Dept. of Zoology,
Faculty of Science, Assiut U. ersity, Assiut, 71516, Egypt

ROLE OF CERAMIDE IN TRANSFERRIN RECEPTOR MOVEMENT IN THE PLANE OF THE PLASMA MEMBRANE

(With 4 Figures)

By

A-B.M. ABDEL-SHAKOR and RANA ALAWADHI*

*Dept of Science, Faculty of Basic Education, Kuwait.

(Received at 15/10/2009)

دور السيراميد في حركة مستقبل الترانسفرين علي سطح غشاء البلازما

أبوبكر محمد الطيب عبد الشكور ، رنا العوضي

الترانسفرين هو بروتين موجود في بلازما الدم ويفرزة الكبد ووظيفته الأساسية هي نقل الحديد الي الخلايا. عندما يرتبط الترانسفرين بمستقبله علي سطح الغشاء الخلوى فان المستقبل يتحرك علي غشاء البلازما ثم يتجمع في تجاويف الكلاثرين الموجودة في غشاء الخلية. بعد ذلك يدخل الترانسفرين مع مستقبلة الي داخل الخلية خلال هذه التجاويف. الهدف من هذا البحث هو التعرف علي الميكانيكية التي يتبعها مستقبل الترانسفرين في الحركة علي غشاء البلازما والتي تقودة الي تجاويف الكلاثرين. أدت نتائج هذا البحث الي التعرف علي الوسيط الليبيدي المعروف بأسم السيراميد (ceramide) علي أنه العامل الأساسي في ميكانيكية حركة مستقبل الترانسفرين. لقد وجد أن مستوي السيراميد يزداد خلال أول دقيقة بعد اتحاد الترانسفرين بمستقبلة كما هو مستنتج من طريقة DGK لقياس مستوي السيراميد والكشف المناعى الخلوي للسيراميد . أوضحت النتائج أن نشاط أنزيم ASMase يزداد ونشاط إنزيم SMS ينخفض خلال الدقيقة الأولي وبذلك يزداد مستوي السيراميد خلال هذه الفترة. وللتأكيد من أن السيراميد له دور في حركة المستقبل وتعرفه علي تجاويف الكلاثرين تم تثبيط نشاط ASMase بواسطة عقار Imiprarmine وأيضا تم معادلة السيراميد النتائج بواسطة الأجسام المضادة للسيراميد. لقد وجد أن الخلايا التي تم تثبيط السيراميد بها يحدث لها عدم إعادة الترانسفرين الي الخارج وتبقي هذه الخلايا محتفظة بالترانسفرين داخلها. تم أيضا استخدام طريقة التجزئة الخلوية (Cell Fractionation) في تتبع حركة مستقبل الترانسفرين علي سطح غشاء البلازما. لقد وجد أن تثبيط السيراميد يؤدي الي إنضمام اعداد كبيرة من المستقبل الي مناطق في غشاء الخلية بعيدة عن تجويف الكلاثرين والتي تعرف بأسم الرافت (Rafts). تعد الخلايا السرطانية أكثر الخلايا تمثيلا لمستقبل الترانسفرين علي أغشيتها الخلوية لذلك فإن نتائج هذا البحث تلقى بعض الضوء علي فهم ميكانيكية عمل مستقبل الترانسفرين والتي يمكن الاستفادة منها في مقاومة الخلايا السرطانية.

SUMMARY

After ligation with transferrin (Tf), transferrin receptor (TfR) is aggregated on cell surface in clathrin coated pits and then internalized into the cell. In the present work, the mechanism of (Tf/TfR) and the role of Ceramide in this mechanism were studied. After Tf/TfR ligation, Ceramide level on plasma membrane outer leaflet was temporarily elevated as clarified by immunocytochemical detection of cell surface Ceramide in human "jurkat" T lymphoma cell line. Ceramide elevation was confirmed by Diacylgelycerol kinase assay for biochemical Ceramide measurement. The fast and brief Cer elevation was the result of acid sphingomyelinase activity, and no detection of neutral sphingomyelinase activity was noticed. Sphingomyelin synthase activity was obviously decreased in the same time frame of Ceramide generation, to maintain the elevated level of Ceramide. Inhibition of Ceramide generation by Imipramine, an acid sphingomyelinase inhibitor, or neutralization of the generated Ceramide by anti-Ceramide mAb lead to miss-internalization of transferrin, instead of being clathrin mediated it becomes, most probably, raft mediated as indicated by altered Tf recycling and cell fractionation studies. Miss-internalization of transferrin leads to abrogation of cell growth and finally apoptotic cell death.

Abbreviations: ASMase – Acid Sphingomyelinase; Cer – Ceramide; DGK - Diacylgelycerol kinase; NSMase – Neutral Sphingomyelinase; SM – Sphingomyelin; SMS – Sphingomyelin synthase; Tf – Transferrin; TfR – Transferrin receptor.

Key words: *Transferrin, ceramide, sphingomyelin, acid sphingomyelinase, clathrin, jurkat cells.*

INTRODUCTION

Transferrin (Tf) is an 80 kDa plasma glycoprotein secreted by the liver, function as iron carrier. When Tf is loaded with iron its binding capacity towards its membrane receptor, TfR is increased, then Tf/TfR complex is aggregated into clathrin coated pits. The receptor and the empty Tf is recycled back to the cell surface after discharge of iron, ready for another round of iron uptake (Huebers and Finch, 1987). The internalization and trafficking events of different receptors and agents via clathrin pathway are complex molecular machinery. Intensive studies were carried out to clarify the protein molecules chairing in this

machinery. Recently lipid molecules attract the attention of the researchers as functioning molecules in clathrin endocytosis pathway (De Camilli *et al.*, 1996; Roth, 1999; Di Paolo *et al.*, 2004; Padron *et al.*, 2006). The role of lipid molecules in this process was additionally clarified by inhibition or mutation in the lipid modifying enzymes leading to blocking of endocytosis events (Hughes and Parker, 2001; Du *et al.*, 2003, 2004). Cells have the ability to direct different molecules to be internalized through different pathways and to different intracellular targets. For example TfR and T cell receptor (TCR) are in close and physical association to each others (Salmeron *et al.*, 1995). Upon activation, TCR is aggregated into lipid rafts (Boniface *et al*, 1998; Monks *et al.*, 1998) whereas; TfR is aggregated into clathrin coated pits. How cells control the movement of specific receptor or agent towards specific membrane domain, however still need to be clarified.

Hydrolysis of Sphingomyelin (SM) results in Cer releasing, which is catalyzed by three sphingomyelinases, acid, neutral and alkaline with the respective pH optimum activity (Kolesnick *et al.*, 2000; Hannun and Obeid, 2008). Another pathway for Cer generation is by de novo synthesis, which involving serine-palmitoyl-CoA transferase as the key enzyme. Among sphingomyelinases, ASMase is the best characterized one; it was shown to be critically involved in many cellular activities (Gulbins and Kolesnick, 2003). ASMase is found in two forms, lysosomal and secretory that are both derived from the same gene but differ in their glycosylation pattern (Schissel *et al.*, 1998). Cer is a fast moving lipid molecules has the tendency to spontaneous fusing with each others forming Cer platforms. Cer molecules in such platforms seem to be tightly backed with each others forming rather stable membrane domains (Veiga *et al.*, 1999; Grassmé *et al.*, 2001 a, b; Megha and London, 2004). This help in trapping and clustering receptor molecules in Cer rich platforms resulting in a high receptor density within a small area of cell membrane (Veiga *et al.*, 1999; Megha and London, 2004). This high receptor density helps them to be in close association with their signaling molecule or to be excluded from undesirable molecule as phosphatases. Another study (Abdel Shakor *et al.*, 2004) clarified that Cer acts as a driving force for Fc gamma RII to be trapped into SM and cholesterol rich membrane domain (membrane rafts). The immediate and fast generation of Cer is the character of ASMase activity whereas other sphingomyelinases seem to act slower (Gulbins and Kolesnick, 2003 and Dumitru and Gulbins, 2006). In the present study, it is shown that Cer is generated within seconds of Tf/TfR interaction, the generated Cer drive the movement of the receptor in the

plane of the plasma membrane towards clathrin coated pits and away from raft trapping. The current work was designed to study the mechanism of TfR movement in the plane of the plasma membrane and the role of the lipid mediator Cer in this process.

MATERIALS and METHODS

Materials

Transferrin Alexa fluor 488 conjugated and goat anti-mouse IgM Alexa fluor 546 were from Iinvitrogen. Anti lyn rabbit IgG from Santa Cruz Biotechnology, anti Cer IgM monoclonal MID15B4 from Alexis, SuperSignal West Pico chemiluminescent substrate (Pierce). C6-NBD-Cer and C6-NBD-SM from Avanti. All other chemicals, if not mentioned, were purchased from Sigma.

Cell culture, Tf treatment and measurement.

Human T lymphocytes, (Jurkat cell line) was routinely cultured in RPMI 1640 supplemented with 10% calf serum in 100% humidified atmosphere with 5% CO_2 at 37°C. After 48 h, inoculated cells were harvested and 2×10^6 cells were kept in serum free medium for 30 min at 37°C for elimination of endogenous Tf. Cells were left as control or treated with 30 μM Imipramine, 10 μg/ml anti-Cer IgM MID15B4 mAb and 10 μg/ml irrelevant mouse IgM for 1 hr before Tf uptaking. Cells were treated for different time points at 37°C with 5 μg/ml of human Tf alexa fluor 488 conjugated. To stopping Tf/TfR movement and internalization, cells were washed trice with ice cold PBS (pH 7.2). Cells were fixed with 2% formaldehyde for 30 min at ambient temperature. For measurement of Tf uptaking, 2×10^6 cells were processed for uptake of alexa fluor 488 conjugated Tf for 5 and 15 min. After fixation, cells washed once with acidic PBS (pH 4.5) to remove the membrane attached Tf. Immediately, cells were washed twice with normal PBS, lysed by 1% NP-40, in Tris buffer pH 7.2. After centrifugation at 5,000 rpm for 5 min, aliquots of supernatant were collected and measured for fluorescence intensity at excitation/emission 495/519 nm. Fluorescence intensity was presented as percentage of the highest mean value.

Cell surface Ceramide immunocytochemistry

Cells were processed for Tf uptaking for different time points as described above, cytospinned onto glass slides. Blocking of the active sites was done by 0.1% BSA for 30 min at room temperature. For visualization of cell surface Cer, cells were treated with 1 μg/ml anti-Cer IgM MID15B4 mAb for 1 h at room temperature followed by Goat anti

mouse IgM Alexa fluor 546, conjugated. Cells were examined with a fluorescent microscopy (BX60-34FFB1; Olympus).

Measurements of ASMase and NSMase activities

Sphingomyelinases activities were assayed based on a previously described method (Okazaki *et al.*, 1989 and Tanaka *et al.*, 1996). Breively, cells (2.5x 10^6) were homogenized in 1 ml lysis buffer containing 10 mM Tris-HCL (pH 7.5), 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethyl-sulfonyl fluoride, 10 µg/ml pepstatin A and 2.5 µg/ml aprotenin. A breive sonication was done for ASMase activity determination. Cell lysates was centrifuged at 12,000 x g for 10 min at 4 °C, supernatant was used as enzyme source. The protein concentration was determined using a protein assay kit (Bio-Rad). 20 µg of protein was mixed with the reaction buffer for NSMase activity, including 100 mM Tris-HCL (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 54 µM C6-NBD-SM and 0.1% Triton X-100 (total volume 100µl). For ASMase activity, the protein was mixed with the reaction buffer including 100 mM acetate buffer (pH 5.0), 54 µM C6-NBD-SM and 0.1 % Triton X-100 (total volume 100 µl). The mixure was incubated for 30 min at 37 °C, the reaction was stopped by adding 2 ml chloroform/methanol (2:1, v/v) and then 900 µl of H_2O and centrifuged at 3,000 x g for 5 min to separate the organic and aquas phases. The lower phase was collected and the solvent was evaporated. Aliquots were then applied to TLC plates (Whatman, LK6DF Silica gel) and NBD-lipids were resolved in a solvent system containing chloroform/methanol/12 mM $MgCl_2$ (65/25/4, v/v/v), the fluorescence was detected and quantified by image analyser, (LAS-2000, Fuji Film, Japan).

Measurement of SMS activity

SMS activity was measured as described previously (Yamaoka *et al.*, 2004) with minor modifications. 5 x 10^6 cells were homogenized in lysis buffer containing 20 mM Tris-HCL (pH 7.5), 2 mM EDTA, 10 mM EGTA, 1 mM phenylmethyl-sulfonyl fluoride, 2.5 µg/ml leupeptin and 2.5 µg/ml aprotenin. Cells were left on ice for 10 min and then homogenised by passing 10 times through 27 gauge needle. The homogenates were centrifuged (2500 rpm, 5 min, 4 °C), the supernatants used as enzyme source. Supernatants (100 µg protein) were incubated with (20 µg) C6-NBD-Cer/phosphatidylcholine (1:10) liposomes in buffer containing 10 mM Tris-HCL (pH 7.5),1mM EDTA (in total volume 100 µl) for 60 min at 37 °C. Reaction was stopped by adding 2 ml chloroform: methanol (2:1, v/v) and then 900 µl of H_2O and centrifuged at 3000 rpm for 5 min to separate the two phases. The lower phase was collected and the solvent was evaporated. Aliquots were then

applied to TLC plates and NBD-lipids were resolved in a solvent system containing chloroform/methanol/12 mM $MgCl_2$ (65/25/4, v/v/v), the fluorescence was detected and quantified, (LAS-2000, Fuji, Japan).

Measurment of Cer

Cer was quantified using diacylglycerol kinase assay (DGK) as described elswere (Bielawska *et al.*, 2001). Briefly human holo Tf 5 µg/ml was added to 2.5 x 10^6 jurkat cells in serum free rpmi 1640 medium for different time points. Total cellular lipids were extracted (Bligh and Dayer, 1959) and dried, then solubilized in 7.5 % octyl-β-D-glucose, 25 mM dioleolphosphatidylglycerol solution. Lipid micelles were mixed with the reaction buffer containing 50 mM Imidazol (pH 6.6), 5 mM LiCl, 12.5 mM $MgCl_2$, 1 mM EGTA (pH 6.6), 19.36 µM DTPA, 3.8 µg/µl DGK membrane from DGK-overexpressed E. coli,100 µM ATP and 4 µ Ci [γ-^{32}P] ATP (Amersham) in total 100µl volume.The mixture was incubated at 37 °C for 30 min. Aliquots of extracted lipids were spotted on TLC plates and lipids were separated in a solvent system containing chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v/v/v/v), radioactive lipids, were visualized and quantified by BAS2000 Image Analyzer (Fuji, Japan). Lipid spots corresponding to Cer-phosphate were identified compared with the known standards. Lipid phosphate level was measured compared with the phosphate level of linear concentrations of NaH_2PO_4 and then Cer levels were normalized to lipid phosphate levels and expressed as (pmol/nmol phosphate).

DAPI staining and apoptotic percentage

Subconfluent cells were washed twice with serum free medium, treated or not with Imipramine, anti-Cer mouse IgM mAb and irrelevant mouse IgM, reseeded at 2×10^5 cells/ml and incubated at the same medium supplemented with 5 µg/ml human holo transferrin in 5% CO_2 at 37°C. Cells were processed for cellular nuclear chromatin changes detection by DAPI staining method, where cells were washed and fixed with 1% glutraldehyde for 30 min, then labelled with 2 µg/ml DAPI. Apoptotic percentage were caculated by counting 200 cells at least for each treatment for three independent expriments.

Cell fractionation

Cell fractionation was carried out using Triton X-100 cold lysis of cells, essentially as described (Korzeniowski *et al.*, 2003). Cells (12.5 × 10^6 per sample) treated or not with Imipramine as previously described were treated with 5 µg/ml human holo Tf for one min at 37 °C. Cells were lysed for 30 min at 0 °C in 200 µl of buffer containing 0.2% Triton

X-100, 100 mM NaCl. 2 mM EDTA, 2 mM EGTA, 30 mM Hepes (pH 7.5), phosphatase inhibitors (1 mM Na_3VO_4, 50 µM phenylarsine oxide, 30 mM p-nitrophenylphosphate) and a cocktail of protease inhibitors. Cells were further disrupted by repeated passages through an 18-Guage needle. After clarification (1.5 minute, 480 x g, 4°C), cell lysates (200 µl) were supplemented with 40% OptiPrep and 10% sucrose (600 µl total volume) and the mixtures was transferred to centrifuge tubes. The samples were overlaid with 30%, 25%, 20% (400 µl of each) and 0% OptiPrep (300 µl) in 0.2% TX-100 lysis buffer containing 10% sucrose, and centrifuged for 3 hours at 170,000 x g, 4°C (RCM 100 ultracentrifuge, Sorvall). Seven fractions of 300 µl were collected from the top of the gradient. Aliquots were subjected to SDS-page and immuno-detection of TfR and Lyn kinase were carried out.

RESULTS

Tf enhances temporary Cer generation in the plasma membrane.

In the present study, early time points of Tf uptaking resulted in cell surface Cer generation. Cer generation was early and temporarily, it was obviously increased after one min of Tf addition and then Cer rendered to its normal level. Cer level tends to increase slightly again after ten min of Tf addition (Fig. 1 A, B). The elevation of Cer level upon Tf uptaking was accessed by immunocytochemical detection of cell surface Cer (Fig. 1A) and was confirmed biochemically by Diacylgelycerol kinase (DGK) assay (Fig. 1B).The kinetics of Tf uptaking and recycling was studied to determine at which stage Cer is produced. jurkat cells started to internalize Tf after two min of Tf addition, and Tf was stayed intracellular till the minute 5 and then the recycling back to the extracellular milieu after the minute 5 (Fig. 1C). Depending on these data, it is clear that Tf attached to cell surface for 1 min and during this time Cer is generated on cell surface before Tf internalization. Upon internalization of Tf, Cer was rapidly disappeared or metabolized. Theses data indicate that Cer might play a role in the movement of TfR in the plane of the plasma membrane.

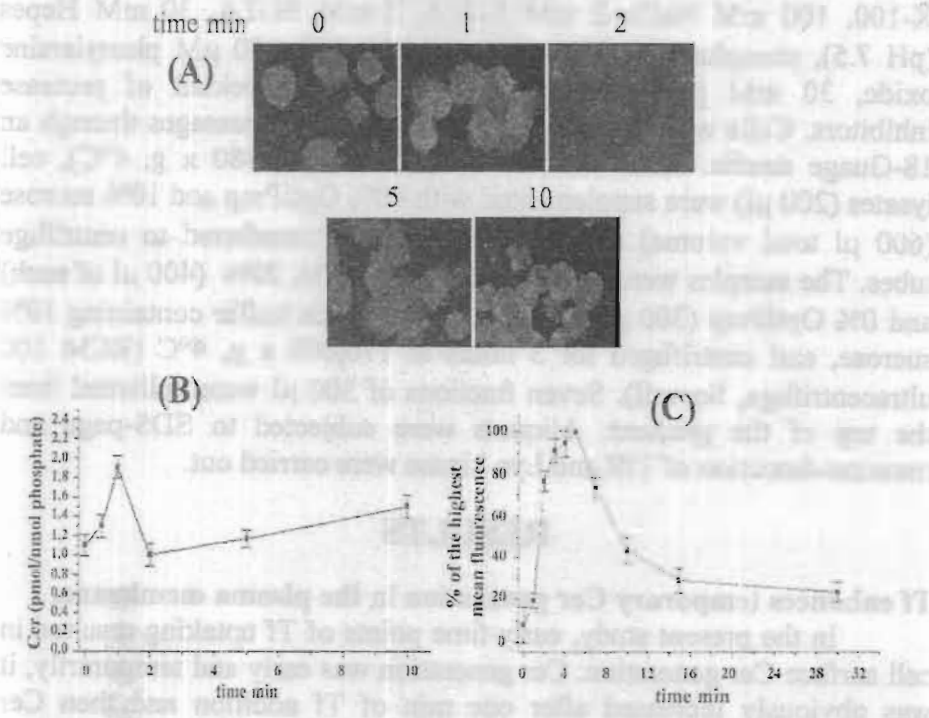

Fig. 1: Temporary and breif Cer elevation upon Tf treatment as shown by immunocytochemical detection of cell surface Cer (A) and by DGK assay (B).Tf uptaking and recycling kinetics in jurkat cells (C).

Activation of ASMase and inhibition of SMS upon Tf uptaking

Looking for the enzymes responsible for Cer generation, ASMase and NSMase activities were investigated during time course of Tf uptaking. ASMase was found to be activated in the early time points of Tf addition to cells (Fig. 2A), reaching its maximum activity at one min and then gradually return almost to its basic level of activity. It seems that ASMase is the only SMase that responsible for Cer generation; hence NSMase had almost no change of its activity (Fig. 2A). SMS activity was decreased dramatically in the same time frame and then returns gradually to the basic level of activity (Fig. 2B). It is clear that Cer generation is occurred by ASMase early and brief activation together with inhibition of SMS activity at the same time to maintain Cer level without metabolizing by SMS. After this time point, Cer seems to be metabolized by the basic activity level of SMS giving SM.

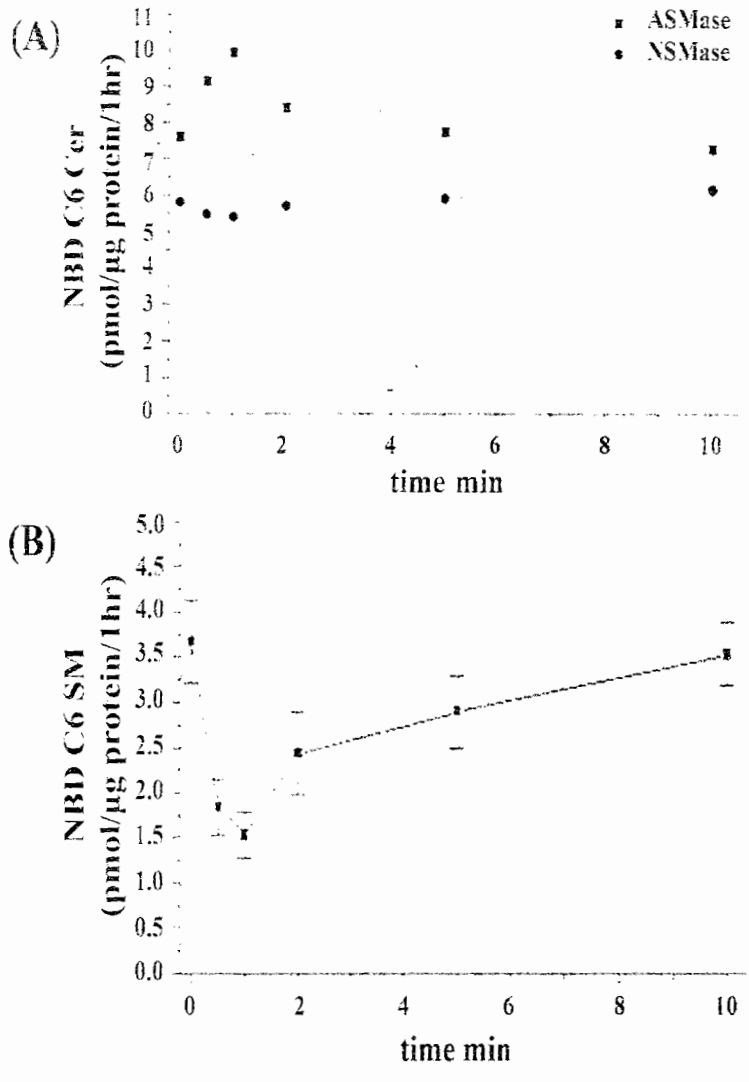

Fig. 2: Activation of ASMase but not NSMase (A) and downregulation of SMS activity upon Tf treatment (B).

Cer is important for Tf/TfR internalization through clathrin and avoiding rafts.

Tf was internalized and remains for relatively short time, almost 5 minutes (at least in jurkat cells) inside cells and then starts to be recycled back to the plasma membrane and then to the extracellular

space after the minute 5 as indicated from the Tf uptaking and recycling kinetic curve (Fig. 1C). To investigate the role of Cer in Tf uptaking and/or recycling, abrogation of Cer generation by inhibition of ASMase activity with Imipramine, and neutralization of cell surface Cer by anti-Cer mAb was carried out. The amount of intracellular Tf was estimated at 5 min and 15 min (the uptaking and recycling times, respectively). The percentage of intracellular fluorescent Tf at 15 min was 89 and 100 of the highest mean fluorescence in anti-Cer and Imipramine treated cells, respectively compared with 18 and 31 in control and irrelevant Ab treated cells, respectively (Fig. 3A). Whereas, Tf internalization (5 min) was not affected under such treatment. This observation lent the attention to the probability that Tf missed its way to the recycling endosomes. For more analysis of Tf recycling alteration under Cer abrogation, TfR redistribution on cell surface following Tf ligation was studied.

It is well known that TfR is one of the receptors that did not trap into membrane microdomains, known as rafts; it is frequently used as non-rafts marker. TfR avoids rafts to be collected and internalized through clathrin machinery. After 1 min of Tf treatment, cell fractionation study of cold Triton, TX-100 cell lysis revealed that, in control cells, TfR mainly concentrated in non-rafts fractions number 4-7, and no detection of TfR could be found in lighter fractions "rafts fractions" number 1 and 2 (Fig. 3B). Inhibition of Cer generation by Imipramine leads to a clear shift of the main bands of TfR towards lighter fractions number 1 and 2 (Fig. 3B), these fractions represents raft fractions as indicated by Lyn tyrosine kinase, one of the raft markers. It seems that Cer generation is so important for TfR to avoid raft trapping and direction to clathrin-coated pits, its proper site of collection and internalization. Taking together, inhibition of Cer generation upon Tf/TfR interaction, leads to trapping of TfR into rafts and then most probably internalized through raft machinery. This result confirms the assumed role of Cer in controlling TfR movement in the plane of the plasma membrane, away from rafts and towards clathrin coated pits.

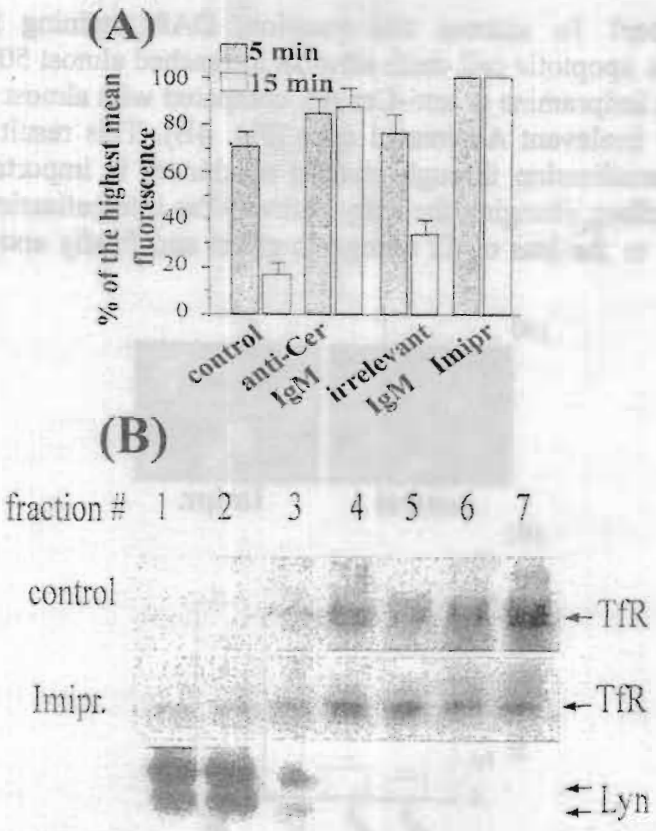

Fig. 3: Fluorescencet intinisty measurments of the intracellular Tf at 5 and 15 min of Tf treatment in control and after Cer abrogation, represented as the percentage of the highest mean fluorescence (A). Cell fractionation study showing the redistribution of TfR on cell surface after 1 min of Tf addition in control and in 30 μM Imipramine treated cells, raft fractions are indicated by Lyn, Tyrosine Kinase (B).

Internalization of Tf through non-clathrin machinery inhibits cell growth.

Tf is an important growth factor for cell growth and division, the importance of Tf as a growth factor is not restricted to its function as iron carrier, but TfΛTfR interaction has its own signaling pathway, which enhances cell growth and division. Hence TfR has its specific signaling and its specific entry pathways, it will be a matter of interest to investigate the role of Tf internalization pathway on cell growth, by other words, is clathrin an obligatory pathway for Tf to be a powerful

growth factor? To address this question, DAPI staining (Fig. 4A) revealed that apoptotic cell death after 24 hr reached almost 50% in cells treated with Imipramine or anti-Cer Ab, compared with almost 6% of the control and irrelevant Ab treated cells (Fig. 4B). This result indicated that Tf internalization through clathrin machinery is important for its mitogenic effect; changing the entry pathway "as internalization through rafts" leads to the loss of Tf mitogenic effect and finally apoptotic cell death.

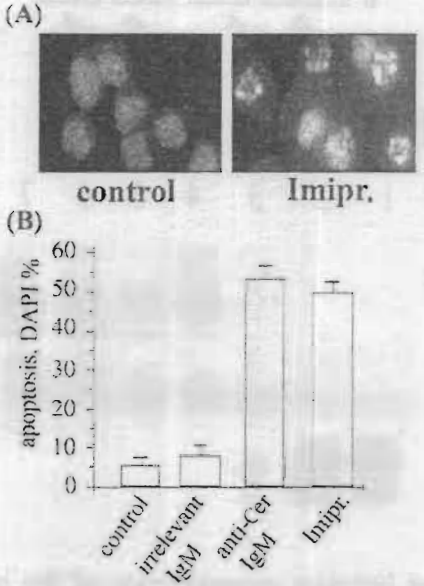

Fig. 4: Fluorescent microscopy of chromatin changes after 24 hrs of cell inoculation using DAPI staining in control and in 30 μM Imipramine treated cells (A). Percentage of apoptotic cell death as determined from DAPI staining after 24 hrs inoculation of cells under different treatments of Cer inhibition, cell with unhomogenous nucleus staining was considered as apoptotic, 200 cells at least were counted for each treatment for three independent experiments (B).

DISCUSSION

There are several mechanisms for internalizing different molecules from cell surface to various intracellular destinations. In general these mechanisms are clathrin coated-pits, non-clathrin coated pits, phagocytosis (for uptake of relatively large particles), caveolae/rafts, membrane ruffling and STEM formation (Surface-

connected Tubules Entering Macrophages) (Maxfield and McGraw, 2004). It is still an open question in the field of membrane trafficking is how distinct ligands follow a specific internalization pathway to different intracellular targets.

The current study through the light on a mechanism that may help in understanding the receptor movement on cell membrane and internalization through specific site of plasma membrane. The present data indicates that, when Tf attaches to its cell surface receptor, TfR, it takes about 1 min before internalization. Within the frame of this time, ASMase is briefly activated hydrolyzing cell surface SM, which is enriched in the membrane outer leaflet (Pomorski and Menon, 2006), giving rise to Cer. To maintain the level of Cer without metabolizing, SMS activity was decreased and then returns to its basic level of activation, returning Cer back to SM. The brief Cer generation upon TfR activation is so important to direct TfR towards clathrin coated pits and avoiding trapping into rafts.

ASMase is the best-characterized sphingomyelinase; it was shown to be involved in many forms of cellular activities. In addition, ASMase was shown to bind to the extracellular leaflet of the plasma membrane, however, it is still unknown whether this form of ASMase is a secretory, lysosomal or a third type of ASMase. Cer is a rapid moving lipid molecule; its release alters the biophysical characters of the membrane (Huang *et al.*, 1996). Cer molecules are spontaneously associated and tightly bind to each others resulting in the formation of Cer-enriched membrane microdomains. These microdomains have the tendency to fuse again with each others forming Cer-enriched macrodomains (Grassmé *et al.*, 2001a, b). Indeed, these Cer-enriched platforms are almost different from rafts (Megha, and London, 2004). Cer-enriched membrane platforms were shown to trap and cluster many receptors leading to sorting of specific proteins in a high density level for perfect signaling (Cremesti *et al.*, 2001; Nurminen *et al.*, 2002; Grassmé *et al.*. 2003). The role of cell surface Cer generation in receptor movement and sorting seems to be used by many receptors and agents (Pfeiffer *et al*, 2001; Grassmé *et al.*, 2002; Göggel *et al.*, 2004; Bionda *et al.*, 2007). The mechanism followed by these receptors to be sorted and clustered is poorly understood. One study mentioned to the importance of the transmembrane region of the receptor that determine trapping or excluding from membrane domains (Bock and Gulbins, 2003). Another study clarified that cell surface Cer is generated in two different membrane domains; one near the receptor and the other is inside rafts. By the spontaneous moving and tight binding of Cer molecules, the two

domains are bind to each other leading to trapping of receptor into rafts (Abdel Shakor *et al.*, 2004). SM found in different subpools on plasma membrane (Linardic and Hannun, 1994).

TfR has been shown in direct and physical association with T cell receptor and important for its activation (Batista *et al.*, 2004). It is well known that TCR is clustered into rafts upon activation, but TfR is aggregated in clathrin coated pits and avoiding rafts. This two different compartmentalization of two receptors what are in close and physical association with each others gives the impression of the presence of specific mechanism that differentiates between the two receptor clustering pathways. In this study, Cer is suggested to be the driving lipid molecule that differentiates between raft and clathrin trapping.

There is direct evidence that SM utilizes clathrin machinery upon cell entry (Koval and Pagano, 1990), but it is still unknown whether clathrin machinery differentiates between different SM pools. In other words, clathrin machinery uptake rafts or non-rafts SM or both. In recent studies (Deinhardt *et al.*, 2006; Masuyama *et al.*, 2009), some cell surface proteins or attached agents already found in lipid rafts but its internalization is performed through clathrin machinery. this means that rafts or non-rafts SM can be internalized through clathrin machinery depending on the protein or agent to be internalized.

In the current study, the part of TfR that trapped into rafts upon Cer inhibition is most probably not internalized through clathrin coated pits because there was altered Tf recycling. The molecular mechanism followed by Cer to direct TfR towards clathrin pits and to avoid rafts however needs to be clarified. There was a detectable slight increasing of Cer level at 10 min after Tf addition to cells; this increasing may be according to a second round of Tf internalization after the fast recycling that occurs at 8-10 minutes. Tf should be internalized through clathrin machinery to recycle back to extracellular space for another round of internalization. Miss-internalization of Tf/TfR leads to accumulation of Tf inside cells without recycling as shown in the present study.

This accumulation of Tf inside cells leads to loss of mitogenic capacity of Tf and growth arrest and finally death. The loss of Tf mitogenic function is more probably not related to iron transportation, because cells can take up iron by different ways as low molecular weight iron chelates, non-receptor mediated Tf internalization and intercellular stores as ferritin, but however cells are in need of Tf/TfR for proliferation (Brock, 1981). It seems that Tf has an additional role unrelated to its iron-donating properties (Brock and Mainou – Fowler, 1983; Bomford *et al.*, 1983). Taken together, Tf/TfR interaction and

their proper internalization pathway through clathrin machinery might itself provide some intracellular signaling that enables cell growth and division.

The present data clarify the role of early and brief Cer elevation in the direction of TfR towards clathrin coated pits and avoiding raft trapping, this role may be applicable for other receptors and agents that internalize via clathrin machinery.

ACKNOWLEDGMENT

Great appreciation to the group of cell biology, Faculty of Science. Zoology Dept. Alexandria University, Damanhour branch, Damanhour, Egypt for supplementation of DGK membranes.

REFERENCES

Abdel Shakor, A.B.; Kwiatkowska, K. and Sobota, A. (2004): Cell surface ceramide generation precedes and controls Fc gamma RII clustering and phosphorylation in rafts. J. Biol. Chem. 279: 36778-36787.

Batista, A.; Millan, J.; Mittelbrunn, M.; Sanchez-Madrid, F. and Alonso, M.A. (2004): Recruitment of transferrin receptor to immunological synapse in response to TCR engagement. J. Immunol. 172: 6709-6714.

Bielawska, A.; Perry, D.K. and Hannun, Y.A. (2001): Determination of ceramides and diglycerides by the diglyceride kinase assay. Anal. Biochem. 298: 141-150.

Bionda, C.; Hadchity, E.; Alphonse, G.; Chapet, O.; Rousson, R.; Rodriguez-Lafrasse, C. and Ardail, D. (2007): Radioresistance of human carcinoma cells is correlated to a defect in raft membrane clustering. Free Radic. Biol. Med. 43: 681-94.

Bligh, E.G. and Dayer, W.J. (1959): A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

Bock, J. and Gulbins, E. (2003): The transmembranous domain of CD40 determines CD40 partitioning into lipid rafts. FEBS Lett. 534: 169-174.

Bomford, A.; Young, S.P.; Nouri-Aria, K. and Williams, R. (1983): Uptake and release of transferrin and iron by mitogen-stimulated human lymphocytes. Br. J. Haematol. 55: 93-101.

Boniface, J.J.; Rabinowitz, J.D.; Wülfing, C.; Hampl, J.; Reich, Z.; Altman, J.D.; Kantor, R.M.; Beeson, C.; McConnell, H.M. and Davis, M.M. (1998): Initiation of signal transduction through the T cell receptor requires the multivalent engagement of peptide/MHC ligands. Immunity. 9: 459-466.

Brock, J.H. (1981): The effect of iron and transferrin on the response of serum-free cultures of mouse lymphocytes to concanavalin A and lipopolysaccharide. Immunology. 43: 387-392.

Brock, J.H. and Mainou–Fowler, T. (1983): The role of Iron and transferrin in lymphocyte transformation. Immunol. Today. 4: 347-351.

Cremesti, A.; Paris, F.; Grassmé, H.; Holle,r N.; Tschopp, J.; Fuks, Z.; Gulbins, E. and Kolesnick R. (2001): Ceramide enables fas to cap and kill. J. Biol. Chem. 276: 23954-23961.

De Camilli, P.; Emr, S.D.; McPherson, P.S. and Novick, P. (1996): Phosphoinositides as regulators in membrane traffic. Science. 271: 1533-1539.

Deinhardt, K.; Berninghausen, O.; Willison, H.J.; Hopkins, C.R. and Schiavo, G. (2006): Tetanus toxin is internalized by a sequential clathrin-dependent mechanism initiated within lipid microdomains and independent of epsin1. J. Cell Biol. 174: 459-471.

Di Paolo, G.; Moskowitz, H.S.; Gipson, K.; Wenk, M.R.; Voronov, S., Obayashi, M.; Flavell, R.; Fitzsimonds, R.M.; Ryan, T.A. and De Camilli, P. (2004): Impaired PtdIns(4,5)P2 synthesis in nerve terminals produces defects in synaptic vesicle trafficking. Nature, 431: 415-422.

Du, G.; Altshuller, Y.M.; Vitale, N.; Huang, P.; Chasserot-Golaz, S.; Morris, A.J.; Bader, M.F. and Frohman, M.A. (2003): Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs. J. Cell Biol. 162: 305-315.

Du, G.; Huang, P.; Liang, B.T. and Frohman, M.A. (2004): Phospholipase D2 localizes to the plasma membrane and regulates angiotensin II receptor endocytosis. Mol. Biol. Cell. 15: 1024-1030.

Dumitru, C.A. and Gulbins, E. (2006): TRAIL activates acid sphingomyelinase via a redox mechanism and releases ceramide to trigger apoptosis. Oncogene, 25: 5612-5625.

Göggel, R.; Winoto-Morbach, S.; Vielhaber, G.; Imai, Y.; Lindner, K.; Brade, L.; Brade, H.; Ehlers, S.; Slutsky, A.S.; Schütze, S.;

Gulbins, E. and Uhlig, S. (2004): PAF-mediated pulmonary edema: a new role for acid sphingomyelinase and ceramide. Nat. Med. 10: 155-160.

Grassmé, H.; Jekle, A.; Riehle, A.; Schwarz, H.; Berger, J.; Sandhoff, K.; Koleśnick, R. and Gulbin,s E. (2001a): CD95 signaling via ceramide-rich membrane rafts. J. Biol. Chem. 276: 20589-20596.

Grassmé, H.; Schwarz, H. and Gulbins, E. (2001b): Molecular mechanisms of ceramide-mediated CD95 clustering. Biochem. Biophys. Res. Commun. 248: 1016-1030.

Grassmé, H.; Jendrossek V.; Bock J.; Riehle A. and Gulbins E. (2002): Ceramide-rich membrane rafts mediate CD40 clustering. J. Immunol. 168: 298-307.

Grassmé, H.; Cremesti, A.; Kolesnick, R. and Gulbins, E. (2003): Ceramide-mediated clustering is required for CD95-DISC formation. Oncogene, 22: 5457-5470.

Gulbins, E. and Kolesnick, R. (2003): Raft ceramide in molecular medicine. Oncogene, 22: 7070-7077.

Hannun, Y.A. and Obeid, L.M. (2008): Principles of bioactive lipid signalling: lessons from sphingolipids. Nat. Rev. Mol. Cell Biol. 9: 139-150.

Huang, H.W., Goldberg, E.M., Zidovetzki, R. (1996): Ceramide induces structural defects into phosphatidylcholine bilayers and activates phospholipase A2. Biochem. Biophys. Res. Commun. 220: 834-838.

Huebers, H.A. and Finch, C.A. (1987): The physiology of transferrin and transferrin receptors. Physiological Reviews, 67: 520-582.

Hughes, W.E. and Parker, P.J. (2001): Endosomal localization of phospholipase D 1a and 1b is defined by the C-termini of the proteins, and is independent of activity. Biochem J. 356: 727-736.

Kolesnick, R.N.; Goñi, F.M. and Alonso, A. (2000): Compartmentalization of ceramide signaling: physical foundations and biological effects. J. Cell Physiol. 184: 285-300.

Korzeniowski, M.; Kwiatkowska, K. and Sobota, A. (2003): Insights into the association of FcgammaRII and TCR with detergent-resistant membrane domains: isolation of the domains in detergent-free density gradients facilitates membrane fragment reconstitution. Biochemistry. 42: 5358-5367.

Koval, M. and Pagano, R.E. (1990): Sorting of an internalized plasma membrane lipid between recycling and degradative pathways in normal and Niemann-Pick, type A fibroblasts. J. Cell Biol. 111: 429-442.

Linardic, C.M. and Hannun, Y.A. (1994): Identification of a distinct pool of sphingomyelin involved in the sphingomyelin cycle. J. Biol. Chem. 269: 23530-23537.

Masuyama, N.; Kuronita, T.; Tanaka, R.; Muto, T.; Hirota, Y.; Takigawa, A.; Fujita, H.; Aso, Y.; Amano, J. and Tanaka, Y. (2009): HM1.24 Is Internalized from Lipid Rafts by Clathrin-mediated Endocytosis through Interaction with {alpha}-Adaptin. J. Biol. Chem. 284: 15927-15941.

Maxfield, F.R. and McGraw, T.E. (2004): Endocytic recycling. Nat. Rev. Mol. Cell Biol. 2: 121-132.

Megha, London E. (2004): Ceramide selectively displaces cholesterol from ordered lipid domains (rafts): implications for lipid raft structure and function. J. Biol. Chem. 27: 99997-10004.

Monks, C.R.; Freiberg, B.A.; Kupfer, H.; Sciaky, N. and Kupfer, A. (1998): Three-dimensional segregation of supramolecular activation clusters in T cells. Nature, 395: 82-86.

Nurminen, T.A.; Holopainen, J.M.; Zhao, H. and Kinnunen PK. (2002): Observation of topical catalysis by sphingomyelinase coupled to microspheres. J. Am. Chem. Soc. 124: 12129-12134.

Okazaki, T.; Bell, R.M. and Hannun, Y.A. (1989): Sphingomyelin turnover induced by vitamin D3 in HL-60 cells. Role in cell differentiation. J. Biol. Chem. 264: 19076-19080.

Padron, D.; Tall, R.D. and Roth, M.G. (2006): Phospholipase D2 is required for efficient endocytic recycling of transferrin receptors. Mol Biol Cell. 17: 598-606.

Pfeiffer, A.; Böttcher, A.; Orsó, E.; Kapinsky, M.; Nagy, P.; Bodnár, A.; Spreitzer, I.; Liebisch, G.; Drobnik, W.; Gempel, K.; Horn, M.; Holmer, S.; Hartung, T.; Multhoff, G.; Schütz, G.; Schindler, H.; Ulmer, A.J.; Heine, H.; Stelter, F.; Schütt, C.; Rothe, G.; Szöllôsi, J.; Damjanovich, S. and Schmitz, G. (2001): Lipopolysaccharide and ceramide docking to CD14 provokes ligand-specific receptor clustering in rafts. Eur. J. Immunol. 31: 3153-3164.

Pomorski, T. and Menon, A. (2006): Lipid flippases and their biological functions. Cell Mol. Life Sci. 63: 2908-2921.

Roth, M.G. (1999): Lipid regulators of membrane traffic through the Golgi complex. Trends Cell Biol. 9: 174-179.

Salmeron, A.; Borroto, A.; Fresno, M.; Crumpton, M.J.; Ley, S.C. and Alarcon, B. (1995): Transferrin receptor induces tyrosine phosphorylation in T cells and is physically associated with the TCR zeta-chain. J. Immunol. 15: 1675-1683.

Schissel, S.L.; Jiang, X.; Tweedie-Hardman, J.; Jeong, T.; Camejo, E.H.; Najib, J.; Rapp, J.H.; Williams, K.J. and Tabas, I. (1998): Secretory sphingomyelinase, a product of the acid sphingomyelinase gene, can hydrolyze atherogenic lipoproteins at neutral pH. Implications for atherosclerotic lesion development. J. Biol. Chem. 273: 2738-2746.

Tanaka, K.; Ogawa, N.; Asanuma, M.; Kondo, Y. and Nomura, M. (1996): Relationship between cholinergic dysfunction and discrimination learning disabilities in Wistar rats following chronic cerebral hypoperfusion. Brain Res. 729: 55-65.

Veiga, M.P.; Arrondo, J.L.; Goñi, F.M. and Alonso, A. (1999): Ceramides in phospholipid membranes: effects on bilayer stability and transition to nonlamellar phases. Biophys. J. 76: 342-350.

Yamaoka, S.; Miyaji, M.; Kitano, T.; Umehara, H. and Okazaki, T. (2004): Expression cloning of a human cDNA restoring sphingomyelin synthesis and cell growth in sphingomyelin synthase-defective lymphoid cells. J. Biol. Chem. 279: 18688-18693.