Chapter 3
The Internalization and Therapeutic Activity of Antibody Drug Conjugates

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3.1 Introduction

Unlike small molecules such as amino acids and ions, antibody conjugate drugs (ADCs), a class of protein macromolecules, usually be transported into the cell in a different way. ADCs need to be wrapped on the surface of cell membrane before enter the cytoplasm as a vesicle structure. a process called endocytosis. Apart from some cells (such as macrophages, monocytes, etc.), endocytosis is used by cells to engulf over 100 um diameter particles [1]. In terms of function mechanism, it can be divided into macropinocytosis, clathrin-mediated endocytosis (CME), caveolin-mediated endocytosis, clathrin- and caveolin-independent endocytosis [2]. Endocytosis of ADCs and antibodies is mainly completed through receptor-mediated endocytosis, that is, through CME [3, 4].

CME widely exists in mammalian cells and plays a role in the process of ingesting some essential nutrients, such as low density lipoprotein (LDL) receptor binding LDL, transferrin receptor binding transferrin [5]. CME could affect the signal pathway by regulating cell surface receptors. It also provides support for intercellular signal transmission in the growth of tissues and organs [6]. The endocytosis process is first mediated by receptors, which recruit adaptor proteins and combine receptor ligands complex with clathrin before transport of them across the cell membrane. After that, the surface of cell membrane invaginates to form a den.

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Vacuoles coated with clathrin would separate from cell membrane and interact with endosomes by GTPase-activating protein [7]. After entering the endosome, the receptor protein complex has two pathways: recycled back to cell surface [8]; retained in late endosomes and shuttled into lysosomes. During this trafficking process, once ADCs enter the lysosome, the antibody would be degraded and cytotoxic agents would be released in the cells.

Although some ADCs in the early research choosing extracellular cleavable linkers to release cytotoxic drugs in the tumor microenvironment, which could exert its activity without internalization [9, 10], FDA approved ADCs and most ADCs in clinical trials must bind to the target cell surface antigen before efficiently internalize into the cytoplasmic to release cytotoxic drugs to kill the target cells. Therefore, the internalization and internalization efficiency of ADCs impact the drug trafficking in tumor cells and the subsequent cytotoxic activity, which closely related to the activity of ADCs. How to evaluate the internalization of ADCs and improve the internalization efficiency of ADCs has important guiding significance for the development of new ADCs in the future.

3.2 Experimental Steps for Evaluating Internalization

Determine rate of internalization and intracellular localization after internalization are two aspects for evaluating internalization.

Determine rate of internalization: Antibodies could not be internalized into cell after binding to cell surface at 4 °C. While being transferred to 37 °C, the internalization of antibodies on the cells could be observed. The fluorescence intensity difference of cell surface antibodies labeled with fluorophores are measured by flow cytometry under two temperatures. The rate of internalization could be calculated.

Intracellular localization of internalized antibodies: ADC is internalized into cells and degraded in lysosomes to release the cytotoxic agents. Lysosomes and antibodies were labeled with different fluorescences and localized by confocal microscope.

3.2.1 Materials

FITC (fluorescein isothiocyanate)-labeled goat anti-human IgG (H + L) second antibodies; Alexa Fluor 555 labeled goat anti-mouse IgG (H + L); Anti-LAMP-1 (lysosome-associated membrane protein) mouse monoclonal antibody; 4′,6-diamidino-2-phenylindole, DAPI; Fixation solution for cells: 4% paraformaldehyde in PBS (phosphate buffer saline); Permeabilization solution ((0.1% TritonX-100 and 0.2% bovine serum albumin (BSA) in PBS); Freezing centrifuge; Flow cytometry and high-resolution confocal microscopy, etc.

3.2.2 Internalization Assay by Flow Cytometry

The cells were centrifuged to remove the medium and incubated with certain concentrations of antibody or ADC in 1% BSA-PBS at 4 °C for 30 mins. PBS was used as negative control.

Wash twice with ice cold PBS to remove antibody or ADC that do not bind to the cell surface. The cells were incubated at 4 or 37 °C for 2 h. After washing twice with PBS, cells were stained with FITC-labeled goats anti-human IgG (H + L) secondary antibody diluted 1:250 and incubated on ice for 30 mins. Wash twice and resuspend in PBS. The mean fluorescence intensity (MFI) on cell surface was detected by flow cytometry. The internalization rate of ADC is calculated by formula below:

$$\text{Internalization}\% = \frac{(1 - (\text{MFI of sample incubated at } 37\,^{\circ}\text{C})/}{(\text{MFI of control sample incubatedat } 4\,^{\circ}\text{C}))} \times 100\%$$

3.2.3 Intracellular Localization Assay by Confocal Microscope

The cells were incubated with certain concentration of ADC or antibody at 37 °C for some time (typically 1 h, 4 h and 24 h), centrifugation at 1000 R / min for 5 mins.

To fix the cells on the slide (make sure the cells do not adhere to each other), add 4% paraformaldehyde after rinse the cells with PBS 3 times. Allow cells to fix for 15 mins and then wash twice with PBS. Add 0.1% TritonX-100, 0.2% BSA in PBS at room temperature and incubate for 15 mins to permeabilize cells. After wash cells 3 times with PBS, anti LAMP-1 mouse monoclonal antibody diluted with 1% BSA-PBS was added to the cells and incubated for 45 mins. Again, wash cells 3 times with PBS, cells were stained with FITC -labeled goat anti-human IgG (H + L) and Alexa Fluor 555-labeled goat anti-mouse IgG (H + L). Allow to incubate in dark for 30 mins and wash slides 3 times in PBS. The nuclei were stained with DAPI in dark for 3 mins before washing with PBS 3 times. Anti-quencher solution was dropped on the slide, covered the slide and visualized the subcellular localization of antibody or ADC by confocal laser scanning microscope (Fig. 3.1).

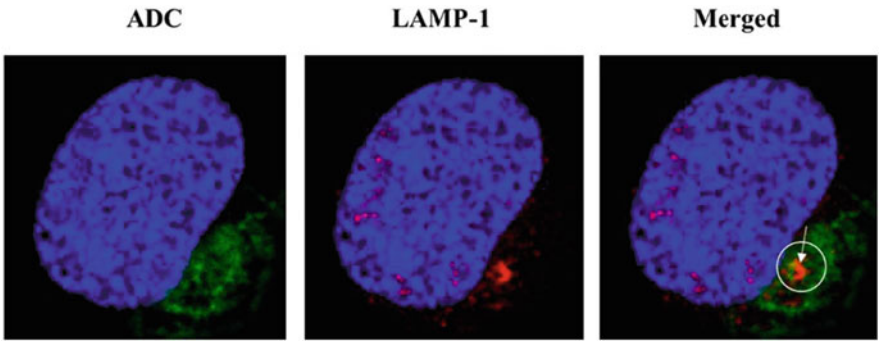

Fig. 3.1 Trafficking and subcellular localization of anti-CD20 ADC. Daudi cells were treated with ADC at 37 °C for 6 h. Cells were fixed as above, ADC and lysosome-associated membrane protein-1 (LAMP-1) were detected with different fluorochrome-conjugated secondary antibodies. Nuclei were stained with DAPI (blue). Arrow indicated co-localization of ADC (green) and lysosomes (red)

3.3 Strategies to Improve Internalization Efficiency

At present, most ADCs target antigens on the cell surface, which means the ADCs needed to be internalized into cells and transported into lysosomes to release cytotoxic drugs and further kill target cells to exert their antitumor activity. Therefore, improving the internalization efficiency of ADCs is a key to the efficacy of them. ADC is composed of targeted antibody, small molecule toxins and linkers. Considering the mechanism of action and structure of ADCs, the key to improve the internalization efficiency depends mainly on the characteristics of specific antibody molecules. There are actually many solutions mainly focus on the construction of antibodies. First of all, choosing the antibodies meets the requirements in antibody screening. The antibody internalization process is related to the characteristics of antigen and the dissociation rate between antibody and antigen. Antibodies with high affinity do not means high internalization efficiency [11]. In addition, modifying the structure of screened antibody to further improve its internalization efficiency is also a hot topic in current ADC research.

3.3.1 Identifying Antibody Binding Epitopes

For specific determined antibody, different binding epitopes of target protein sometimes have a great impact on internalization of antibody. CD56 is over expressed in neuroblastoma, multiple myeloma and small cell lung cancer. Anti-CD56 humanized ADC—IMGN901 from Immunogen is mainly focus on small cell lung cancer and has been in clinical trials. CD56 was proved to be one of optional targets for ADCs [12]. Feng et al. [13] identified two full human antibodies targeting CD56—m900 and m906. The affinities of these two antibodies were similar, however, their

binding epitopes were different. While m900 binds to membrane-distal domain of the extracellular region of CD56, m906 binds to membrane-proximal domain. Compared with m900, m906 showed higher internalization efficiency and induced significant down-regulation of cell surface CD56 more efficiently after binding to CD56. Two ADCs were constructed by conjugating both antibodies with pyrrolobenzodiazepine (PBD) respectively. m906 ADC also displayed more potent killing activity. However, the difference of receptor-mediated internalization efficiency caused by different binding epitopes on same antigen is correlated with the characteristic of target protein. It has been found a similar phenomenon in HER2, a fully studied ADC target. For example, extracellular binding epitope of anti-HER2 pertuzumab is far away from the membrane and does not overlap with that of trastuzumab. Similarly, pertuzumab showed better HER2 internalization efficiency as m906 [14]. This phenomenon is more obvious in a study on anti-HER3 antibodies. Antibody A2 binds to membrane-proximal domain, while A3 binds to the membrane-distal domain. The experiment showed that A2 could induce internalization of receptors, but not A3 [15]. This suggests that for some targets, such as CD56, HER2 and HER3, antibodies binding to membrane-distal domain of the extracellular region have higher internalization efficiency. One potential explanation is that the dimerization of these receptors is influenced by the binding epitopes of antibodies.

3.3.2 Antibody Drug Conjugate Toxins

Depending on the characteristic of antigen, whether the antibody is coupled with small molecule cytotoxic agents will affect the internalization of antibody. For some antigens, such as CD30, the internalization efficiency of anti- CD30 ADC brentuximab vedotin is same as antibody [16]. While for other antigens, such as CD20, the internalization efficiency of antibody has been significantly improved [17], and difference of linkers will also affect the internalization of antibody [18]. This shows that even if the binding characteristics of antigen and antibody are clarified in the early stage, the conjugation process of ADC would also affect its internalization efficiency.

3.3.3 Application of Bispecific Antibody

Application of bispecific antibody to ADC also improves the internalization and lysosome transportation of ADC. This bispecific ADC usually in the form of full antibody could retain its longe half-life in the blood. When targeting two antigens as a bispecific ADC, one target with lower internalization could be dragged into the cell by the high internalization of another target. This drag-type ADC can not only improve the internalization efficiency of low internalization antigens, but also reduce the possibility of antigens recycling to cell surface. Devay et al. [19] combined

amyloid precursor like protein 2 (APLP2) and HER2 to form a bispecific antibody. APLP2 could effectively interact with HER2 to generate a complex which are internalized and transported into lysosomes [20]. In another anti-proprotein convertase subtilisin/kexin type 9 (PCSK9) antibody, APLP2 could bind antibody and promot its degradation after entering lysosome [21]. In order to evaluate the internalization efficiency of APLP2 antibody conjugate drug alone, it was compared with anti-Trop 2 ADC. In vitro cytotoxicity in SKOV3 cells showed that, although the expression level of Trop-2 on cell surface was twice that of APLP2, anti-APLP2 ADC showed lower EC50 value and higher cell killing efficiency. This suggests that the improvement of the lysosome travel efficiency of ADC could indeed improve the efficacy of ADC. Combination of APLP2 and HER2, a target with low internalization efficiency, a bispecific ADC was formed. The lysosomal degradation of HER2 has also been significantly improved due to the high lysosomal targeting efficiency of APLP2 in the cell. This phenomenon has been observed in a variety of cell lines with different APLP2: HER2 ratio.

De Goeij et al. [22] constructed a bispecific ADC targeting CD63 and HER2 in a same way. While the anti-HER2 binding domain provides tumor specificity, anti-CD63 binding domain can promote internalization. CD63, also known as lysosome associated membrane protein 3 (LAMP3), belongs to four transmembrane protein superfamily. It exists on some cell surfaces and is mainly expressed on intracellular vesicles, such as the membrane of endosomes and lysosomes. CD63 regulates the transportation of other proteins in the endocytic pathway [23]. Targeting CD63 can promote internalization and lysosomal transport efficiency. Compared with monovalent control antibodies targeting HER2 or CD63, this bispecific structure could be transported into lysosomes of HER2 positive tumor cells more effectively. This bispecific ADC also exhibited excellent tumor killing in in vitro and in vivo experiments. To avoid this bispecific ADC targeting cells expressing only CD63, such as platelets and granulocytes in peripheral blood, the affinity of anti-CD63 antibody was attenuated, which could maintain the specificity and improve the safety of this bispecific ADC [23, 24].

Choosing a target widely expressed and could be internalized into lysosome targets efficiently to drag other tumor associated antigen are solutions above. In order to reduce the non-specific killing of this ADC, it necessary to modify the affinity of high internalization binding domains or allow the ADC binding antigens in a pH-dependent manner and exhibit its activity in a tumor microenvironment expressed two antigens simultaneously. Construction of a bispecific ADC with two tumor associated antigens is another idea. Although simultaneously expressing two tumor associated antigens shrink the applicable tumor targets, it also reduced the non-specific killing effect resulted by widely expressed target.

Similarly, Regeneron Pharmaceuticals constructed a bispecific ADC targeting HER2 and prolactin receptor (PRLR) [25]. PRLR is expressed in some kind of breast cancer and is related to the pathogenesis of breast cancer. Therefore, as same as HER2, PRLR is also a tumor associated antigen [26, 27]. The expression level of PRLR on the surface of breast tumor cells is lower than that of HER2. However, unlike HER2, which is often recycling to the cell surface [28], PRLR could be

continuously transported into lysosomes and degraded [29]. This characteristic of PRLR make this bispecific ADC could drag the anti-HER2 part with low internalization efficiency into lysosomes and then release small molecule cytotoxic agents more effectively. Compared with anti-HER2 or anti-PRLR ADC, the bispecific ADC targeting PRLR and HER2 also kill breast tumor cells more effectively.

The ligand of mesenchymal epithelial transition factor (MET), also known as c-Met, is hepatocyte growth factor (MET) secreted by stromal cells. MET is associated with drug resistance in various cancers. Lee et al. [30] combining the MET with HER2 or EGFR. Although it was not conjugated to obtain ADC, this bispecific antibody could also exhibit drag function in presence of MET, and promote the degradation of the whole bispecific antibody. MET and EGFR are tumor associated antigens in this bispecific format. Overexpression of both proteins plays an important role in tumor growth and metastasis. Since the signal pathways of MET and EGFR are interrelated, inhibiting one target alone would activate another pathway in tumor cell, targeting both antigens could achieve a better therapeutic effect [31]. In a study of EGFR- MET bispecific antibody, it has been found that this bispecific antibody could promote the interaction between EGFR and MET and dissociate heat shock protein 90 (Hsp90) from MET-EGFR complex. As a chaperone, HSP90 plays a protective role in the process of lysosomal degradation. It is reasonable for the degradation of EGFR and MET simultaneously after this bispecific antibody binding to both targets and the good antitumor activity of this antibody.

3.3.4 Application of Biparatopic Antibody

Biparatopic ADC is also a kind of ADC with bispecific structure. it does not target two antigens at the same time, but different epitopes on the same antigen. Until now, targeting different epitopes of same target simultaneously mainly focus on EGFR and HER2, which all belong to ErbB family.

One successful example is an anti-HER2 biparatopic ADC in clinical research. This biparatopic antibody format was not used in ADC at first. Robert et al. [32] found that a antibody targeting two nonoverlapping epitopes of carcinoembryonic antigen (CEA) exhibited higher binding activity and in vivo antitumor activity compared with the original antibody. Another research shows that combining several non-competitive anti EGFR antibodies could synergistically reduce the EGFR on tumor cell surface and show better antitumor activity and longer survival time in tumor-bearing mouse model [33, 34]. Further research have shown that the combination of multi-epitope antibodies makes the receptors on the cell surface interact cross linked and form a larger antibody-antigen complex [35]. Spangler et al. [36] found that multi-epitope antibodies could inhibit the antigen in endosomes recycling back to cell membrane and promote the down-regulation of EGFR on the cell surface after receptor-antigen is internalized.

As same as EGFR, the combination of anti-HER2 multi-epitope antibodies would also down-regulate cell surface receptors and promote internalization [37]. Based on

this principle, MedImmune identified a full human anti-HER2 antibody—39S whose binding epitope is different from trastuzumab or pertuzumab. Fusing the scFv form of Trastuzumab at the N- terminal of heavy chain of 39S obtained a new antibody. Since the HER2 binding epitope of 39S is nonoverlap with trastuzumab, this new antibody is a tetravalent anti-HER2 biparatopic antibody. The relative molecule mass of antibody is larger than conventional antibody, about 240 kDa [38]. Although the relative molecular weight of this biparatopic antibody is large, its internalization efficiency on BT-474 cells is higher than that of trastuzumab or pertuzumab, even higher than that of mixture from both trastuzumab and pertuzumab. Therefore, this biparatopic format is not equivalent to the mixing of antibodies targeting different epitopes.

Further mechanism studies showed that once the biparatopic antibody binding with the different epitopes on HER2, the N-terminal tetravalent structure of biparatopic ADC (MEDI4276) would induce HER2 receptor clustering on the cell surface, which form a larger HER2- ADC complex cluster. The clustering of HER2 receptor at the cell surface could promote the internalization of receptor and inhibit its recycling from lysosome to the cell surface, which improves ADC enter lysosomal trafficking of ADC significantly [39]. It has been found that down-regulation of HER2 in some T-DM1 resistant cell lines. In addition, heterodimerization of HER2 and HER3 is another reason for T-DM1 resistance. Benefit from high internalization efficiency of MEDI4276 and the hindrance of HER2-HER3 dimerization by binding epitope of 39S, MEDI4276 could kill T-DM1-resistant HER2 low expression tumor cells, which could expand the application scope of anti-HER2 ADC in future clinical trials.

3.4 Conclusion

The internalization efficiency of ADC is a key factor for its activity. Due to the low internalization efficiency of some target proteins, they are not considered as a suitable target for ADCs. The internalization efficiency of screened antibodies specific for different epitopes of some target proteins might be quite different, and the activities of ADCs derived from them could be quite different. Therefore, choosing an appropriate antibody as the target part of ADC could effectively improve its internalization efficiency and enhance its antitumor activity. In addition, multi-specific or bispecific antibodies are currently hot research topics in antibody drugs. ADCs can also have multi-target characteristics. The combination of high and low internalization efficiency targets not only strengthens the specificity of ADC, but also strengthens its antitumor activity, which could widen the pharmaceutical application of ADCs.

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