cancer in mice using a semi-quantitative analysis under *in situ* fluorescence microscopy. Furthermore, we evaluated mice receiving either serial injection of IgG or mixed injections of IgG and Fab using multicolor fluorescence microscopy. With these two different antibody injection strategies, we hypothesized that the microdistribution of antibody could be modified and these methods had the potential for preferential delivery of anti-cancer drugs to either the tumor periphery or its center.

Materials and Methods

Reagents

Trastuzumab (Herceptin®), an FDA-approved humanized IgG1 anti-HER2 antibody, which has a complimentary determination region (CDR) against HER2 grafted on a human IgG1 framework, was purchased from Genentech Inc. (South San Francisco, CA). Amino-reactive rhodamine green (RhodG-NHS), Alexa568-NHS, and TAMRA-NHS were purchased from Invitrogen Corporation (Carlsbad, CA). Trastuzumab-Fab was made using the papain digestion method. Briefly, 5mg/mL trastuzumab-IgG was incubated with papain beads (Immobilized papain, Pierce, Rockford, IL) in a PBS-based digestion buffer (pH 7) containing 7.88 mg of Cysteine • HCl for 31 hours at 37°C, a process monitored by the UV absorption profile of a TSK G3000SWxL column during HPLC (Tosoh Bioscience LLC, Montgomeryville, PA; 0.067 sodium phosphate-0.16 M KCl, pH 6.8; 1 ml/min). When trastuzumab-IgG was totally digested into Fab, the reaction solution was purified with dialysis using a Slide-A-Lyzer Dialysis Cassette (Pierce) against PBS, 1X pH 7.2 (0.066M)

without magnesium and without calcium overnight at 4°C. After purification, the purity of trastuzumab-Fab was >97% as measured by HPLC.

Synthesis of RhodG, Alexa568 and TAMRA-conjugated Trastuzumab and Trastuzumab-Fab

At room temperature, 1 mg (6.85 nmol) of trastuzumab in Na₂HPO₄ was incubated with 68.5 nmol of RhodG-NHS, 47.95 nmol of Alexa568-HNS, or 68.5 nmol of TAMRA-NHS, at pH 8.5 for 15 minutes, and 1 mg (20 nmol) of trastuzumab-Fab was incubated with 60 nmol of RhodG-NHS at pH 8.5 for 15 minutes. The mixture was purified with a Sephadex G50 column (PD-10; GE Healthcare, Piscataway, NJ). Trastuzumab-RhodG, -Alexa568, -TAMRA and trastuzumab-Fab-RhodG were kept at 4°C in the refrigerator as stock solutions. The protein concentrations of samples were determined with Coomassie Plus protein assay kit (Pierce Biotechnology, Rockford, IL) by measuring the absorption at 595 nm with a UV-Vis system (8453 Value UV-Visible Value System; Agilent Technologies, Santa Clara, CA) using standard solutions of known concentrations of trastuzumab or trastuzumab-Fab. The concentration of RhodG, Alexa680 and TAMRA was then measured by absorption at 503, 679, 679 nm, respectively, with the UV-Vis system to confirm the number of fluorophore molecules conjugated with each trastuzumab molecule. The number of fluorophore molecules per trastuzumab/trastuzumab-Fab was adjusted to approximately 3 for trastuzumab-RhodG -Alexa568, and -TAMRA, and 1 for trastuzumab-Fab-RhodG.

Cell line and cell culture

An established human ovarian cancer cell line, SKOV3 was used for generating intraperitoneal disseminated tumors. SKOV3 over-expresses HER2/neu, the target for trastuzumab (20). The cell line were grown in RPMI 1640 medium (Invitrogen Corporation, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen Corporation, Carlsbad, CA), 0.03% L-glutamine at 37 °C, 100 units/mL penicillin, and 100 µg/mL streptomycin in 5% CO₂.

Tumor model

All procedures were approved by the National Cancer Institute Animal Care and Use Committee. The intraperitoneal tumor implants were established by intraperitoneal injection of 2×10^6 SKOV3 cells suspended in 200 µL of PBS in female nude mice (National Cancer Institute Animal Production Facility, Frederick, MD). Experiments with tumor bearing mice were performed 30–35 days after inoculation.

***In situ* fluorescence microscopy after injection of either labeled trastuzumab or trastuzumab-Fab**

After establishing the intraperitoneal dissemination model, groups of mice (n=3–4 per group) were injected i.p. with 15, 50, or 150 µg of trastuzumab-RhodG i.p., or 15 and 50µg of trastuzumab-Fab-RhodG diluted with 300 µL PBS. After 24 hours, mice were sacrificed with carbon dioxide, and then the abdominal cavities were exposed and the peritoneal membranes were spread on glass slides. *In situ* fluorescence microscopy was performed using an Olympus BX61 microscope (Olympus America, Inc., Melville, NY). DIC images were used to identify the tumor nodules and fluorescence images were obtained with blue filter settings; a band pass filter from 470 to 490 nm and a band pass filter from 515 to 550 nm were used for excitation and emission light, respectively. Finally, 52 nodules in the 15-µg trastuzumab group, 62 nodules in the 50-µg trastuzumab group, 51 nodules in the 150-µg trastuzumab group, 52 nodules in the 15-µg trastuzumab-Fab group, and 54 nodules in the 50-µg trastuzumab-Fab group were identified and analyzed.

***In situ* multicolor fluorescence microscopy of serial trastuzumab injections**

After establishing the intraperitoneal dissemination model, groups of mice (n=3, each) were injected i.p. with 30 µg of trastuzumab-TAMRA diluted with 300 µL PBS. After 19 hours, the same mice were injected i.p. with 30 µg of trastuzumab-RhodG. diluted with 300 µL PBS. Following an additional 5 hours, the mice were sacrificed with carbon dioxide, and then the abdominal cavities were exposed and the peritoneal membranes were spread on glass slides. *In situ* multicolor fluorescence microscopy was performed using an Olympus BX61 microscope with blue filter settings and green filter settings (a band pass filter from 533 to 588 nm and a band pass filter from 608 to 683 nm). As a control, a mixed-injection group of mice received i.p. a mixture of 30 µg of trastuzumab-TAMRA and 30 µg of trastuzumab-RhodG. and were also evaluated 24 hours after injection. Finally, 34 nodules were identified in the serial-injection group, while 27 nodules were identified in the control mixed-injection group.

***In situ* multicolor fluorescence microscopy of a mixed-injection of trastuzumab and trastuzumab-Fab**

After establishing the intraperitoneal dissemination model, groups of mice (n=3, each) were injected i.p. with a mixture of 50-µg trastuzumab-Alexa568 and 50-µg trastuzumab-Fab-RhodG diluted with 300 µL PBS. Twenty four hours later, the mice were sacrificed with carbon dioxide, and the abdominal cavity was exposed; the peritoneal membranes were spread on glass slides. *In situ* multicolor fluorescence microscopy was performed using an Olympus BX61 microscope with blue filter settings and green filter settings. Finally, 40 nodules were identified and analyzed in this mixed-injection group.

Semi-quantitative image analysis

All fluorescence images were analyzed with ImageJ software (<http://rsbweb.nih.gov/ij/>). Linear regions of interest (ROI) were placed on the long axis of each tumor nodule (Fig. 1a). Tumor nodules, whose long-axes were covered by fat tissue, were eliminated from image analysis because fat absorbs and scatters fluorescence from tumor nodules. The ROIs were divided evenly into 3 portions; peripheral 1, central, and peripheral 2, and then mean signal intensities were calculated in each portion (P1, C, and P2, respectively) (Fig. 1b). After obtaining mean signal intensities, central/peripheral signal intensity ratios (C/P ratios) were calculated using the following formula:

$$C/P\text{ratio} = \frac{C}{(P1+P2)/2}$$

Statistical analysis

Statistical analyses were performed using a statistics program (GraphPad InStat, version 3.06, GraphPad Software, La Jolla, CA) on a Windows computer. Mann-Whitney test was performed to compare the mean C/P ratio between the 2 groups, while a one-way analysis of variance (ANOVA) with post test (Kruskal-Wallis test with post-test) was used to compare the mean C/P ratio among the 5 groups of single injection study. Values of $p < 0.05$ were considered statistically significant.

Results

***In situ* fluorescence microscopy after single-injection of either trastuzumab or trastuzumab-Fab**

Fifty-two nodules in the 15- μ g trastuzumab group, 62 nodules in the 50- μ g trastuzumab group, 51 nodules in the 150- μ g trastuzumab group, 52 nodules in the 15- μ g trastuzumab-Fab group, and 54 nodules in the 50- μ g trastuzumab-Fab group were identified and analyzed in this study. Tumor sizes (long-axis) were 0.73 ± 0.28 mm (mean $\pm$ SD), 0.71 ± 0.30 mm, 0.64 ± 0.22 mm, 0.71 ± 0.24 mm, and 0.62 ± 0.21 mm, respectively, and showed no significant difference among all the groups. The C/P ratios in each group were 0.91 ± 0.06 , 0.88 ± 0.17 , 1.23 ± 0.25 , 0.90 ± 0.07 , and 1.01 ± 0.15 , respectively. *In situ* fluorescence images of 15- μ g, 50- μ g trastuzumab and 15- μ g trastuzumab-Fab groups showed accumulation of the agent predominantly in the periphery while 150- μ g trastuzumab and 50- μ g trastuzumab-Fab showed more homogeneous accumulation throughout tumor (Figs. 2a, 2b). The C/P ratios were highest for the 150- μ g trastuzumab among the all groups. The 50- μ g trastuzumab-Fab group also showed a higher C/P ratio than the other 3 groups (15-, 50- μ g trastuzumab group and 15- μ g trastuzumab-Fab group), while these remaining 3 groups showed no significant difference. These data are summarized in Figure 2c. These results indicated that the high dose of antibody distributed more centrally than the lower and middle doses of antibody, and trastuzumab-Fab distributed more centrally than that of same gram amount of IgG.

***In situ* multicolor fluorescence microscopy of serial injections of trastuzumab**

Thirty-four nodules were identified in the serial-injection group, while 27 nodules were identified in the control mixed-injection group. Tumor sizes (long-axis) were 0.56 ± 0.28 mm (mean $\pm$ SD) and 0.56 ± 0.39 mm, respectively. In the serial-injection group, the mean C/P ratio of the 2nd injection (trastuzumab-RhodG) was statistically higher than the C/P ratio of the first injection (trastuzumab-TAMRA), and 31/34 nodules (91.2 %) showed higher C/P ratios after the 2nd injection compared with the first (Fig. 3c). In contrast, no statistical difference was identified between the C/P ratios of simultaneously-injected trastuzumab-RhodG and trastuzumab-TAMRA (Fig. 3d). These different distribution patterns were clearly visualized in composite fluorescence images (Figs. 3a, 3b). These findings indicate that the second injection of trastuzumab distributed more centrally compared to that of the first injection of trastuzumab in over 90% of the tumor nodules.

***In situ* multicolor fluorescence microscopy of the mixed injection of trastuzumab and trastuzumab-Fab**

Forty nodules were identified and analyzed in this mixed-injection group. Tumor size was 0.58 ± 0.19 mm. Both fluorescence images and C/P ratio analysis showed that trastuzumab-Fab distributed more centrally than the co-injected trastuzumab, and 37/40 nodules (92.5 %) showed higher C/P ratios for trastuzumab-Fab than the co-injected trastuzumab (Figs. 4a, 4b). In the mixed-injection of trastuzumab and trastuzumab-Fab group, trastuzumab-Fab showed more central accumulation and less peripheral accumulation than the single injections of the same amount of trastuzumab-Fab (Figs. 2b, 4a). Moreover, in the mixed injection group trastuzumab-Fab showed a higher C/P ratio than trastuzumab-Fab alone (Fig. 4c). These finding indicate that in the mixed injection, trastuzumab-Fab distributed more centrally in the tumor than the same amount of co-injected trastuzumab or individually injected trastuzumab-Fab.

Discussion

In this study, we evaluated the microdistribution of intraperitoneally-injected fluorescence-labeled trastuzumab and trastuzumab-Fab in a mouse model of peritoneally disseminated ovarian cancer. When trastuzumab was administered in low and intermediate doses (15 and 50 µg per mouse), the agent distributed peripherally (Fig. 2a). This finding can be explained by mechanical and physiological barriers within solid tumor nodules, including increased interstitial pressure, collagen, and tight-junction. In addition to these factors, Weinstein and colleagues also proposed the binding-site barrier theory to explain such a nonuniform antibody microdistribution (21); According to this theory, the high affinity interaction between antibodies and antigens interferes with the penetration of the antibody into the tumor.

The injected antibodies first interact with tumor antigens at periphery of each tumor and can be trapped in the periphery due to mechanical and physiological barriers which hamper antibody penetration, resulting in a doughnut-like accumulation. Such nonuniform accumulations were overcome by either increasing the dose of the full antibody or by using a Fab in our study. Increasing the dose allows more antibodies to interact with the tumor nodules, and can overcome the binding-site barrier effect (13). Fab fragments (50 kDa), have been reported to diffuse faster and penetrate more deeply into tumor nodules due to their smaller molecular weight (6,22). Also, the faster off-rate of Fab can overcome the binding-site barrier (22). These lead to a more uniform microdistribution of Fab within tumor nodules than is seen with the full antibody, although insufficient dose of Fab may still result in a peripheral dominant accumulation (Fig. 2b). ^{R1-1}

Serial injections of trastuzumab showed that the latterly injected trastuzumab distributed more centrally and less peripherally^{R1-2} than the trastuzumab injected first (Fig. 3). This finding is difficult to explain by mechanical barriers alone, because such barriers may not be influenced by antibody-antigen complexes in the peripheral region of the tumor. Therefore, we assumed that this serial injection strategy neutralizes the binding-site barrier; since interactions between primarily-injected antibodies and tumor antigens mainly occur in peripheral region (Fig. 2), unbound available antigens may decrease especially in peripheral region. Thus, the latterly-injected antibody may be free from the binding-site barrier effect and then distribute more deeply into tumor nodules.

When the mixture of trastuzumab and trastuzumab-Fab was injected, trastuzumab-Fab showed more central accumulation and less peripheral accumulation than the same amount of co-injected trastuzumab or trastuzumab-Fab alone (Figs. 2b, 4). These findings can partly be explained by the different permeability of IgG and Fab. However, less accumulation in the peripheral region after exposure to Fab is difficult to explain. A possible explanation of this finding is the higher off-rates of Fab from antigen compared with the full IgG (K_d, 4 nM) (23); since IgG has two-binding sites per antibody, while Fab has only one, the off-rate of Fab is generally considered faster than that of IgG (22). Therefore, while both IgG and Fab bind to peripheral antigens at the same time, Fab does not remain bound, resulting in an IgG-dominant accumulation in the periphery of nodules. Since this leads to decreased unbound antigen in the periphery, Fab may be less affected by the binding-site barrier effect and distribute more centrally than IgG.

Our results suggest that serial injections of IgG and mixed injections of IgG and Fab can be used to modify antibody microdistribution within tumors. This has the potential for selective delivery of anti-cancer drugs to either the periphery or center of small peritoneal disseminations. That is to say, if only one antibody is loaded with an anti-cancer agent, it could be possible to concentrate the drug in either the center or periphery even in

submillimeter tumor nodules. Such precise drug delivery may have implications for improving cancer therapies since it is known that regional differences exist within tumors with regard to angiogenesis (periphery) and hypoxia (center), between the periphery and center of tumor nodules (24,25).

In conclusion, our study demonstrates that the microdistribution of intraperitoneally-injected fluorescence-labeled antibodies in peritoneally disseminated tumor nodules varies with dose, antibody structures, and administration methods (single, serial, mixed). These findings may have implications for improving the uniformity of antibody distribution to improve cancer therapy or conversely exploiting differences in the biology of the periphery and central parts of tumors to more effectively deliver the right drug to the right place.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Image analysis procedures for C/P ratio calculation. Linear region of interest (ROI) is placed on fluorescence images of each tumor nodule along its long-axis based on the DIC images (a). Linear ROIs are divided evenly into 3 portions and signal intensities of each pixel are measured (b). Mean signal intensities are calculated in each portion (P1, C, and P2). Finally, a central/peripheral signal intensity ratio (C/P ratio) is calculated using the formula described in the method section.

Fig. 2.

In situ fluorescence microscopic images of trastuzumab-injected groups (a) and trastuzumab-Fab-injected groups (b), and C/P ratios (means and SDs) in each groups (c). Fluorescence images of 15-µg, 50-µg trastuzumab and 15-µg trastuzumab-Fab groups show peripheral dominant antibody accumulation, while 150-µg trastuzumab and 50-µg trastuzumab-Fab groups show relatively homogenous accumulation throughout tumor. C/P ratios demonstrate that the 150-µg trastuzumab group has the highest among all groups. 50-µg trastuzumab-Fab group also shows higher C/P ratio than the other 3 groups (15-, 50-µg trastuzumab and 15-µg trastuzumab-Fab group), while these 3 groups show no significant difference. Scale bars on DIC images indicate 500µm. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

Fig. 3. *In situ* fluorescence microscopic images of serial injection group (a) and mixed-injection group (b) of trastuzumab-rhodamine green (RhodG) and -TAMRA. Scatter plots of C/P ratios in each group are also shown (c: interval-injection group, d: non-interval-injection group). C/P ratios of each set of tumor nodules are connected in scatter plots. Composite image of serial injection group clearly shows that the secondarily injected trastuzumab-RhodG (green) distributes more centrally than trastuzumab-TAMRA (red) which was injected first. C/P ratios demonstrate that the 2nd injection of trastuzumab-RhodG demonstrates a statistically higher C/P ratio than the trastuzumab-TAMRA which was injected first. In contrast, the mixed injection group shows the same accumulation patterns between two trastuzumabs in both fluorescence images and C/P ratio analysis. Scale bars on DIC images indicate 500 μ m. *** $p < 0.0001$, NS: Not significant.

Fig. 4.

In situ fluorescence microscopic images (a) and scatter plot of C/P ratios in mixed-injection of trastuzumab and trastuzumab-Fab (b). C/P ratios of the same tumor nodules are connected in the scatter plots. Both fluorescence images and C/P ratio analysis show that trastuzumab-Fab distributed more centrally than co-injected trastuzumab. Comparison of C/P ratios of trastuzumab-Fab with/without co-injection of trastuzumab (c, also see Fig. 2) shows that co-injection of trastuzumab alters the trastuzumab-Fab accumulation so that it has a more central distribution pattern. Scale bars on DIC images indicate 500 μ m. *** $p < 0.0001$.