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Effect of EDEM1 overexpression on the generation and assembly of major histocompatibility complexes

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ABSTRACT

Background. A better understanding of the role of endoplasmic reticulum degradation-enhancing alpha-mannosidase – like protein 1 (EDEM1) in endoplasmic reticulum associated degradation (ERAD) may open new therapeutic approaches in autoimmune diseases.
Aim. To study ERAD and EDEM1 in the generation and assembly of MHC I and the potential role in the pathophysiology of autoimmune diseases.
Materials and methods. HEK293T cell line (human embrionic kidney cells), A375 cell line (amelanotic melanoma cells) and THP-1 cell line (leukemic monocytes used both as undifferentiated and differentiated) underwent transient transfection with EDEM1 and mock transfection with pTriEx. Western blot experiments assessed the total cellular MHC I levels in cell lysates, while expression on the cellular surface was quantified by flow cytometry of fixed cells. Results were analysed using the FACS Calibur and CellQuest Pro dedicated software. Experiments were done twice with duplicate probes for the Western blot assay and triplicate probes were used for flow cytometry. GraphPad Prism was used for data analysis.
Results. MHC I plasma membrane routing and expression was similar in HEK293T and A375 both in mock transfected and non-transfected cells. Western blot assay for EDEM1 transfected cells showed bands corresponding to the total MHC I that migrated at 42kDa mass in non-transfected and mock transfected Hek293T, A375 and undifferentiated THP-1 cells. Mock transfected differentiated THP-1 cells showed a reduction of total MHC I. EDEM1 transfected Hek293T, A375 and undifferentiated THP-1 cells displayed higher levels of total MHC I, while differentiated THP-1 cells showed a marked reduction. Flow cytometry assay showed significantly reduced cell surface MHC I levels in Hek293T cell line. We observed a modest reduction of MHC I complexes on the cellular surface in undifferentiated THP-1 EDEM1 transfected cells, while there was no significant change in the A375 EDEM1 transfected cell line, as well as the differentiated THP-1 EDEM1 transfected cells.
Conclusion. The impact of ERAD's EDEM1 in MHC I reduction may have an important role in autoimmune disease, making ERAD an interesting therapeutic target.

Keywords: endoplasmic reticulum associated degradation, EDEM1, MHC I

INTRODUCTION

The endoplasmic reticulum (ER) is a multifolded membraneous structure and the site of lipid and protein biosintesys (1). It is estimated that aproximately one-third of the secreted and membrane proteins are synthesized and assembled in their native structure inside the ER lumen (2). Therefore, it is essential that a quality control system exists in order to maintain crucial cell homeostasis environment. Accumulation of unfolded intermediaries or misfolded proteins in the ER lumen leads to ER stress and triggers several stress sensors that act as the effectors of unfolded protein response (UPR). UPR is characterized by several cytoprotective pathways that ultimately lead to ER stress mitigation. This result is achieved by either increasing the protein folding capacity in the ER or decreasing the protein folding load through the activation of ER associated degradation (ERAD) or, when everything else fails, trigger apoptosis (3). The end result is to reestablish cell homeostasis.

Three main effectors are described for UPR: activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and PKR-like ER-localized eIF2α kinase (PERK), all residents of the ER and

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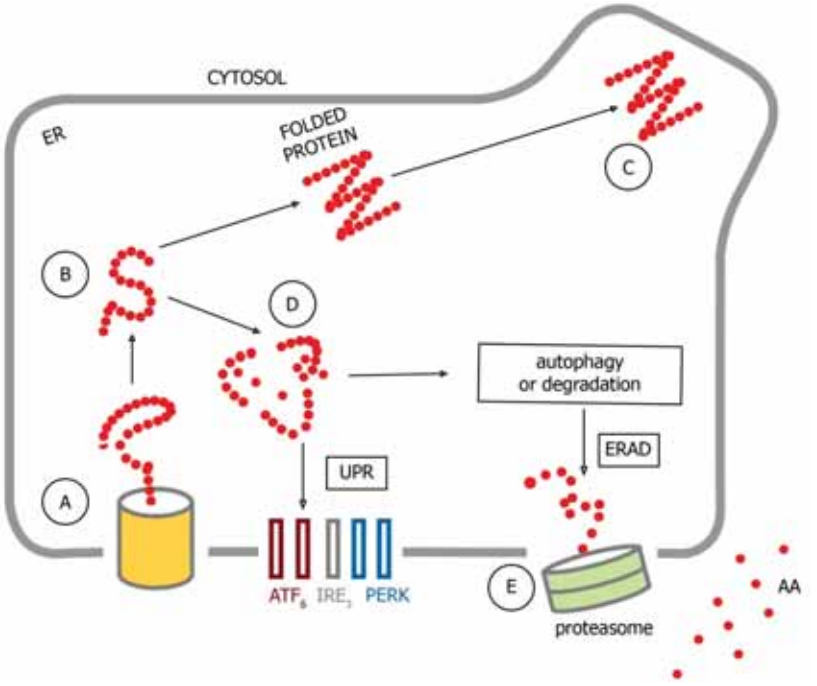

FIGURE 1. *In ERAD, misfoled proteins are translocated from the ER lumen in the cytosol, a process known as retrotranslocation, where they are ubiquitylated and undergo proteasomal degradation.*
A – translocation of the nascent protein in the ER; B – folding intermediaries; C – transport of the native protein to the dedicated compartement; D – misfolded intermediaries aggregate and trigger UPR; E – misfolded proteins are retrotranslocated in the cytosol (reproduced after Douglas M. Cyr et al) (4)

maintained in an inactive state. Phosporylation of IRE1 generates X-box binding protein 1 spliced (XBP-1s), which in turn activates chaperones, leading to degradation of misfolded intermediaries. ER degradation-enhancing α-mannosidase-like protein 1 (EDEM1) was initially tagged as an ER stress inducing gene targeting XBP-1s (5). More recent studies show that overexpression of EDEM1 leads to degradation of misfolded subtrates, making EDEM1 an important member of ERAD. Current understanding is that EDEM1 contribues to degradation of misfolded substrates along with ER chaperone calnexin and the UPR-induced XBP-1 target E3 ubiquitin ligase hydroxymethylglutaryl-coenzyme A reductase degradation 1 (HRD1)-suppressor of Lin12-like (SEL1) complex (5).

Major histocompatibility complex class I (MHC I) present antigens, both self and non self, to CD8+ T lymphocites, and are important players in immune tolerance and immune response. As most of the proteins, MHC I heavy chains are secreted, folded and loaded in the ER. Interactions between MHC I-peptide complexes and CD8+ T cells and antigen presenting cells trigger the appropriate immune outcome. Therefore, the generation of peptides and their loading on the MHC I molecules through a multistep process called antigen processing and presenting machinery is crucial. Studies suggest that due to its high heterogenity, MHC I is prone to misfold. Because MHC I is ubiquitous, it is believed that misfolding may have a potential impact on virtually every cell of the organism (6), and thus, the disfunctionality of ERAD and its incapacity of MHC I peptide loading potentially leads to an altered immune response and the premises of pathology.

AIM

The purpose of this study was to demonstrate the role of EDEM1 in the ERAD. Particularly, we tried to address the function of EDEM1 in the intracellular secretion and cell surface expression of the MHC I in several immortalised cell lines.

MATHERIALS AND METHODS

Cells and cell culture

cDNA of mouse EDEM1 cloned into pCMV-Sport was a kind gift from Dr. K.Nagata and Dr. N. Hosokawa (Institute of Frontier Medical Science,

Kyoto University). Experiments were performed on two adherent cell lines: HEK293T cells (human embrionic kidney origin) and A375 cells (amelanotic melanoma cells from European Collection of Animal Cell Cultures, Porton Down, UK). The rationel for these cell lines was their ease of transfection and high transfection effficiency and the possibility to study ERAD both in young cells and in neoplasia-like cell lines.

HEK293T and A375 cell lines were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine and 50 UI/ml Penicillin/Streptomycin in the 37° incubator infused with 5% CO_2.

Suspension cells THP-1 (acute leukemia monocytes) were used for their potential to express both MHC I and MHC II, once differentiated to macrophages. Cells were cultured in RPMI-1640 Medium supplemented with 10% heat incativated fetal bovine serum, 12.5 mM HEPES, 50 UI/ml Penicillin/Streptomycin and 100 mM sodium pyruvate in the 37° incubator infused with 5% CO_2.

Experiments on THP-1 cell line were performed both in undifferentiated cells and differentiated to macrophages. The differentiation protocol used phorbol 12-myristate 13-acetate (PMA) at a final concentration of 10ng/ml, followed by 48h incubation at 37ºC, in 5% CO_2 atmosphere, without changing the medium.

Overexpression of EDEM1

HEK293T, A375, undifferentiated and differentiated THP-1 cells were plated in 6 well plates at $5x10^5$ cells/well. All cell lines underwent transient transfection with EDEM1 and mock transfection with the pTriEx 1.1 vector, using PEI reagent for HEK293T cell line and Lipo2000 reagent for A375 and THP-1 cell lines respectively. Plates were incubated at 5% CO_2 37°C. Cells were harvested after 48h transfection and further analysed.

Intracellular MHC I secretion assay

Western blot experiments were performed in order to assess the intracellular expression of MHC I in transfected cell lines which were compared to mock transfected and nontransfected cells (control). For this experiment cells underwent lysis, poliacrilamide gel separation in nonreducing conditions and were transferred on nitrocelulose membrane. Monoclonal anti-MHC I antibodies (W6/32 hybridoma) were used for the assay. We performed all Western blot experiments on nonreduced, non denaturated cell lysates, because previous optimisation tests enabled us to determine that these are optimal conditions to expose MHC I to antibodies. For validation of the results, we performed Western blot on reduced and denaturated (95°C for 5 min) samples from the same lysate in order to certify the transfection with EDEM1 (anti-EDEM1 antibodies) and appropriate sample loading control (anti-calnexin antibodies). Western blot experiments were performed twice using duplicate probes.

MHC I expression on the cellular surface assay

Flow cytometry experiments were performed in order to assess MHC I expression on the cellular surface. FACS Calibur with its dedicated software (CellQuest Pro) was used. Cells were incubated 30 minutes on ice with specific antibodies coupled with FITC mouse anti-human HLA-ABC from BD Pharmingen. Flow cytometry experiments were done twice using triplicate samples.

Data analysis

Data was analysed using GraphPad Prism software. Unpaired t test was used for statistics.

RESULTS

1. EDEM1 overexpression and total MHC I levels

1.a. Adherent cells

Western blot experiments revealed an increase of MHC I total levels in both HEK293T and A375 cell lines, as expressed by the appereance of distinct electrophoretic bands corresponding to the 42 kDa molecular mass of the MHC I molecule.

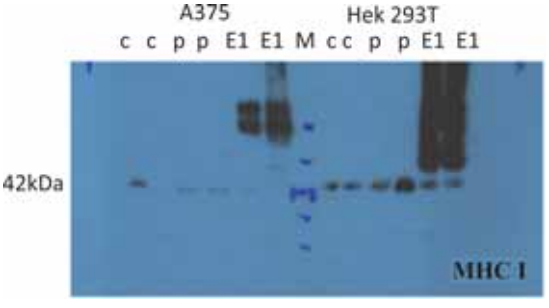

FIGURE 2. *Total MHC I levels increase (multimers) in EDEM1 transfected HEK293T and A375 cell lines (c – control samples, p – pTriEx transfected samples, E1 – EDEM1 transfected samples, M – mass marker)*

1.b. Cells in suspension

MHC I total levels were markedly increased in undifferentiated EDEM1 transfected THP-1 cells as

compared to control non tranfected undifferentiated cells. The Western blot assay revealed bands of higher intensity corresponding to the 42kDa MHC I mass in undifferentiated EDEM 1 transfected THP-1 cell line. An important reduction of total MHC I levels occurs after differentiaton of THP-1 to macrophages. We observed that both non transfected, mock transfected and EDEM1 transfected THP-1 differentiated cells had low levels of MHC I expressed as reduced intensity bands migrating at MHC I molecular mass.

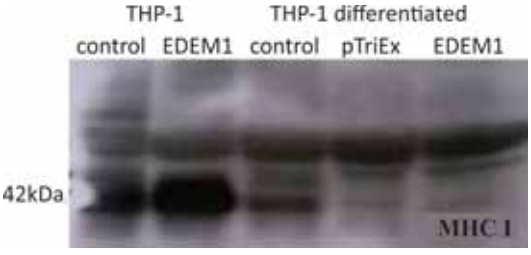

FIGURE 3. *Thick lines corresponding to the MHC I molecular mass of 42 kDa. Marked decrease of MHC I total levels is observed in the differentiated THP-1 cell line*

2. EDEM1 and cell surface MHC I complexes

Flow cytometry experiments on EDEM1 transfected HEK293T cell lines revealed a significant reduction of surface MHC I complexes compared to the mock transfected HEK293T cells (74.25±0.32% for EDEM1 transfected cells compared to 93.00 ± 0.7% mock transfected with pTriEx). However, results were modest in the amelanotic melanoma cells EDEM1 transfected cells compared with the mock transfected group (95.23 ± 0.85% for A375 mock pTriEx and 97.35±1.12% for A375 EDEM1 transfected).

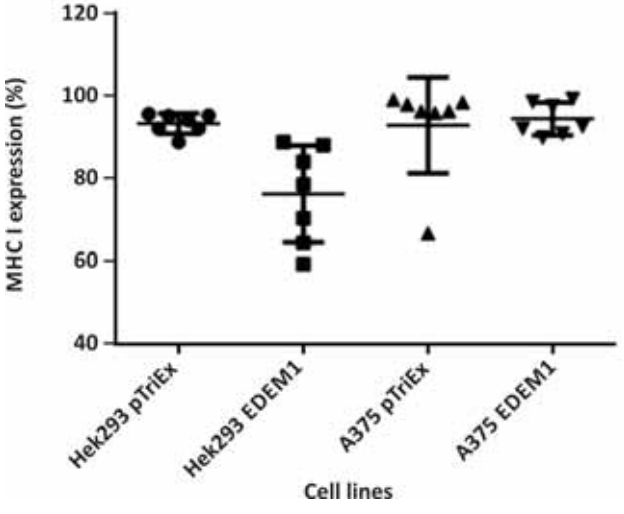

FIGURE 4. *Cell surface MHC I complexes are reduced in HEK293T EDEM1 transfected cell as compared to the mock transfection group (p = 0.0001)*

Experiments of flow cytometry showed a modest decrease of cell surface MHC I complexes in the undifferentiated THP-1 group. We observed no significant changes of cellular surface MHC I complexes in the differentiated cell lines.

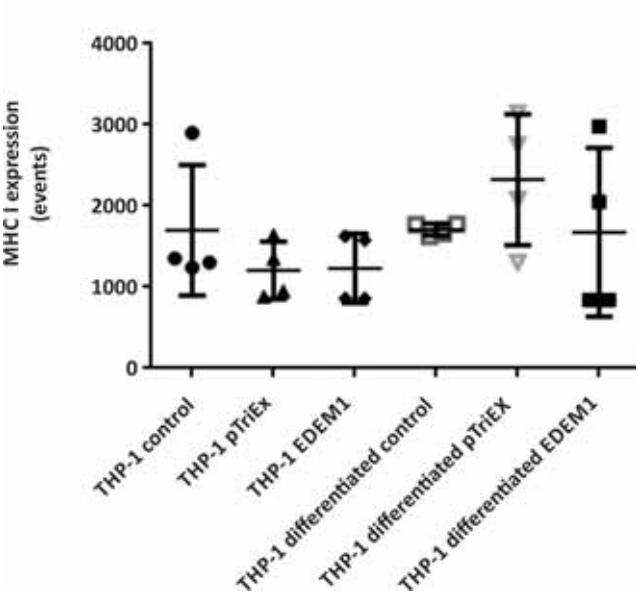

FIGURE 5. *Modest reduction of cell surface MHC I complexes observed in the undifferentiated THP-1 EDEM1 transfected cell line compared to control group (p < 0.0001). No differences were observed in the differentiated EDEM1 transfected THP-1 cells compared to control group*

DISCUSSION

Overexpression of EDEM1 has different effects on the intracellular levels of MHC I

The main steps of ERAD pathway are substrate recognition, substrate targeting, retrotranslocation, ubiquitylation and proteasome degradation. ER degradation-enhancing α-mannosidase-like lectins (EDEMs) are key palyers of ERAD in recognition and targeting of the misfolded proteins in order to be retrotranslocated. The question of whether EDEMs have substrate specificity remains to be answered, but current knowledge is that EDEM1 may receive substrates from the calnexin cycle (7,8). Guiliano et al demonstrated in their study that HRD1 along with EDEM1 have specific affinity for misfolded mono- and dimers of HLA-B27, an MHC I allele. The study highlights several important implications: firstly, misfolding and agreggation of HLA-B27 may be the basis for inflammatory disease. Secondly, the EDEM1 and the HRD1-SEL1 complex may be important steps in the degradation of misfolded HLA-B27 di-

mers and thirdly, the manipulation of ERAD through EDEM1 may be a therapeutic option for diseases such as ankylosing spondylitis (5).

Our study revealed that overexpression of EDEM1 in HEK293T, A375 and undifferentiated THP-1 cell lines leads to a total increase of intracellular MHC I. However, this process was not observed in the differentiated THP-1 cell line. The explanation may be that once differentiated to macrophages, THP-1 have a decreased basal secretion of MHC I in favour of the MHC II, which is important for their function as antigen presenting cells. Our results are in accord with previously published reports only as far as a part of MHC I is concerned, namely the HLA-B27 allele. However, other pathways may be involved in the processing of the various MHC I alleles.

Overexpressed EDEM1 leads to a modest decrease of MHC I complexes at the cellular surface in suspension cells

We observed a reduced expression on the cellular surface of the MHC I complexes after transient transfection with EDEM1 of all the cell lines which were tested. Our hypothesis is that in the HEK293T, A375 and undifferentiated THP-1 cell lines, MHC I is restricted to the intracellular environment, in the ER or in Golgi vesicles through yet unknown mechanisms. In the differentiated THP-1 cells, we hypothesise that the low cell surface expression may be due to the reduced surface-routing of MHC I and, possibly, due to the fact that the differentiated THP-1 cells have a primary function to present antigens on MHC II. The importance of expressing MHC I on the cell surface resides in the presentation of endogenous material with pathogen potential to cytotoxic lymphocytes and subsequent generation of an immune response. One study lead by van de Weijer ML et al explains the stages of MHC I formation, peptide coupling and intracellular transport towards the surface. MHC I forms starting from a protein that is co-translationally inserted in the ER membrane, where it is coupled with ER chaperones calnexin and BiP in order to stabilize its conformation until it binds to β2micro-globulin. Upon coupling to the β2microglobulin, the nascent MHC I polypeptide dissociates from calnexin and BiP and interacts with ERp57, tapasin, calreticulin and a transporter associated with antigen processing, forming the MHC I peptide-loading complex. As calnexin, calreticulin and ERp57 are key factors for the proper folding of nascent proteins, van de Weijer suggests that viral infection may trigger ER stress by inhibiting these components and trigger ERAD with the ultimate scope of eluding MHC I presentation. (9) However, literature lacks information on the exact mechanism through which EDEM1 may be directly involved in the presentation of MHC I.

The role of ERAD in pathology and potential therapeutic promises

The current opinion suggests that more than 60 diseases may have the ERAD inability to mitigate misfolded proteins as physiopathologic basis, among the most studied being the noplastic and autoimmune diseases. As discussed earlier, ER stress induces UPR, which activates several cytoprotective pathways in order to restore cell homeostasis. However, it is thought that these same pathways are involved in apoptosis. Moreover, it seems that certain cancer cells have different sensitivity in ER stress-induced death, for example in multiple myeloma or proliferative mielopathies. Cancerous cells are prone to ER stress apoptosis, leading to the theory that there may be a therapeutic window for ER stress inducers to clear out malignant cells selectively (10). In their study on the functional value of proteasome-independent MHC I tumor peptide generation, Ebstein et al point out the importance of targeting of the ubiquitin-proteasome-system (UPS) in the treatement of T cell malignancies, as UPS is responsible for the generation of MHC I epitopes (11).

Among the most studied autoimmune disease linked to ERAD are rheumatoid arthritis and ankylosing spondylitis. Colbert et al suggest that the high polymorphism of HLA-B27 may be responsible for the misfolding and inefficient peptide loading, which may result in ERAD of the heavy chains. He states that there may also be a deficient response of cells with activated UPR to innate immune stimuli. This altered response may influence pro-inflammatory cytokine production responsible not only for maintaining the inflammation, but also for the further HLA-B27 misfold. Thus, Colbert et al hypothesise that manipulation of more ERAD components, than solely UPR, may prove useful to therapy (6). Yamasaki S et al show that rheumatoid arthritis may be studied from the perspective of a “hyper-ERAD disease”, arguing that while disruption of the ERAD leads to apoptosis and induction of neurodegenera-

tive diseases, the upregulation of ERAD may prove to inhibit apoptosis and can induce synovial cells to proliferate and sustain the inflammed hostile environment of rheumatoid arthritis joints (12).

CONCLUSION

The importance of ERAD study resides in the prospects for targeted therapy in diseases with high potential of disability and morbidity. The impact of ERAD's EDEM1 in MHC I reduction may have an important role in autoimmune disease, making ERAD an interesting therapeutic target.

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