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Differential effect of cross-linking the CD98 heavy chain on fusion and amino acid transport in the human placental trophoblast (BeWo) cell line

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Abstract

CD98 (otherwise known as 4F2) is an integral membrane protein with multiple functions including amino acid transport, integrin activation, cell fusion and cell activation. The molecular mechanisms coordinating these multiple functions remain unclear. We have studied CD98 heavy chain (hc) function in a human placental trophoblast cell line (BeWo). We show that cross-linking of CD98hc by incubation of cells in the presence of functional monoclonal antibodies causes cellular re-distribution of the protein from the cytoplasm to the plasma membrane as measured by flow cytometry, western blotting and quantitative immuno-electron microscopy. The latter technique also indicated that CD98hc is trafficked between cell surface and cytoplasmic pools in vesicles. Increased cell surface CD98 correlates with increased cellular fusion in BeWo cells. In addition, we show reduced LAT 1 surface expression and neutral amino acid transport in the presence of the CD98 mabs. The results thus suggest that the function of CD98 in cell fusion is distinct from its role in cellular nutrient delivery.

Keywords: CD98; LAT 1; Cell fusion; Amino acid transport

1. Introduction

Syncytialized trophoblast forms during implantation and is then maintained at the villous maternal–fetal interface throughout pregnancy. Understanding the mechanism that leads to formation, maintenance and turnover of the syncytiotrophoblast is clearly important for understanding both normal and abnormal pregnancy (including pre-eclampsia). However, cell fusion is a very rare event in cell biology and in humans is typically observed only in the placenta (syncytiotrophoblast), in skeletal muscle (striated muscle fibre) and in bone formation (osteoclast) as well as during the fusion of gametes at fertilization. It is also recognised pathologically as a mechanism induced or required by some viruses to engage with host cells.

CD98 is a multifunctional transmembrane protein originally found on the surface of activated T cells [1] but now known to be present on virtually all cells, being expressed at particularly high levels in actively proliferating tissue. Recent reports describe the possible role of CD98 in the regulation of cell differentiation, adhesion, growth, apoptosis and cancer development [2–5]. Often these diverse cellular functions require activation of integrin molecules but there is little information about the exact mechanisms underlying them and more generally of CD98 intracellular pathways [6–8].

The CD98 antigen forms a dimeric structure consisting of a type 2 glycosylated integral membrane protein of around 80 kDa (heavy chain) and one of at least six proteins with apparent molecular mass of 40 kDa (light chain) linked by disulfide-bond. Depending on which light chain is part of the heterodimer many amino acids, including several essential amino acids, are transported across the plasma membrane in a Na+-independent (system L, neutral amino acids transport) and Na+-dependent (system y+L, neutral and cationic amino acid transport) manner. The heavy chain is ubiquitous but variously glycosylated according to the tissue of origin while the non-glycosylated light chains are expressed differentially according to tissue origin [9,10]. Transfection studies in mammalian cells have indicated that while CD98hc can be expressed on the plasma membrane on its own, trafficking of the light chain to

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the cell surface is possible only in the heterodimer form and apparently independently of disulfide linkage [7,11].

CD98 has been found to be identical to fusion regulatory protein-1 (FRP-1) and its expression is necessary for virus-induced cell fusion [12] and for osteoclast formation [13,14], it is therefore of particular interest that CD98 is expressed on the cytotrophoblasts and on the plasma membrane of the syncytiotrophoblast, the site of exchange of nutrients, lipids and minerals between the mother and the fetus, which results from the fusion of the underlying cytotrophoblast cells [15,16].

While some antibodies against CD98/FRP-1 are known to suppress virus-induced cell fusion and CD98-mediated cell fusion of monocytes [13,17], others cross-linking CD98 stimulated cell aggregation and growth [3,4,18] indicating that CD98/FRP-1 molecules are able to regulate cell fusion.

Forskolin, a cell permeable diterpenoid isolated from Coleus forskohlii, stimulates adenylate cyclase activity to increase intracellular cyclic adenosine monophosphate (cAMP) thus activating cAMP-dependent protein kinase and other cAMP receptor proteins. BeWo cells, a well established choriocarcinoma cell line, can undergo fusion and morphological differentiation similar to the formation of syncytiotrophoblast by the cytotrophoblast in the placenta. Forskolin is known to increase this cellular fusion in BeWo cells, and in other fusogenic cell lines [19–22].

Our laboratory has previously shown that manipulation of CD98 cellular expression by antisense oligonucleotides [20] has an effect on amino acid transport and on cellular fusion in BeWo cells; in this paper we show that two monoclonal antibodies against CD98 (mab4F2 and mabAHN18) can affect its surface expression, cell differentiation and amino acid transport.

2. Materials and methods

2.1. Primary antibodies

4F2 was a generous gift from Dr. T. Sethi; AHN18 was from Chemicon International. Goat anti-human CD98 (C-20), rabbit anti-human CD98 (H-300), normal goat IgG and normal rabbit IgG (isotype-matched controls) were from Santa Cruz Biotechnology Inc. Rabbit anti-rat LAT 1 (AHP735) and mouse IgG_1 were from Serotec; 98C01 was from Stratech Scientific Ltd. and mouse IgG_{2a} was from Dako.

2.2. Secondary antibodies

Goat anti-rabbit IgG horseradish peroxidase-conjugated was from Dako. Donkey anti-goat IgG fluorescein isothiocyanate-conjugated was from Jackson immuno research laboratories and rabbit anti-goat IgG r-phycoerythrin-conjugated was from Sigma. Protein A–15 nm gold complex was from British Biocell.

2.3. Cell culture

BeWo cells were cultured at 37 °C as monolayers in F-12K Nutrient Mixture (Kaighn's modification) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine (all GIBCO), 100 U ml^{-1} penicillin and 100 U ml^{-1} streptomycin (Sigma) in an humidified atmosphere of 5% CO_2 and 95% air. Confluent cells were harvested by trypsinisation with trypsin-EDTA in HBSS w/o Ca^2 and Mg^2 (GIBCO), viable cells counted by the trypan blue (Sigma) method, resuspended in serum-free medium and stained with either with 10 μl of vibrant DiO cell labelling solution (1 mM, Molecular probes) per 10^6 /ml cells for 30 min at 37 °C or with MitoTracker Deep Red 633 (Molecular probes) at a concentration of 25 nM per 10^6 /ml cells for 15 min 37 °C in the dark shaking gently. After extensive washing with warm serum-free medium each group of cells was resuspended in complete growth medium and plated either on their own or in a 50% mixture in six well culture plates (BD Falcon). When the cells reached 65–70% confluence forskolin (Sigma) or vehicle (dimethyl sulfoxide, DMSO) was added in fresh medium at a final concentration of 100 μM for 24 h, unless otherwise indicated. In some wells, after 5 h of culture in forskolin or DMSO, 4F2 or AHN18 monoclonal antibody or the respective isotype matched control (mouse IgG_{2a} or IgG_1) was added at a final concentration of 20 μg/ml (19 h).

2.4. Preparation of cell fractions, SDS-PAGE and Western Blotting (WB)

Confluent cultures from six well plates were washed with ice-cold PBS and then lysed by manual agitation at 4 °C in ice-cold buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 10 μl protease inhibitor mixture, (Sigma). We used classical cell fractionation [23,24] to explore the distribution of CD98hc. Thus the lysed cell homogenate (H) was centrifuged at low speed (800×*g*) for 15 min at 4 °C. Pellet (P1) was suspended in 200 μl buffer containing 50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA and 10 μl protease inhibitor mixture for 15 min on a rocker and then sonicated three times for 30 s. Using the same buffer, supernatant (S1) was sonicated three times for 30 s (200 μl buffer) and clarified by centrifugation at high speed (17000×*g*) for 15 min at 4 °C, allowing us to collect supernatant S2. WB was then carried out on P1, containing crude plasma membrane and heavy organelles, and S2, containing purified membranes including small intracellular vesicles. For WB, precisely 10 μg of protein was solubilised in NuPAGE sample buffer with reducing agent (Invitrogen), warmed for 10 min at 75 °C and then run on 4–12% Novex Bis-Tris NuPAGE gels with MOPS running buffer (Invitrogen). The proteins were transferred to nitrocellulose membranes, blocked using 5% (w/v) non fat dry milk in 0.01 M PBS (Sigma) with 0.05% (v/v) Tween 20 for 1 h at room temperature and then incubated with rabbit anti human CD98 (H-300, 1:200) overnight at 4 °C. Goat anti rabbit horseradish peroxidase-conjugated antibody was used for secondary labelling. Immunoreactive bands were identified by SIGMA*FAST* 3,3′-Diaminobenzidine tablets (Sigma) according to the manufacturer's instructions. SeeBlue Plus2 pre-stained protein standards were from Invitrogen. As a negative control we used a mouse M1 cell homogenate (10 μg of protein).

2.5. Flow cytometry: surface staining on intact cells

Cells from six wells plates were detached with trypsin-EDTA (GIBCO). Aliquots of 1×10^6 cells were washed in PBS and re-suspended in 250 μl of FACS buffer (PBS, 1% fetal calf serum, 0.1% NaN_3) with goat anti-human CD98 (C-20, 1:20) or isotype control IgG or no primary antibody. Cells were incubated for 45 min on ice, followed by three washes with FACS buffer. Samples were then incubated with donkey anti-goat IgG fluorescein isothiocyanate-conjugated (1:50) or rabbit anti-goat IgG r-phycoerythrin-conjugated secondary antibody (1:20) for 45 min on ice and washed three times. Samples were finally resuspended in FACS buffer and 2% paraformaldehyde (PFA) and analyzed by flow cytometry using FACSCalibur (BD Biosciences) and Cell Quest software and/or EPICS Altra (Beckman Coulter) and EXPO32 software.

2.6. Flow cytometry: surface and intracellular staining

Cells suspensions were fixed in 2% PFA for 20 min at room temperature, washed once in PBS, permeabilised with 1% saponin in FACS buffer for 15 min at room temperature and then stained following the surface staining protocol. After the final wash samples were fixed again in 2% PFA before analysis.

2.7. Immunogold electron microscopy

Cells were prepared for electron microscopy by standard methods [25]. Briefly, cell pellets were post-fixed in osmium tetroxide (1% w/v in 0.1 M sodium phosphate buffer), contrasted with uranyl acetate (2% w/v in distilled water), dehydrated through increasing concentrations of ethanol (70–100%) and embedded in LR Gold resin (Agar Scientific UK). Ultra-thin sections (50–80 nm) were prepared by use of a Reichert Ultracut S microtome and mounted on 200-mesh

nickel grids. For immunogold detection of CD98 and LAT 1 sections were incubated with either goat anti-human CD98 (1:100) or rabbit anti-rat LAT 1 (1:50) polyclonal antibody for 2 h and for 1 h with Protein A–15 nm gold complex. For control sections, the primary antibody was omitted and replaced with a matching dilution of the respective non-immune serum. Sections were then lightly counterstained with uranyl acetate and lead citrate. All antibodies were diluted in 0.1 M phosphate buffer containing 1% w/v egg albumin. The sections were viewed with a JEOL 1010 transmission electron microscope (JEOL, Peabody, MA, USA) and representative micrographs were prepared.

The area of each cellular compartment of interest was determined by point counting morphometry and the number of gold particles over each compartment was counted.

2.8. Amino acid influx

After aspirating culture medium, each well was washed with pre-warmed (37 °C) PBS and cells were depleted of intracellular amino acids by incubating in PBS at 37 °C for 30 min to minimize any transtimulation effects. The influx of amino acid was initiated by replacing the PBS with pre-warmed Na^+-free PBS containing 2 μM L-^{3}H-leucine, followed by 3 min incubation at 37 °C. Na^+-free PBS was prepared by replacing NaCl, $NaHCO_3$ and NaH_2PO_4 with choline chloride, choline bicarbonate and KH_2PO_4, respectively. Other additions are described in the figure legends. All uptake studies were conducted under initial rate conditions. Following aspiration of isotope solution, cells were quickly washed with ice-cold PBS containing 10 mM unlabelled L-leucine. Then 0.1 M NaOH and 0.1% sodium dodecyl sulphate solution were added for solubilisation and aliquots were taken for liquid scintillation counting and protein determination.

2.9. Statistical analysis

Results are presented as means±S.E. Significance of the differences between means was assessed using two-tailed Student's t test. Values of $p<0.05$ were considered significant.

3. Results

3.1. Surface expression of CD98 increases significantly in cells cultured in the presence of CD98 mabs 4F2 and AHN18

BeWo cells represent a useful tool for investigating the molecular mechanism of syncytialisation in relation to the expression of CD98 and its regulation.

We sought to correlate CD98 expression in BeWo cells with fusogenicity; previously Kudo et al. [26] had observed a time-dependent increase in CD98 mRNA in BeWo cells following forskolin treatment. We determined levels of expression of CD98 in BeWo cells cultured with/without forskolin by single colour flow cytometry (FACS) and found a significant increase of CD98 expression on intact BeWo cells after 24 h (Fig. 1A).

Monoclonal antibodies against different epitopes of CD98 have been used by several groups to either promote or inhibit function of CD98 [3,4,13,14,17,18,27–31]. To date there is no report of investigations on the effect of CD98 monoclonal antibodies on BeWo cells either in relation to fusion or amino acid transport. We cultured BeWo cells in the presence/absence of monoclonal antibody 4F2 (IgG_2), AHN18 (IgG_1) and 98CO1 (IgM). While the latter (data not shown) or the isotype matched negative controls did not have any effect in our system (Fig. 1A), the two other monoclonal antibodies significantly increased CD98 expression at the surface of intact cells both in control and in forskolin treated cells (Fig. 1B, pooled data). Since in permeabilized cells there was no significant change in the total expression (surface plus cytoplasm) of CD98 (Fig. 1C, pooled data), this indicated that over the time course of these experiments, culture of cells with the cross-linking antibodies caused a shift in the relative cellular distribution of CD98 rather than being the result of increased synthesis of CD98 protein.

3.2. Relocation of CD98 molecules in response to cross linking with the CD98 mabs 4F2 and AHN18

Further studies were carried out on BeWo cells cultured for 24–26 h in medium containing DMSO (control cells) or forskolin with/without 4F2 or AHN18 monoclonal antibodies (19 h). For preliminary investigation we used western blotting (WB). Fig. 2A shows the consistency of protein loading for each of the conditions used. Immunoblots of cell fractions showed in the presence of forskolin a redistribution of CD98hc following mab4F2 incubation (Fig. 2B). The quantitation by densitometry of these results is shown in supplementary file Fig. S1.

We therefore used immuno-electron microscopy to analyse the cellular localisation of CD98 molecules. Although CD98 molecules were mostly individually distributed throughout the cells, we noted pockets of clustering which were particularly frequent either in forskolin (Fig. 3A) or in forskolin plus monoclonal antibody-treated cells (Fig. 3B, C). Especially interesting were some areas of almost circular clustering which resembled vesicle assembly. However, since fixation procedures, necessary for optimal antigen detection (for both CD98 and LAT 1, see Materials and methods) at the EM level are sub-optimal for cellular morphology, at this stage we are unable to prove vesicle involvement.

When the number of gold particles were determined with respect to their cellular distribution in different cellular compartments (membrane, cytoplasm, and nucleus) we observed a marked increase of immunolabelling by gold particles in the plasma membrane in BeWo cells previously cultured in the presence of cross-linking antibody (Fig. 3D). This is in agreement with the results obtained by both flow cytometry and WB. However, the analysis of total CD98 immunoreactivity per unit area also showed a two fold increase in BeWo cells cultured in the presence of cross-linking antibody compared to cells treated with forskolin only (data shown in supplementary file Fig. S2). This is in apparent contrast with the total modest increased observed with FACS (surface and intracellular staining) but probably is due to both the different number of cells examined with the two techniques (8 and 10,000) and the inclusion of nuclear staining in the EM total. An alternative explanation for the discrepancy relates to different accessibility of CD98 epitopes with the different methods used.

3.3. Quantitation of cellular fusion

We used the Vibrant DiO cell-labelling solution (Fig. 4A), a lipophilic tracer weakly fluorescent in water but highly fluorescent and quite photostable when incorporated into membranes, and Mitotracker deep red 633 (Fig. 4B), a cell-permeant mitochondria selective dye, to uniformly label suspended BeWo cells. Flow cytometry of a 50:50 mixed cell

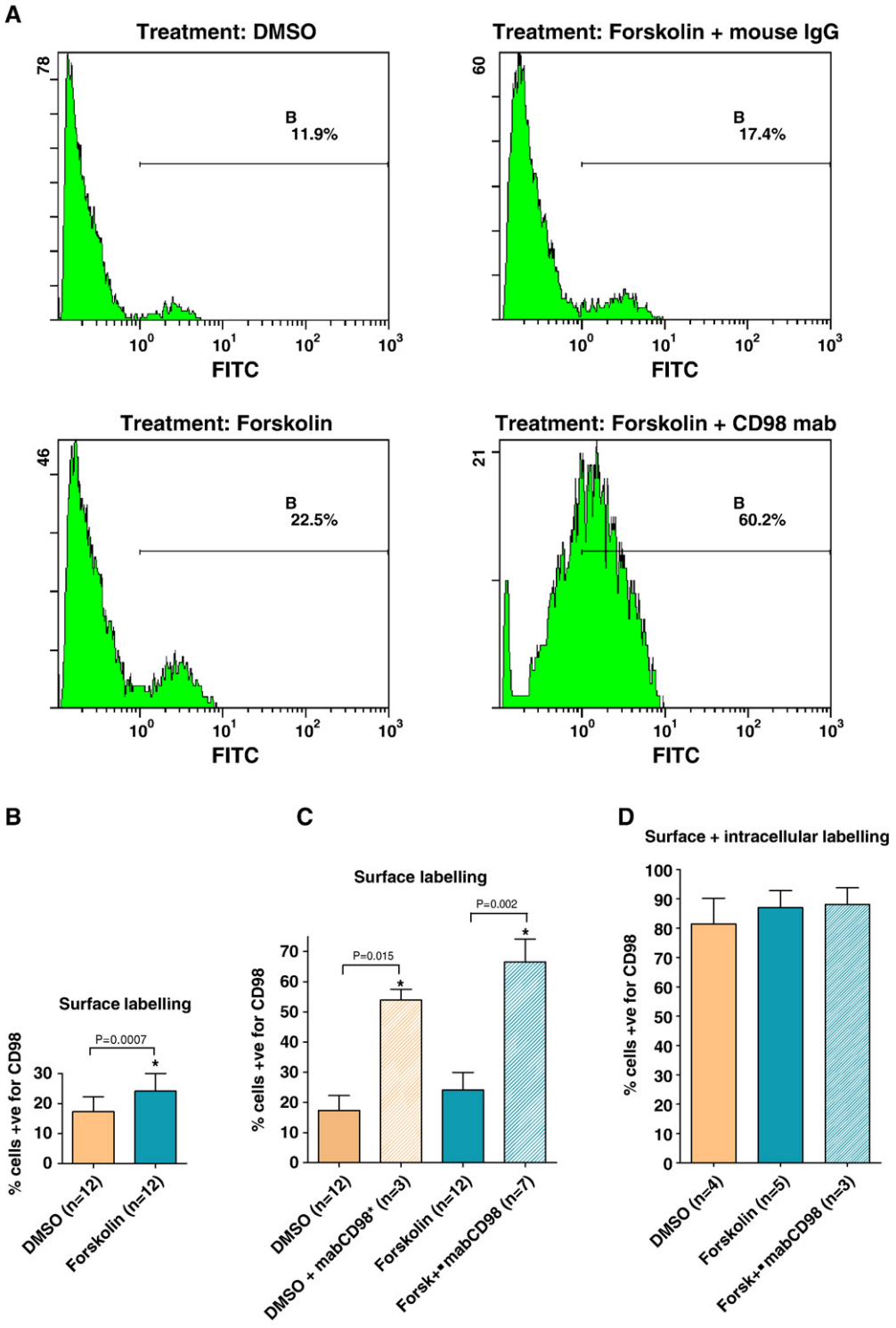

Fig. 1. Single colour Flow cytometry for CD98. BeWo cells were cultured for 24 h in medium containing DMSO, Forskolin or Forskolin plus mab 4F2▪ or mab AHN18▪ or isotype-matched negative control mouse IgG (19 h incubation) as described in Material and methods. After treatment, cells were labelled with goat anti-human CD98 and rabbit anti-goat IgG conjugated with FITC or PE. (A) Histograms (flow cytometry) showing the percentage of cells positive for CD98 surface expression in the different samples; notice that in the forskolin plus mab CD98 sample 60.2% of cells are positive compared with only 17.4% in its isotype-matched IgG control. Single representative experiment. (B,C) Labelling of surface antigens on intact BeWo cells; (D) labelling of surface and intracellular antigens after cell permeabilization. ▪Pooled results. Statistical analysis: two tailed paired *t* test; *n* = number of experiments, each experiment performed in duplicate or triplicate ± SEM.

population from cells stained either with DiO or Mitotracker red and then cultured together allowed us to quantify cellular fusion/stable aggregation represented by two positive cells (Figs. 4C, 5). Two colour flow cytometry showed a statistically significant increase of cellular fusion after forskolin treatment of BeWo cells ($p = 0.0013$, two tailed paired t test) which was clearly enhanced, although not statistically significant, when the cells were also cultured with CD98 mabs (Fig. 5A).

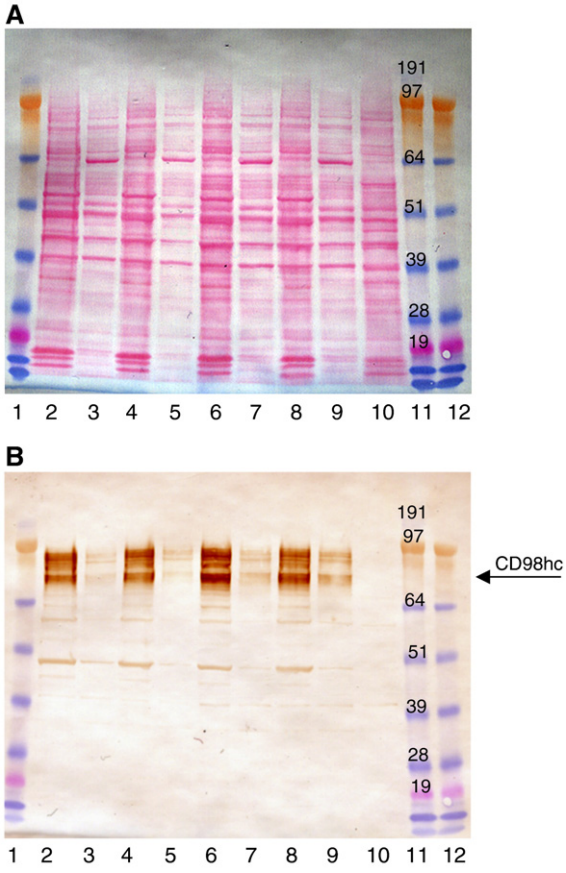

Fig. 2. Analysis of BeWo cell fractions. (A) SDS-PAGE, whole membrane after transfer and staining with Ponceau Red. Lanes 1, 11 and 12 are protein standards; lane 10 is a negative control cell line (M1). Lanes 2 and 3 are respectively P1 and S2 from DMSO treated cells; lanes 4 and 5 are P1 and S2 from DMSO plus mab 4F2 treated cells; lanes 6 and 7 are P1 and S2 from forskolin treated cells; lanes 8 and 9 are P1 and S2 from forskolin plus mab4F2 treated cells (see Materials and methods). Note the consistency of protein loading (10 μg). (B) Immunoblot of data shown in (A) after incubation with rabbit antihuman CD98 (Santa Cruz, 1:200), anti-rabbit-HRP and DAB. Arrow indicates the 80 kDa band corresponding to CD98hc. Note the absence of CD98 immunoreactivity in the negative control (lane 10).

3.4. CD98 and cellular fusion

We then employed a three colour flow cytometry protocol by indirectly labelling the goat polyclonal anti human CD98 we routinely use to detect expression of CD98 molecules with a secondary antibody conjugated with PE (phycoerythrin). Although partial spectral overlap between DiO and PE required careful selection of appropriate compensation settings we were able to show a parallel increase of CD98 expression and cellular fusion on intact cells (Fig. 5B, C).

3.5. Anti-CD98 monoclonal antibodies affect LAT 1 surface expression

CD98 has been shown to form heterodimers with several light chains, each dimer considered the minimal functional unit for transport activity. The heterodimer formed by CD98 and LAT 1 localizes at the cell surface and mediates the Na^+-independent transport of large neutral amino acids (L-type amino acid transporter 1) [9]. It is generally assumed that the heavy chain is mainly involved in the trafficking of the complex to the plasma membrane, whereas the transport process itself is catalysed by the light chain. We wondered if cross-linking of CD98 with the monoclonal antibody could, by influencing LAT 1 trafficking to the cell surface, alter amino acid transport into the cells. Fig. 6 shows the distribution of LAT 1 molecules in the forskolin treated (Fig. 6A) and in the forskolin plus monoclonal anti-CD98 treated (Fig. 6B) cell samples. Surprisingly, when we counted the gold particles we observed a clear decrease of LAT 1 membrane expression (Fig. 6C) (total LAT 1 immunoreactivity for cell area was also decreased following exposure to the monoclonal anti-CD98, data shown in supplementary file Fig. S3).

3.6. Cross-linking CD98 affects system L-amino acid transport

LAT 1, the light chain of system L, transports medium-size and large neutral amino acids and does not show Na^+ dependence. Since this transporter shows preference for neutral non-polar amino acids such as L-leucine, we measured the influx of 2 μM L-leucine in a Na^+ free buffer into control and forskolin treated BeWo cells, the latter cultured in the absence/presence of CD98 monoclonal antibodies. In order to separate the transport pathways contributing to total Na^+-independent flux, the synthetic amino acid 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) was added at a concentration of 10 mM. The BCH-inhibitable component (i.e. system L-mediated influx under the conditions used) was significantly reduced following forskolin plus mab CD98 treatment (Fig. 6D). Exposure to monoclonal antiCD98 significantly inhibits both expression (Fig. 6C) and function (Fig. 6D); quantitatively the inhibition of surface immunoreactivity is greater. Possible explanations include (a) the inclusion of non-functional light chains in the immune-EM assay; (b) the participation of other LAT isoforms (e.g. LAT2).

4. Discussion

In this study we consider the functional relationship between CD98 expression and fusion and amino acid transport by cross-linking CD98 with monoclonal antibodies in a human trophoblast cell line.

Since the early 1990's monoclonal antibodies capable of either enhancing or inhibiting virus induced fusion and entry in host cells have been isolated. We are interested in investigating the involvement of CD98 in trophoblast fusion, and for this reason examined the effects of CD98 monoclonal antibodies in the absence/presence of forskolin on BeWo cell fusion.

We used the monoclonal antibodies 4F2 and AHN-18 both previously shown to have widespread effects in different systems [18,32–34]. A third antibody, 981CO (or IPO-T10) did not have any effect in our system and data are not included.

In cytotrophoblast primary cultures in vitro, the cellular processes involved in syncytium formation are associated with a concomitant increase in intracellular cAMP [35]. Previously

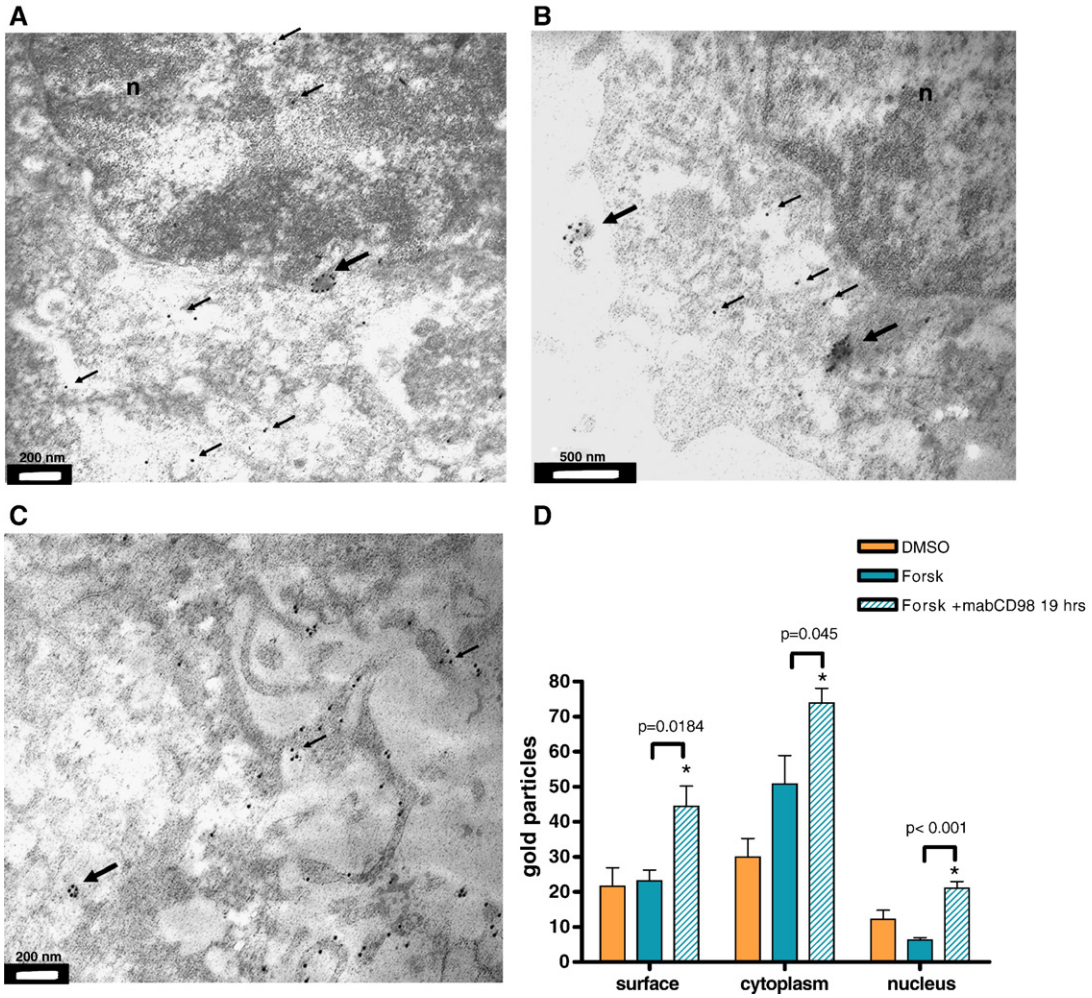

Fig. 3. Immuno-electron microscopy for CD98. (A) Electron micrographs of forskolin (×20K) and (B,C) forskolin and mab4F2 (×15K–20K) treated cells (24 h culture, three representative fields). Single gold particles (thin arrows), clusters of gold particles on membrane process and cytoplasm (thick arrows). (D) Quantitation of immunogold staining for CD98, 8 representative cells analysed: graph shows number of gold particles in different cellular compartment; ▪Pooled results. Statistical analysis: paired *t* test, two tailed.

Kudo et al. [36] used transfection generated green–red BeWo cells, forskolin treatment and fluorescence-activated cell sorting (FACS) to monitor the extent of cell fusion (syncytialisation). Since one aim is to transfer and reproduce results from the cytotrophoblast model to the primary cell line, where GFP type transfection is not feasible, we used two well separated

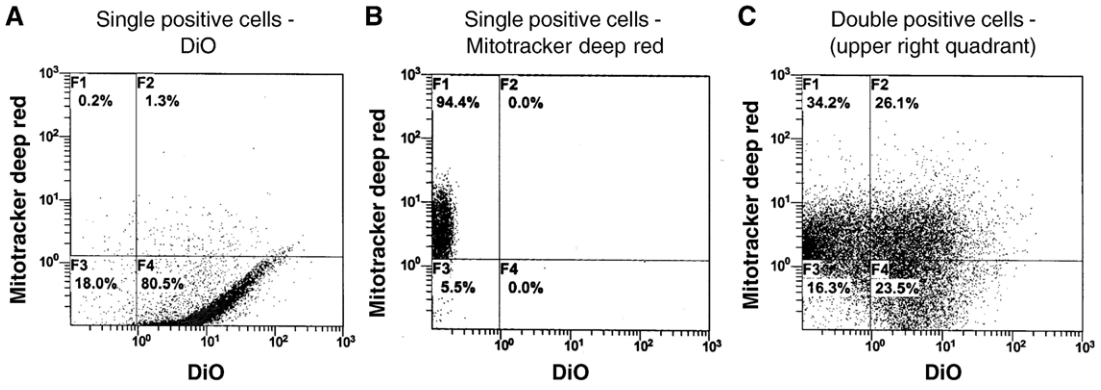

Fig. 4. Use of flow cytometry to detect cell fusion. Examples of BeWo cells labelled with (A) DiO (em.501 nm), (B) Mitotracker deep red 633 (em.665 nm) or (C) a 50:50 mixture of single labelled cells after forskolin treatment (two positive cells in the upper right quadrant). Acquisition with EPICS Altra (Beckman Coulter) flow cytometer.

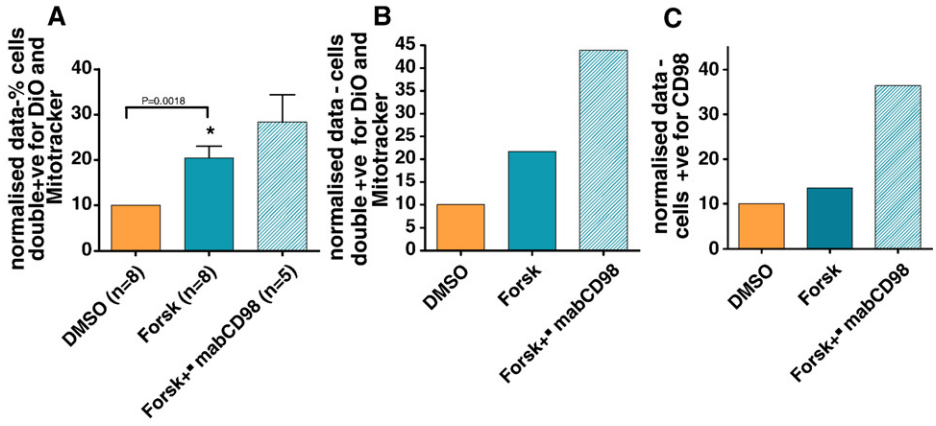

Fig. 5. Flow cytometry of cell fusion. (A) Two colour flow cytometry for detection of fused (two positive) cells with/without monoclonal antibodies. BeWo cells were pre-stained with DiO or Mitotracker deep red 633 dyes. Single colour cells or a 50:50 mixture of both cells were then cultured for 24 h in medium containing DMSO or forskolin or forskolin plus mabs CD98 (19 h incubation). Statistical analysis: paired *t* test, two tailed. (B,C) Three colour Flow Cytometry for detection of fusion and CD98 expression. Pre-stained cells, after forskolin and mabs CD98 treatment, were labelled with goat anti-human CD98 and rabbit anti-goat conjugated with PE (em.575 nm) for simultaneous detection of fusion (B) and CD98 expression (C). ▪Pooled results. Graphs show data normalised to DMSO control (DMSO = 10%). (B) and (C) single representative experiments.

fluorescent dyes to study cell fusion events in a similar fashion. The dyes used have low cytotoxicity, are adequately (if not perfectly) retained after fixation with 2% PFA and have well-separated emission spectra. Other studies similarly using flow cytometry protocols with BeWo cells noted artefacts due to cell aggregation after trypsinisation [21]. Our cells were harvested

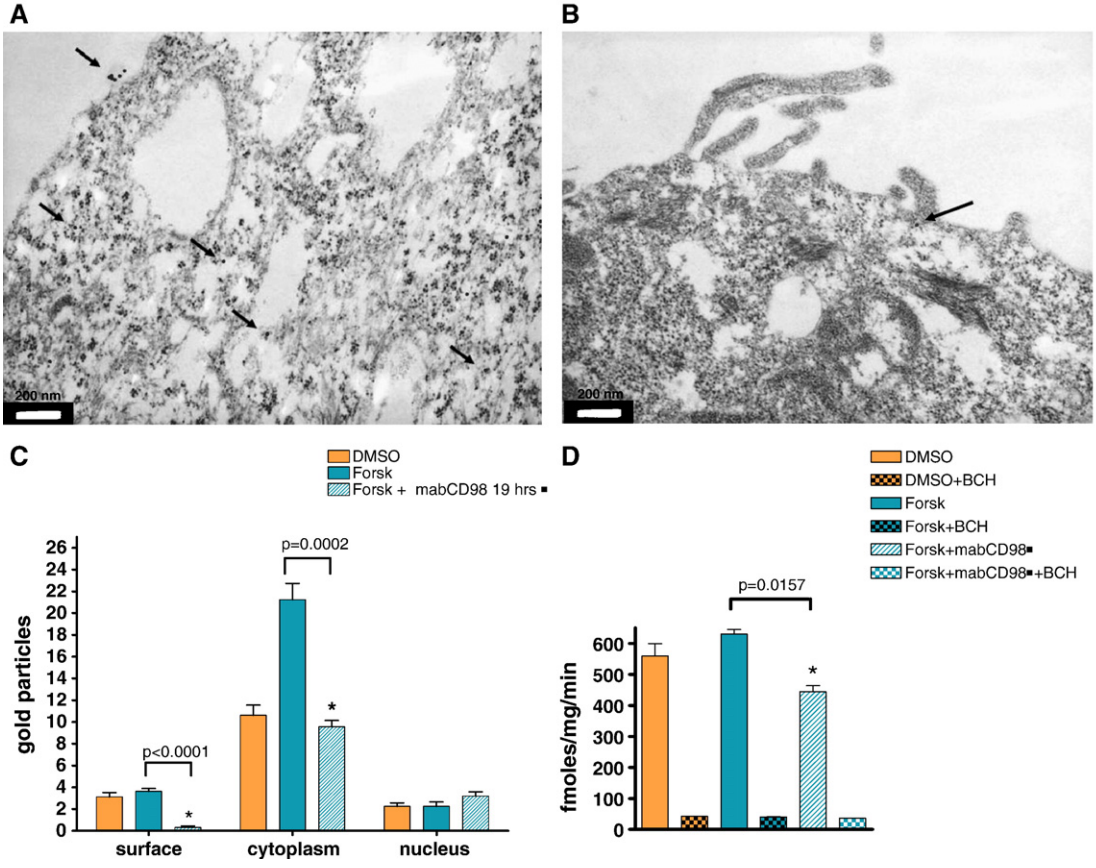

Fig. 6. Distribution and function of LAT 1. (A,B,C) Immuno-electron microscopy for LAT 1: (A) Electron micrographs of forskolin (×25K) and (B) forskolin and mab4F2 (25K) treated cells (24 h culture, two representative fields). (C) Quantitation of immunogold staining for LAT 1: number of gold particles in different cellular compartments, 8 representative cells analysed. ▪Pooled results. Statistical analysis: paired *t* test, two tailed. (D) 3H-L-Leucine (2 μM) influx in Na+ free buffer after 24 culture: in order to separate the transport pathways contributing to total Na+-independent flux, the synthetic amino acid 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), a system L-specific analogue, was added at a concentration of 10 mM. The BCH inhibitable component (i.e. system L-mediated influx) was significantly reduced following forskolin plus mabCD98 treatment. ▪Pooled results.

for flow cytometry after 24 h of culture when syncytium formation, although present, was limited; we found that gentle but thorough re-suspension of our pellets, while causing disruption of larger syncytia and elimination of the transient aggregates due to the procedure, did maintain small syncytia and stable aggregation of cells, which is the first step in syncytialisation. We were easily able to analyse between 10,000 and 20,000 cells, which is not possible with normal routine fluorescent microscopy, and results were reproducible; in addition, in a preliminary study with primary cytotrophoblasts these dyes did not have adverse effects (data not shown).

Finally, we examined the widespread transport system L, which accounts for a sodium-independent exchange of large neutral amino acids and is composed of the CD98 heavy chain and the LAT 1 light chain, in the absence/presence of CD98 mabs.

Our results showed the following:

(1) A statistically significant, although limited, increase in CD98 surface expression is already present after 24 h (by FACS); however, the addition of either of the two monoclonal antibodies against CD98, for the last 19 h of the 24 h culture, caused an unexpected large increase of CD98 membrane expression while total protein expression (both surface and intracellular) was not appreciably raised. Although in this study we assumed (but have not proved) dimerization of CD98 molecules through cross-linking by monoclonal antibodies, other groups, investigating CD98 stimulation of integrin dependent cell adhesion or homotypic cell aggregation [4,34], have shown with these antibodies that CD98 dimerization rather than a direct physical effect of antibody binding to CD98 is required for functional activities; significantly in these experiments monovalent antibody fragments were unable to stimulate function.

(2) The appearance in all samples, but in particular in forskolin treated samples, of round clusters of immunogold particles corresponding to CD98 molecules, both in the cytoplasm and surface of the cells, and which strongly suggested assembly in osmophilic vesicular structures (in situ cellular localization of CD98 molecules by immunoelectron microscopy). And, confirming previous experiments with other techniques, a statistically significant increase of CD98 plasma membrane distribution in the presence of monoclonal antibodies. This finding again points to a mechanism of activation/facilitation of molecular re-distribution of CD98 in the presence of a ligand. This raises the question ‘what could be the physiological cross linker in vivo?’ One possibility we are currently investigating is that of galectin-3, previously suggested as an endogenous linker for CD98. Moreover, galectin-3 and CD98 have both been proposed as modulators in a process leading to activation of integrin-mediated adhesion, in fusion and in inflammatory responses and both have been reported to induce calcium influx [3,4,37–41].

(3) Forskolin treatment of BeWo cells causes a significant increase in cellular fusion (plus 46% ±17); this is further increased by 24% when the cells are also cultured with CD98 mabs (by two colour FACS). More importantly, increased cell fusion is matched by increased surface expression of CD98 (by three colour FACS on the same set of cells). This result strengthens the hypothesis that CD98 is involved in BeWo cell fusion.

(4) The light chain LAT 1 is uniformly distributed in the cells, without clusters and, at least as detected with our antibody, much less abundant than the CD98 heavy chain (~ 1/4) both in control and in forskolin-treated cells (in situ cellular localization of LAT 1 molecules by EM). Intriguingly, cross-linking the heavy chain of the dimer has a significant effect on the expression of the light chain, an effect which is especially pronounced at the plasma membrane, where little immunoreactivity is detected. In addition, L-leucine influx is reduced in the presence of the CD98 mabs. It has been suggested that the light chain needs co-expression with the CD98 heavy chain in order to reach the plasma membrane, and that the extracellular domain of CD98 is necessary to ensure proper translocation to the plasma membrane [7,11]. Expression levels of CD98 and LAT 1 are raised in many primary human cancers; recently, Storey et al. [42] using an adenoviral expression system showed that LAT 1 protein levels in hepatic cells could be increased three fold, resulting in increased L-type amino acid transport. The LAT 1 protein complexed with the CD98 heavy chain was expressed on the cell surface and, at the same time levels of the total CD98 protein complex were also increased 2.4-fold. We hypothesize that, if CD98 is necessary for LAT 1 trafficking to the plasma membrane and if CD98 protein is more abundant than LAT 1 (a likely pre-requisite for a molecule which forms different heterodimers), increased levels of LAT 1 would draw free cytosolic CD98 for trafficking to the cell surface (producing the increased surface expression of both exogenous LAT 1 and endogenous CD98 and the increased Na+-independent system L mediated influx observed). However, by ligating the heavy chain with its monoclonal antibody we may interfere, by steric hindrance, with the formation and stability of the disulphide bond and/or non-covalent interactions between the light and heavy chains, resulting in minimal trafficking to the surface of the heterodimeric complex. The free LAT 1 protein unable to bind the heavy chain will eventually be broken down (as indicated by reduced total levels), and neutral amino acid transport will diminish as confirmed by our data on L-leucine influx.

In conclusion in this study we attempt to gain new insights into the mechanism and functional impact of the CD98 heterodimeric protein; we suggest that CD98’s two functions, as mediator of cellular fusion and as a component of a family of amino acid transporters, are distinct. This answers a hitherto open question [43]. The limiting factor for both functions is CD98 availability, albeit in the latter indirectly since it is required only for LAT 1 surface localization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2006.11.020.

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