Intra- and trans-cellular delivery of enzymes by direct conjugation with non-multivalent anti-ICAM molecules

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ARTICLE INFO

Article history:
Received 30 March 2016
Received in revised form 16 July 2016
Accepted 25 July 2016
Available online 27 July 2016

Keywords:
Drug targeting, monomeric vs. multivalent targeting
Receptor-mediated endocytosis
Transcytosis
Antibody-enzyme conjugate
ICAM-1

ABSTRACT

Intercellular adhesion molecule 1 (ICAM-1) is a cell-surface protein overexpressed in many diseases and explored for endocytosis and transcytosis of drug delivery systems. All previous evidence demonstrating ICAM-1-mediated transport of therapeutics into or across cells was obtained using nanocarriers or conjugates coupled to multiple copies of anti-ICAM antibodies or peptides. Yet, transport of therapeutics linked to non-multivalent anti-ICAM ligands has never been shown, since multivalency was believed to be necessary to induce transport. Our goal was to explore whether non-multivalent binding to ICAM-1 could drive endocytosis and/or transcytosis of model cargo in different cell types. We found that anti-ICAM was specifically internalized by all tested ICAM-1-expressing cells, including epithelial, fibroblast and neuroblastoma cells, primary or established cell lines. Uptake was inhibited at 4 °C and in the presence of an inhibitor of the ICAM-1-associated pathway, rather than inhibitors of the clathrin or caveolar routes. We observed minimal transport of anti-ICAM to lysosomes, yet prominent and specific transcytosis across epithelial monolayers. Finally, we coupled a model cargo (the enzyme horseradish peroxidase (HRP)) to anti-ICAM and separated a 1:2 antibody/enzyme conjugate for non-multivalent ICAM-1 targeting. Similar to anti-ICAM, anti-ICAM-HRP was specifically internalized and transported across cells, which rendered intra- and trans-cellular enzyme activity. Therefore, non-multivalent ICAM-1 targeting also provides transport of cargoes into and across cells, representing a new alternative for future therapeutic applications via this route.

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1. Introduction

Uptake of therapeutic drugs by cells [1–3] and drug transport across cell linings that separate body compartments [4–7], are paramount events influencing the success of many therapeutic interventions. To induce these types of transport, many strategies target drugs or their carriers to cell-surface receptors [2,3]. For this purpose, targeting molecules (antibodies, peptides, aptamers, etc.) are coupled either directly to a pharmaceutical agent (drug conjugates) or on the surface of drug-load ed carriers [1–3]. Upon their binding to cells, receptor-mediated endocytosis is triggered, which results in the formation of membrane-enclosed vesicles that carry the drug conjugate or carrier within cells [1–3]. Following uptake, drug conjugates or carriers are typically trafficked to endo-lysosomal compartments, where pH- or enzyme-dependent cleavage may release the drug [1–3]. For drugs that cannot permeate the endo-lysosomal membrane or that are degraded in these compartments, additional strategies are required to enable cytosolic escape [1,2]. In other instances, receptor-mediated uptake results in transport across cellular barriers, such as the case of endothelial and epithelial linings that separate some body compartments [4,5,8,9]. The mechanisms that regulate receptor-mediated transport into or across cells include classical pathways, such as clathrin- and caveolae-dependent endocytosis and transcytosis, whose abundant biological information renders them readily exploitable in drug delivery [1–3]. In contrast, some clathrin- and caveolae-independent pathways have also been described, but their regulation and use in drug delivery is much less characterized [2,3,10]. An example is that of the transport pathway associated to intercellular adhesion molecule-1 (ICAM-1). ICAM-1 is a cell surface glycoprotein, whose expression on various cell types is up-regulated during most pathological conditions, thereby enabling drug targeting to diseased tissues [11].

Efficient uptake of therapeutics via ICAM-1 has been shown both in cell culture and in vivo [8,9,12–17]. Multivalent binding to ICAM-1 triggers uptake via Cell Adhesion Molecule (CAM)-mediated endocytosis, a non-classical pathway distinct from clathrin- and caveolae-mediated endocytosis, phagocytosis, and macroinocytosis [12,18]. Drug conjugates and carriers of various sizes, shapes, and chemistries have been
shown to enter cell via this route upon multivalent binding to ICAM-1, providing therapeutic and/or imaging activity [19–23]. After uptake by cells, multivalent anti-ICAM conjugates and carriers were observed to traffic both to lysosomes and across cells, where they could deliver therapeutics [8,9,19,24–26].

Contrary to multivalent binding of conjugates and carriers to ICAM-1, decade-old reports had indicated that binding of non-multivalent anti-ICAM molecules to ICAM-1 did not induce endocytic transport [12,13]. However, using protocols more amenable than those previously available, we recently observed that anti-ICAM is endocytosed by endothelial cells [25]. Following uptake, a significant amount of anti-ICAM molecules recycles back to the plasmalemma, resulting in low endo-lysosomal accumulation [25], which had been misinterpreted as lack of uptake in previous works. This finding may now provide an opportunity for direct conjugation of therapeutic or imaging agents to anti-ICAM molecules able to induce CAM-mediated transport. This holds significance because direct conjugation may offer a simpler formulation from a manufacturing perspective and may lead to distinct biodistribution, metabolism, clearance, etc., expanding the range of applications of ICAM-1 targeting.

The present study addressed several questions that remained unanswered: Does endocytosis of anti-ICAM molecules occur in cells other than endothelial, to enable delivery in other tissues? Do anti-ICAM molecules transcytose across cell linings, as previously observed for multivalent carriers? Are anti-ICAM molecules able to transport a drug cargo into and/or across cells?

2. Materials and methods

2.1. Antibodies and reagents

Mouse anti-human ICAM-1 (anti-ICAM) was clone R6.5 (American Type Culture Collection, Manassas, VA, USA). Mouse IgG, anti-horseradish peroxidase (HRP), anti-human lysosomal-associated membrane protein 1 (LAMP-1), FITC- or Texas Red (TxR)-labeled secondary antibodies were from Jackson ImmunoResearch (West Grove, PA, USA). HRP-conjugated antibodies and chemiluminescent detection reagents for Western blot were from Genetec Electric Healthcare Bio-Sciences (Pittsburg, PA, USA). Lightning Link® HRP conjugation kit was from Innova Biosciences (Cambridge, UK). Tetramethylbenzidine (TMB0, substrate for HRP) and Pierce Iodogen iodination tubes were from Thermo Scientific (Pittsburg, PA, USA). Lightning Link® HRP conjugation kit was from Innova Biosciences (Cambridge, UK). Tetramethylbenzidine (TMB0, substrate for HRP) and Pierce Iodogen iodination tubes were from Thermo Fisher Scientific (Rockford, IL, USA). Na125I was from Perkin Elmer Analytical Sciences (Wellesley, MA). All other reagents were from Sigma Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Human epithelial adenocarcinoma Caco-2 cells (a gift from Dr. Jerrold Turner, University of Chicago) were cultured at 37 °C in Dulbecco’s Modified Eagle Medium ( GibcoBRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and antibiotics. Other cells used were head and neck cancer A431 cells (ATCC, Manassas, VA), skin fibroblasts (a gift from Dr. Edward Schuchman, Mount Sinai School of Medicine), SH-SY5Y neuroblastoma (ATCC, Manassas, VA), and human umbilical vein endothelial cells (HUVECs), whose culture conditions have been reported elsewhere [15,26]. Cells were seeded on gelatin-coated glass coverslips or Transwell® inserts (polycarbonate terephthalate, 0.4-μm pore size; BD Falcon, Franklin Lakes, NJ). The permeability of Caco-2 monolayers was assessed by measuring the trans epithelial electrical resistance (TEER), using an EVOM™ volt-ohm meter and STX100 electrodes (World Precision Instruments, Sarasota, FL). Where specified, cells were pretreated with 10 ng/mL tumor necrosis factor–alpha (TNFα; BD Biosciences, Franklin Lakes, NJ, USA) to mimic an inflammation status [11].

2.3. ICAM-1 expression and cellular binding of anti-ICAM

Cells were incubated after fixation with 2% paraformaldehyde (to avoid uptake), or live, with 75–140 pmol/L anti-ICAM or IgG for the indicated times and temperatures. Cells were washed, fixed and cell-associated antibody was immunostained using green FITC–labeled secondary antibody [25,27]. Samples were analyzed using an Olympus IX81 microscope (Olympus Inc., Center Valley, PA), via phase contrast (to discern the cell borders) and fluorescence (to visualize antibodies). Micrographs were taken using an ORCA–ER camera (Hamamatsu, Bridgewater, NJ) and SlideBook™ 4.2 software (Intelligent Imaging Innovations, Denver, CO). Images were analyzed using Image–Pro 6.3 (Media Cybernetics Inc., Bethesda, MD) to quantify the sum and mean fluorescence intensity, from which background fluorescence was subtracted [25,27]. Measurements were done cell by cell for all micrographs, so that the level and homogeneity of binding of anti-ICAM on the surface of each cell in the population can be estimated, which relates to the level and homogeneity of ICAM-1 cell expression.

2.4. Endocytosis of anti-ICAM

Cells were incubated at 37 °C with 75 pmol/L anti-ICAM or IgG for 30 min at 37 °C to allow binding (pulse), followed by washing and incubation at 37 °C up to 5 h (chase) to allow uptake. Uptake at 4 °C or in the presence of 3 mM amiloride (to inhibit CAM endocytosis), 50 pmol/L monodansylcadaverine (MDC; to inhibit clathrin pits), or 1 μg/mL filipin (to inhibit caveoli) served to evaluate the pathway [12]. Cells were fixed and antibodies bound on cells were immunostained in red using TxR-goat secondary antibody. Cells were then permeabilized and incubated with FITC–goat secondary antibody, to label all (bound and internalized) antibodies in green. Hence, the cell-surface bound fraction was double-labeled in green and red (yellow), while internalized counterparts were single-labeled in green [25,27]. Samples were analyzed by fluorescence microscopy and quantified to obtain the internalized fluorescence as an absolute value or a percentage compared to the total cell-associated fluorescence [25,27].

2.5. Lysosomal trafficking of anti-ICAM

Cells were incubated at 37 °C with 140 pmol/L FITC-labeled anti-ICAM or IgG using the pulse-chase protocol described above. Incubations were conducted in the absence vs. presence of 300 μmol/L chloroquine, to inactivate lysosomal hydrolases. Cells were fixed, permeabilized, and LAMP-1–positive lysosomes were immunostained with a Texas Red (TxR)-labeled antibody. Co-localization of green anti-ICAM with red lysosomes was quantified by fluorescence microscopy, as described [25]. Degradation of anti-ICAM was estimated by comparing its total fluorescence at any given time to that of the initial 30 min pulse [25].

2.6. Tranacellular transport of anti-ICAM

125I–anti-ICAM or control 125I-IgG (70 pmol/L; 56 nCi/mL) were added to the chamber above cell monolayers grown on Transwell® filters, verified to be confluent (see Cell culture section). After incubation at 37 °C for the indicated times, the radioisotope content in the chamber above the cells (non-bound antibodies), the chamber below the cell (transported antibodies), and the cell fraction (cell-associated antibodies) were measured using a gamma counter. Results were corrected by subtracting the level of free 125I–iodine of each fraction, measured by trichloroacetic acid (TCA) precipitation [9]. The number of antibody molecules associated with and transported across cells, the percentage of molecules transported from this total, and the apparent permeability coefficient (Papp) were determined as follows:
Antibody molecules transported/mm² = \frac{([\text{CPM}_\text{basolateral} - \text{antibody Specific Activity}]/\text{MW}) \times N_\text{mol}}{\text{mm}²}\n\%
\text{Antibody transported} = 100 \times \frac{[\text{CPM}_\text{basolateral} - \text{CPM}_\text{basolateral + CP膜}_{\text{cell fraction}}]}{[\text{CPM}_\text{added} - \text{Vol}] / (A \times t \times \text{CPM}_\text{added})},

where CPM is the 125Iodine counts-per-minute in the upper chamber (CPMadded), the cell fraction (CPMcell fraction), or the lower chamber (CPMbasolateral), the antibody Specific Activity is the CPM/g of protein, MW is molecular weight (g/mol), Nmol is Avogadro’s number, A is the surface area of the filter membrane (cm²), mm² also refer to this area, Vol is volume of medium in the upper chamber (mL), and t is time of incubation (s) [9].

2.9. Endocytosis of anti-ICAM-enzyme conjugates.

Control or TNFα-activated cells were incubated with anti-ICAM-HRP conjugates (separated or non-separated fractions) or IgG-HRP conjugates (75 pmol/L antibody and 150 pmol/L HRP) for 30 min at 37 °C (pulse), followed by washing and incubation at 37 °C (chase). Incubations were performed in the absence vs. presence of 3 mmol/L amiloride, to infer CAM-mediated uptake. Cells were washed, fixed, and the antibody counterpart was immunostained in red. Cells were then permeabilized and all (bound + internalized) antibody molecules were immunostained in green. In parallel, we immunodetected bound vs. internalized HRP counterpart. In both cases, the cell-surface bound fraction was double-labeled in green and red (yellow), while internalized counterparts were single-labeled in green. Samples were analyzed by fluorescence microscopy to quantify internalized fluorescence, as an absolute value or a percentage of the total cell-associated amount [25, 27].

2.10. Transcellular transport of anti-ICAM-enzyme conjugates.

Anti-ICAM-HRP conjugates (separated or non-separated fractions) vs. IgG-HRP, unconjugated HRP, or unconjugated anti-ICAM (70 pmol/L) were added to the apical Transwell® chamber above the cells and incubated at 37 °C for the indicated times. Additional controls consisted of incubating anti-ICAM-HRP conjugates in the presence of anti-ICAM competitor vs. IgG, or in the presence of 20 pmol/L EIPA to inhibit the CAM pathway. The paracellular transport was evaluated by monitoring the TEER. The apical, cell, and basolateral fractions were collected and measured for HRP activity as described above. The percent of transport was calculated from the total activity found in the cell fraction + basolateral fraction.

2.11. Statistical analysis

Data were collected as mean ± standard error of the mean (S.E.M). For each experimental condition analyzed by fluorescence microscopy, the number of independent samples was ≥ 4. For enzyme activity assays, the number of independent samples was ≥ 6. Significance was determined using the Student’s unpaired t-test, assuming a p-value of 0.05.

3. Results

3.1. Binding and endocytosis of anti-ICAM by non-endothelial cells

Endocytosis of anti-ICAM molecules has been recently demonstrated using primary endothelial cells [23]. Here, we examined if this is also the case for non-endothelial, epithelial colorectal adenocarcinoma Caco-2 cells, an established cell line. Fluorescence immunostaining showed that anti-ICAM specifically bound to and was internalized by both
Endocytosis of anti-ICAM was also confirmed in squamous carcinoma A431 cells, neuroblastoma SH-SY5Y, and primary skin fibroblasts, where the level of binding and total uptake correlated well with their respective levels of ICAM-1 expression (compare lower binding, total uptake and expression in A431 and SH-SY5Y cells vs. higher binding, total uptake and expression in fibroblasts; Supplementary Figs. 5, 6 and 7). Therefore, endocytosis of anti-ICAM molecules also occurs in non-endothelial cells.

3.2. Lysosomal trafficking of anti-ICAM in non-endothelial cells

We next examined lysosomal transport of anti-ICAM, which in endothelial cells was markedly low compared to anti-ICAM coated onto nanocarriers [25]. We observed that anti-ICAM also exhibited low colocalization with lysosomal LAMP-1 in Caco-2 cells: 14% and 27% colocalization at 1 h and 5 h (Fig. 2A, B). Since anti-ICAM degradation within lysosomes could have impaired our ability to observe colocalization, we repeated these experiments in the presence of chloroquine, a weak base that impairs lysosomal degradation. In the presence of chloroquine, lysosomal colocalization of anti-ICAM decreased to ~16% at 5 h, showing that the drug indeed impacts this compartment (Fig. 2B). Yet, total immunodetectable anti-ICAM did not vary with time (Supplementary Fig. 3), with only 11% signal decay in control cell medium and 2% decay in chloroquine by 5 h (Fig. 2C). This result confirms minor lysosomal trafficking and degradation, as for endothelial cells [25].

3.3. Transport of anti-ICAM across non-endothelial (epithelial) cell monolayers

Transport across cell barriers has been reported for multivalent anti-ICAM carriers using endothelial and epithelial models [8,9]. Hence, we examined if anti-ICAM molecules can also undergo this route. We used Caco-2 monolayers grown on Transwell® inserts after verifying their confluence by presence of tight junctions and TEER (Supplementary Fig. 8A). Immunofluorescence and radioligand tracing showed that anti-ICAM bound specifically to cell monolayers, e.g., 12-fold over IgG (Supplementary Fig. 8B, C). Specific, tight monolayer transport to the basolateral chamber under the cells was observed: e.g., 3 × 10^9 anti-ICAM molecules were transported per mm^2 of epithelium by 24 h (15-fold over IgG; Fig. 3A). This represented ~80% of all anti-ICAM initially associated to cells (cell + basolateral fractions), a 4-fold greater apparent permeability coefficient (P_{app}) vs. IgG (Supplementary Fig. 9A, B). Anti-ICAM transport was inhibited by an amiloride derivative (50–95% inhibition by 5 h; Fig. 3B) and did not cause any changes to the monolayer permeability (~100% of control TEER), nor it led to increased (albumin) protein leakage, in contrast with H_2O_2 which opens the cell junctions (Fig. 3C, D).

Fig. 1. Uptake of anti-ICAM by Caco-2 cells. (A) Anti-ICAM or control IgG were incubated for 30 min at 37 °C or 4 °C with control vs. TNFα-activated Caco-2 cells. Non-bound antibody was then removed and cells were incubated for additional 30 min to allow endocytosis of pre-bound antibody. Cells were fixed and incubated with Alexa-labeled secondary antibody, accessible only to surface-bound counterparts. Cells were then permeabilized to label total cell-surface + internalized antibody with FITC-labeled secondary antibody. Hence, cell-surface antibody appears yellow (green + red; arrowheads) vs. green internalized counterparts (arrows). Scale bar = 10 μm. Dashed lines mark the cell borders, observed by phase contrast. (B) The percentage of anti-ICAM internalization at 37 °C and different time points was expressed as the fraction of internalized antibody vs. total (surface-bound + internalized) antibody. (C) The percentage of anti-ICAM uptake (1 h, 37 °C) was assessed in absence (control; Ctr) vs. presence of amiloride (Amil), filipin (Fil), or monodansylcadaverine (MDC), and normalized to control cells. Data are Mean ± S.E.M. * Compares each time point to the preceding one, for control cells; † compares each time point to the preceding one, for TNFα-activated cells; ‡ compares control vs. TNFα at respective time points; †† compares uptake at 37 °C vs. 4 °C; ††† compares inhibitor-treated vs. control cells (p < 0.05, Student’s t-test).
3.4. Conjugation of a model enzyme to anti-ICAM

Since anti-ICAM molecules were observed to enter and cross cells, we explored if anti-ICAM could similarly mobilize a cargo. While cargo delivery into and across cells has been documented for anti-ICAM conjugates and nanocarriers displaying multiple anti-ICAM copies [12–17], it has never been shown for anti-ICAM molecules. We conjugated anti-ICAM to a model cargo, HRP, using a commercial kit for conjugation at a 1:2 antibody-to-enzyme molar ratio. We first verified the conjugate by SDS-PAGE followed by Coomassie blue staining and Western blotting (Fig. 4A). Coomassie staining revealed the expected ~150 kDa band (Lane 2) and ~40 kDa band (Lane 3) for unconjugated anti-ICAM and HRP, and a predominant ~230 kDa band for the conjugate mixture (arrow in Lane 1), expected for a conjugate carrying 2 HRP molecules per anti-ICAM molecule. Both antibody and HRP components were immunodetectable on this band (arrows in Lanes 4 and 7), similar to unconjugated anti-ICAM and unconjugated HRP (Lanes 5–6 and 8–9). Apart from this predominant band, faint bands of ~150 kDa and ~40 kDa were present in the conjugate mixture (Lane 1), positive for either anti-ICAM or HRP, but not both (Lanes 4 and 7). Hence, these are unconjugated traces. In addition, two bands above ~230 kDa (Lane 1) were positive for anti-ICAM and HRP (Lanes 4 and 7), suggesting the

Fig. 2. Lysosomal trafficking of anti-ICAM in Caco-2 cells. (A) Caco-2 cells cultured on coverslips were incubated with FITC-labeled anti-ICAM or IgG for 30 min at 37 °C (binding pulse), followed by washing and incubation at 37 °C (chase). As a control for degradation, cells were additionally incubated with chloroquine. Cells were fixed and permeabilized, and LAMP-1-positive lysosomes were immunostained with TxR-anti-LAMP-1. Antibodies that colocalize with lysosomes appear double-labeled with TxR and FITC (yellow, arrowheads), while non-colocalized antibodies are single-labeled in green FITC (arrows). Scale bar = 10 μm. Dashed lines mark the cell borders, observed by phase contrast. (B) Colocalization of green antibody with red-labeled lysosomes was calculated from micrographs. (C) Degradation of FITC-anti-ICAM was estimated by comparing total fluorescence remaining over time to cell-associated fluorescence after the first 30 min incubation. Data are Mean ± S.E.M. * Compares presence vs. absence of chloroquine; ° compares each time point to the preceding one (p < 0.05, Student’s t-test).

Fig. 3. Transepithelial transport of anti-ICAM across Caco-2 monolayers. 125I-anti-ICAM or 125I-IgG were added above Caco-2 monolayers cultured on Transwell inserts, to permit transport to the basolateral chamber. (A) Basolateral 125I was measured to calculate the amount of antibodies transported per mm² (see Methods). IgG is shown for 24 h. (B) Transepithelial transport of 125I-anti-ICAM in the absence or presence of EIPA (5 h), shown as antibody molecules transported per mm² and rate of transport (Papp). (C) TEER was measured during transport of 125I-anti-ICAM across Caco-2 cells and compared to H2O2 treatment, which causes intercellular junction opening. TEER is expressed as a percentage of the values measured for untreated, control cells. (D) Paracellular protein leakage, measured as the Papp of 125I-albumin crossing the cell monolayer in the absence or presence of H2O2 or anti-ICAM. Data are Mean ± S.E.M. * Compares anti-ICAM vs. IgG; ° compares against control cells (p < 0.05, Student’s t-test).
presence of larger conjugates. However, the Coomassie blue intensity of these bands was significantly lower, indicating that they represent a minor fraction vs. the predominant ~230 kDa conjugate.

We then used AF4 coupled to MALS, RI, and UV absorbance detectors to characterize the conjugate mixture [28]. The representation of the differential weight fraction vs. molecular weight in Fig. 4B shows that unconjugated HRP and anti-ICAM have monodisperse peaks with average molecular weights of 43 kDa and 155 kDa, respectively. The anti-ICAM-HRP conjugate mixture contained several species. A minor (~10% of the population) peak averaging 196 kDa was found, equivalent to a mixture of 1:1 antibody:enzyme conjugate and unconjugated anti-
ICAM. Unconjugated HRP was not detected. The main peak (~40% of the population) had molecular weight between 190 and 340 kDa, which corresponds to antibody-to-enzyme molar ratios of 1:1 to 1:4, all with a single anti-ICAM molecule. The average molecular weight of this population was 233 kDa, corresponding to a 1:2 antibody:enzyme conjugate, as seen by SDS-PAGE. Another ~40% peak with average molecular weight of 686 kDa was present, equivalent to large conjugates and/or aggregates. Since this population is likely to contain species bearing more than one antibody molecule (similar to multivalent anti-ICAM carriers and opposite to the 233 kDa population), we conducted experiments comparing the AF4-separated 233 kDa vs. 686 kDa fractions vs. the unseparated conjugate mixture.

3.5. Specific binding and uptake of active anti-ICAM-enzyme conjugates by cells

We validated that anti-ICAM-HRP conjugates bind specifically to cells through the antibody counterpart, rendering HRP-specific activity. Using fixed Caco-2 cells, we tested the conjugate mixture since uptake was not to be traced yet. Both the anti-ICAM and HRP counterparts of the conjugate were visibly bound to cells: 5.8 × 10⁷ and 9.5 × 10⁷ fluorescence A.U., respectively (Fig. 5A, B) with >95% signal colocalization (Supplementary Fig. 10). This was comparable to binding of anti-ICAM (1.3-fold the level of anti-ICAM-HRP), and specific over HRP (<10% the level of anti-ICAM-HRP) and control IgG-HRP conjugates (<1% the level of anti-ICAM-HRP; Fig. 5A, B). Unconjugated anti-ICAM did not display an HRP signal and unconjugated HRP did not display anti-ICAM signal (<10% compared to anti-ICAM-HRP; Fig. 5A, B), resulting in no colocalization (<2%; Supplementary Fig. 10). Binding of anti-ICAM-HRP conjugates to cells was reduced by the presence of competing anti-ICAM molecules in the cell media (47% decreased binding) but not IgG (3% decreased binding; Fig. 5B and Supplementary Fig. 11). Further validating specificity (Supplementary Fig. 12A), binding of anti-ICAM-HRP conjugates was almost undetectable in non-activated vs. high in TNFα-activated endothelial cells (67-fold greater in the latter), as expected due to a reported 50–100 fold greater ICAM-1 expression in TNFα-activated vs. non-activated cells [29].

In addition, anti-ICAM-HRP rendered measurable cell-bound HRP activity: 127 pmol/L by 1 h, as opposed to IgG-HRP or HRP that had 1% and 3% the activity of anti-ICAM-HRP given their lack of binding (Fig. 5C). Although anti-ICAM molecules bound to cells, they did not render HRP activity (7% the activity of anti-ICAM-HRP), as expected. Anti-ICAM competitor diminished the cell-bound activity of anti-ICAM-HRP conjugates (42% of control), in contrast to IgG (95% of control). Hence, anti-ICAM-HRP conjugates bind specifically to ICAM-1 on cells while preserving the activity of the enzyme cargo.

Fig. 6. Uptake and activity of anti-ICAM-HRP conjugates in Caco-2 cells. The anti-ICAM-HRP conjugate mixture was separated by AF4 into a form corresponding to 1:2 antibody-to-enzyme molar ratio (233 kDa) and a form representing larger multimolecular or aggregated conjugates (686 kDa). (A) Caco-2 cells were incubated with the conjugate mixture vs. each conjugate fraction for a 30 min, washed, and incubated with medium for up to 1 h to track uptake. Similar to unconjugated antibodies (Fig. 1), either surface-bound Anti-ICAM or HRP were immunostained to fluoresce in yellow (arrowheads), whereas internalized anti-ICAM or HRP were immunostained to fluoresce in green alone (arrows). Scale bar = 10 μm. Dashed lines mark the cell borders, observed by phase contrast. The percentage of internalization was obtained as in Fig. 1, and normalized to that of the conjugated mixture used as a control (Ctr). (B) Incubations were in the absence or presence of permeabilization. Non-bound conjugates were removed and HRP substrate was added to measure HRP activity ( pmol/L HRP) in permeabilized cells (total internalized and cell surface-bound conjugate) vs. non-permeabilized cells (surface-bound fraction). The difference between these two measurements is internalized activity. (C) The percentage of HRP activity internalized with respect to the total cell-associated activity was calculated in cells treated or not with amiloride. Data are Mean ± S.E.M. * Compares each conjugate fraction against the control mixture; †compares unconjugated counterparts to anti-ICAM-HRP conjugate; ‡compares permeabilized to non-permeabilized cells; †compares amiloride vs. control; (p < 0.05, Student’s t-test).
amiloride to control (Fig. 6A). The 233 kDa conjugate was internalized to an equivalent degree as unseparated conjugates (100% of the uptake observed for the mixture when tracing anti-ICAM and 91% when tracing HRP). Uptake of the 686 kDa conjugate was slightly lower (87% and 69% compared to the unseparated mixture when tracing anti-ICAM or HRP), expected due to its larger size. HRP activity was also measured, without cell permeabilization (to measure cell-surface bound activity) vs. with permeabilization (to measure surface-bound + internalized HRP), so that the difference renders the internalized enzyme activity. As expected, total anti-ICAM–HRP conjugate activity measured after permeabilization was 3.7-fold greater than in the absence of permeabilization (surface-activity); hence, 68% of the total HRP activity provided by anti-ICAM–HRP was located within cells (Fig. 6B, C). In contrast, minimal enzyme activity was detected in cells incubated with unconjugated HRP or anti-ICAM molecules, whether or not the cells were permeabilized (e.g., 10–30-fold lower than for anti-ICAM–HRP; Fig. 6B). Looking at the internalized activity alone (permeabilized — non-permeabilized cells), this was ~9-fold greater for cells incubated with anti-ICAM conjugates vs. unconjugated HRP or anti-ICAM (Supplementary Fig. 13). This demonstrates that internalized enzyme activity is specifically delivered by anti-ICAM conjugates. In fact, inhibiting CAM endocytosis using amiloride resulted in a marked reduction in the internalized HRP activity of the conjugate (25% of control; Fig. 6C).

3.6. Specific transport of active anti-ICAM–enzyme conjugates across cell monolayers

Finally, we examined whether anti-ICAM molecules can transport an active cargo across cell monolayers. By measuring the HRP activity of the basolateral fraction, we found that the 233 kDa conjugate, corresponding to species that display a single antibody, delivered 10-fold greater HRP than the 686 kDa conjugate and 2.6-fold greater than the unseparated mixture (24 h; Fig. 7A). The same was true for the rate of transport, Papp, which was 11-fold and 2.4-fold greater for 233 kDa conjugates relative to 686 kDa and unseparated conjugates (Fig. 7B).

Amiloride-derivative lowered the HRP activity at the basolateral chamber by 60%, and the corresponding Papp by 92% (Fig. 7C). No changes were observed in the permeability barrier during transport of anti-ICAM–HRP conjugates (99% of control TEER; Fig. 7C), suggesting that transport occurred via the vesicular CAM pathway, not paracellular leakage.

4. Discussion

Previous studies exploiting endocytosis or transcytosis of therapeutic by targeting ICAM-1 had used nanocarriers or conjugates coupled to multiple copies of anti-ICAM targeting moieties, as multivalent binding was thought necessary to induce transport [8,9,17,19–27]. This work demonstrates for the first time that non-multivalent binding to ICAM-1 can also be used to carry active cargoes into and across cells.

First, we verified specific endocytosis of (non-multivalent) anti-ICAM molecules in a variety of cells, including two primary cell types (fibroblasts and endothelial cells [25]) and three established cell lines (A431, Caco-2, SH-SY5). These cells belong to different tissues (epithelial, connective, and neural), display normal vs. disease (cancer) phenotypes, and were activated with TNFx or non-activated to mimic inflammation vs. control conditions. Therefore, endocytic uptake of anti-ICAM seems to be a universal phenomenon among cells that express this marker, as previously observed for endocytosis of multivalent anti-ICAM carriers [8,9,12,17,26]. Such common uptake of (non-multivalent) anti-ICAM was not expected, because the natural ligands of ICAM-1 bind to cells in a multivalent manner: in nature ICAM-1 is bound by leukocyte $\beta_2$ integrins, whereby each leukocyte expresses...
multiple copies of these molecules on their surface [11]. Since receptors have evolved signaling mechanisms specifically triggered by their natural ligands, varying the binding valency was expected to change the response, as shown for receptors of immunoglobulins, transferrin, and folate [24–30]. However, this aspect may be less important in the case of ICAM-1, as this molecule has been shown to exist on the cell-surface as a monomer, dimer, tetramer, and larger-order oligomers [11]. If (non-multivalent) anti-ICAM binds to oligomeric ICAM-1, this may still be “sensed” by cells as a multimeric interaction as the receptor is already oligomerized. Nevertheless, this remains to be investigated.

In addition, lysosomal trafficking and degradation by anti-ICAM molecules was minimal in Caco-2 cells, in agreement with previous findings for anti-ICAM molecules in endothelial cells [25]. This is in contrast to multivalent anti-ICAM carriers, which had been shown to efficiently reach lysosomes (~80% of the internalized fraction by 5 h [19, 25]). Whether this differential lysosomal trafficking of non-multivalent anti-ICAM vs. multivalent anti-ICAM carriers depends on their relative affinity toward the receptor remains to be determined (e.g., their affinity has been reported to be in the nmol/L vs. the pmol/L level respectively [29]). In any instance, this property of multivalent anti-ICAM carriers offers an advantage for treatments necessitating lysosomal delivery, such as recombinant enzymes used to treat lysosomal storage disorders [19,20,24], while direct drug coupling to anti-ICAM molecules may offer an alternative destination for treatment of other maladies. In this regard, the final destination of anti-ICAM molecules may depend on the biology of the cell type involved. For instance, cell receptors involved in the transport of ligands across cellular barriers possess properly polarized surfaces (apical vs. basolateral) and specific machinery to distinguish both interfaces and enable transcytosis [35]. However, transcytosis is not biologically necessary for tissue cells that do not form a part of these barriers, nor do they have apical vs. abluminal surfaces, and only intracellular routing or recycling is possible in these cells [35]. In agreement with this, we have observed recycling of anti-ICAM molecules when cells were plated on glass coverslips, where transcytosis is not possible due to the lack of a basolateral space [25]. Instead, this work shows that anti-ICAM molecules are transcytosed across cells that have both apical and basolateral interfaces. Thus, it is expected that this strategy can afford transcellular delivery of therapeutics across cellular barriers vs. endocytosis within tissue cells. This was demonstrated in the present study for model (HRP) enzyme conjugates using Transwell® vs. coverslip models.

Dual trafficking (into and across cells) has been also observed for multivalent anti-ICAM carriers [8,9,12,19]. In both cases (anti-ICAM molecules or anti-ICAM carriers) Transwell® experiments indicate that a fraction of internalized materials traffics across the cell barrier while another fraction remains associated with the cells [8,9,12,19]. The mechanisms that regulate this are currently unknown and will be the focus of our future studies. However, this phenomenon is in agreement with current knowledge about dual transport of natural ligands across cellular linings, such as transferrin, insulin, LDL, etc. [2,3,35,36]. Interestingly, both anti-ICAM molecules and anti-ICAM carriers endured transcytosis albeit different degrees of lysosomal trafficking [25]. This may be due to the different models used to study lysosomal vs. transcytosis transport (coverslips vs. Transwells®) and/or may indicate that transcytosis is a predominant route for the CAM pathway, where lysosomal transport only occurs when transcytosis is not possible (in coverslips).

In conclusion, ICAM-1 targeting may offer interesting delivery versatility, depending on the modality of drug coupling pursued. In this work, direct conjugation of a model enzyme (HRP) to anti-ICAM rendered species bearing a single antibody (233 kDa conjugate) vs. larger species (686 kDa conjugate), both of which were internalized and transcytosed by cells. Yet, transcytosis seemed more efficient for the former species, in agreement with the fact that non-multivalent vs. multivalent anti-ICAM differ in terms of their intracellular retention [25]. Importantly, this strategy rendered significant enzyme activity within cells and transported across cells, illustrating its potential in the context of therapeutic delivery.

5. Conclusion

Whereas previous studies on active ICAM-1 targeting had focused on subcellular transport of multivalent anti-ICAM carriers or highly multimeric protein conjugates [18–27], this study focused on anti-ICAM molecules, demonstrating that: (1) induction of CAM-mediated endocytosis by anti-ICAM is a common phenomenon among ICAM-1-expressing cells, regardless of origin and disease state, (2) uptake of anti-ICAM does not lead to lysosomal transport but to transcytosis across cellular barriers, and (3) coupling anti-ICAM to cargo molecules in a manner that renders non-multivalent conjugate species provides intra- and trans-cellular delivery of active cargo. Therefore, non-multivalent ICAM-1 targeting serves as an alternative to multivalent forms previously investigated for therapeutic applications, providing transport of active cargoes into and across cells.

Disclosure

The authors report no conflicts of interest in this work.

Acknowledgments

This work was supported by a National Science Foundation Graduate Research Fellowship to R.G. (DGE-0750616), and funds awarded to S.M. by the National Institutes of Health (grant R01-HL098416) and the National Science Foundation (CBET-1402756). We thank Dr. Jerrold Turner (Department of Biological Sciences, University of Chicago, IL) for kindly donating Caco-2 cells for this study, and Dr. Edward Schuchman (Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine, NY) for generously sharing wild-type skin fibroblasts.

Abbreviations

AF4 asymmetric flow field-flow fractionation
Amil amilodipine
BSA bovine serum albumin
Caco-2 human colorectal adenocarcinoma cells
Fil filipin
FITC fluorescein isothiocyanate
ICAM-1 intercellular adhesion molecule-1
HRP horseradish peroxidase
HUNVEC human umbilical vein endothelial cells
IgG immunoglobulin G
MALS multi-angle light scattering
MDC monodansylcadaverine
PBS phosphate buffered saline
QELS quasi-elastic light scattering
RI refractive index
RI Texas red
TNFα tumor necrosis factor alpha.
AF4 asymmetric flow field-flow fractionation

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2016.07.042.

References
