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Depletion of membrane cholesterol causes ligand-independent activation of Fas and apoptosis

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Abstract

Fas is a member of the tumour necrosis factor receptor superfamily. Fas-mediated apoptosis is an essential mechanism protecting against skin cancer. Activation of Fas by specific ligand or agonistic antibodies leads to the formation of a membrane associated death-inducing signalling complex comprising aggregates of Fas, the Fas-associated death domain protein (FADD), and caspase-8. It has recently been suggested that activity of Fas is not only regulated by its cognate ligand but also by the association of this receptor with cholesterol-enriched lipid domains in the plasma membrane (lipid rafts). We report here that disruption of lipid rafts by cholesterol-depleting compounds (methyl-β-cyclodextrin, filipin III, cholesterol oxidase, and mevastatin) leads to a spontaneous clustering of Fas in the non-raft compartment of the plasma membrane, formation of Fas–FADD complexes, activation of caspase-8, and apoptosis. We propose that in some cell types exclusion of Fas from lipid rafts leads to the spontaneous, ligand-independent activation of this death receptor, a mechanism that can potentially be utilized in anticancer therapy.

Keywords: Apoptosis; CD95; Cholesterol; Fas; Keratinocytes; Lipid rafts

Cholesterol is an abundant component of plasma membranes of eukaryotic cells and is an essential regulator of membrane fluidity, permeability, receptor function, and ion channel activity [1–4]. The lateral distribution of cholesterol in the membranes is not uniform and its content is particularly high in the submicroscopic areas also enriched in gangliosides and sphingolipids. These microdomains known as lipid rafts, act as molecular platforms that spatially organize membrane receptor molecules [3,5]. The association of receptors with lipid rafts often enhances the efficacy of signalling, as has been shown for B- and T-cell antigen receptors [5,6]. However, other membrane receptors (e.g., epidermal growth factor receptor, EGFR) seem to be silenced rather than activated after redistribution to lipid rafts [7].

Recently, some proteins from the tumour necrosis factor receptor (TNFR) superfamily (TNFR1, CD40, and CD95/Fas) have been shown to interact with lipid rafts [8–12]. This issue is especially interesting in view of the central importance of these receptors in the regulation of inflammatory processes and apoptosis.

Fas (also known as APO-1 or CD95) is the most important membrane death receptor responsible for induction of apoptosis in a variety of cell types. Activation of Fas can be achieved either in the specific ligand (FasL), or non-specifically by ultraviolet radiation, cytotoxic drugs or reactive oxygen species [13–19]. Redistribution of Fas to cholesterol-enriched lipid domains has been proposed to be an important regulatory step during activation of this death receptor. In spite of the fact that some authors found no relationship between Fas signalling and association with rafts [20], several studies on lymphoid cells revealed that activation of Fas by FasL or stimulatory antibodies produces redistribution of Fas to lipid rafts [9,10,21]. Membrane cholesterol depletion by methyl-β-cyclodextrin (MβCD), a widely used tool for lipid raft disruption, blocks the ligand-induced apoptosis and prevents Fas clustering in response to antibody stimulation [12,21]. It has been proposed that association with rafts favours a spontaneous

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formation of Fas microaggregates which amplify receptor signalling [12].

In epidermal keratinocytes Fas-mediated apoptosis is a main protective mechanism eliminating premalignant cells during progression of skin cancer. Blockade of Fas-mediated apoptosis results in an increased incidence of squamous cell carcinoma [22]. On the other hand, excessive activation of Fas can also be deleterious and is involved in some serious skin disorders, such as toxic epidermal necrolysis and graft-versus-host disease [23,24]. Taking into account this central role of Fas in skin pathology, we decided to study the effects of cholesterol depletion on apoptosis and Fas activity in keratinocyte cell line HaCaT. These cells are widely used in the study of epidermal biology and constitute a spontaneously transformed, non-tumorigenic line derived from human keratinocytes.

Materials and methods

Cell culture, cholesterol depletion, and cell survival assay. HaCaT cell line was originally obtained from Dr. Mark Pittelkow (Mayo Clinic, Rochester, MI, USA) and the cells were routinely cultured in Dulbecco's modified essential medium (DMEM) with 10% foetal calf serum (FCS). Before the treatment with cholesterol-depleting agents the cells were switched to the serum-free DMEM. The following cholesterol modifying agents were used: methyl-β-cyclodextrin (MβCD), filipin III, cholesterol oxidase, and hydroxymethyl glutaryl-coenzyme A reductase inhibitor, mevastatin (all from Sigma–Aldrich, St. Louis, MO, USA). For the clonogenic assay the HaCaT cells were seeded on 10 cm petri dishes at a concentration 300 cells/dish and allowed to adhere overnight. The following day the cells were treated with the cholesterol modifying agents as indicated and cultured in DMEM with 10% FCS for additional 2 weeks. Cells were fixed in formaldehyde and stained with crystal violet. Visible colonies larger than 2 mm in diameter were counted manually.

Apoptosis assays. Cell death detection ELISA (Roche Diagnostics) was used to measure the enrichment of mono and oligonucleosomes released into the cytoplasm according to the protocol provided by the manufacturer. Induction of apoptosis-related caspase-8 activity was measured with dedicated, commercially available fluorescent assay (HTS Caspase 8 Activity Assay, Oncogene Research Products) as suggested by the producer.

Confocal laser scanning microscopy. The cells were cultured on the LabTek chamber slides to approximately 80% confluence. At different times after the treatment the cells were fixed in cold acetone, rehydrated, and stained with mouse anti-Fas (Dako, Glostrup, Denmark) with FITC–labelled cholera toxin B subunit (CTx–FITC) or rabbit anti-FADD antibody (H-181, Santa Cruz Biotechnology) followed by secondary FITC- or Texas red labelled antibodies (Jackson Laboratories). Fluorescence was detected by confocal laser scanning microscopy using 488 and 568 nm excitation lines from argon–krypton laser (Olympus FluoView Confocal System).

Fas immunoprecipitation. Fas immunocomplex precipitation was performed according to previously published protocols [21,25]. Briefly, cells were suspended in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and Roche's complete protease inhibitor), lysed by two passes through a 21-gauge needle, pre-cleared, and immunoprecipitated overnight at 4 °C with 1 μg polyclonal goat anti-Fas antibody (Santa Cruz Biotechnology) and protein G–Sepharose (Amersham–Pharmacia Biotech). Immunocomplexes were resolved by SDS–PAGE, blotted on PVDF membranes, and probed with murine anti-Fas (clone B-10, Santa Cruz) and rabbit anti-FADD (H-181, Santa Cruz). Secondary anti-mouse and anti-rabbit antibodies labelled with 700 and 800 nm IRDyes, respectively, were used for blot detection in the infrared Odyssey imaging system (Li-Cor Lincoln, NE).

Sucrose gradient ultracentrifugation. Non-raft portions of the membranes were solubilized in 1% Brij 98 for 5 min at 37 °C, essentially as described previously [9,26]. The lysates were fractionated on the discontinuous sucrose gradient at 45,000*g* overnight at 4 °C. Light fractions containing rafts (R) and heavy non-raft (NR) fractions containing other membrane fragments and soluble proteins were immunoprecipitated with anti-Fas antibody and the complexes were resolved by SDS–PAGE and blotted with anti-Fas and anti-FADD antibodies.

Results and discussion

In the course of preliminary experiments we noted that in HaCaT cells apoptosis could be induced by a lipid raft disrupting agent, MβCD alone (Fig. 1 and manuscript in preparation). This finding was unexpected and contrasted with the earlier data on lymphoid cells where MβCD inhibited rather than stimulated apoptosis [9,12,21]. In order to determine whether the induction of apoptosis was specific to MβCD or rather induced by cholesterol depletion, we treated the cells with other agents known to specifically deplete membrane cholesterol: filipin III, cholesterol oxidase, and a cholesterol synthesis blocker, mevastatin. As shown in Fig. 1 all substances induced apoptosis in a time- and concentration-dependent manner. Non-specific detergents (Triton X-100, Brij 98) that solubilize membrane lipids but spare lipid rafts induced membrane disruption and necrosis, but never apoptosis at any concentration tested (0.001–1% for 5 min, 2 h) (data not shown).

MβCD-induced apoptosis was associated with an increased caspase-8 activity, which suggested involvement of membrane death receptors, possibly Fas (Fig. 1E). Further support to this hypothesis was provided by fluorescence imaging of Fas receptor (Fig. 2). All tested cholesterol-depleting agents caused a striking aggregation of Fas in the membranes that was perceptible from 5 to 10 min (MβCD, filipin, and cholesterol oxidase) to 1 h (mevastatin) after treatment and peaked at approximately 2 h later. Fas aggregation seemed to be functional, since receptor clusters co-localized with FADD immunoreactivities (Fig. 2H). To further substantiate this claim, we immunoprecipitated DISC in MβCD-treated cells by an anti-Fas antibody and separated protein complexes by SDS–polyacrylamide gel electrophoresis. Western blotting revealed increased amounts of FADD in anti-Fas precipitates indicating DISC formation after MβCD treatment (Fig. 3A).

Our data suggested that in keratinocytes, unlike in the cells of lymphoid origin, depletion of cholesterol initiates the ligand-independent Fas clustering and DISC formation. In HaCaT cells the behaviour of Fas

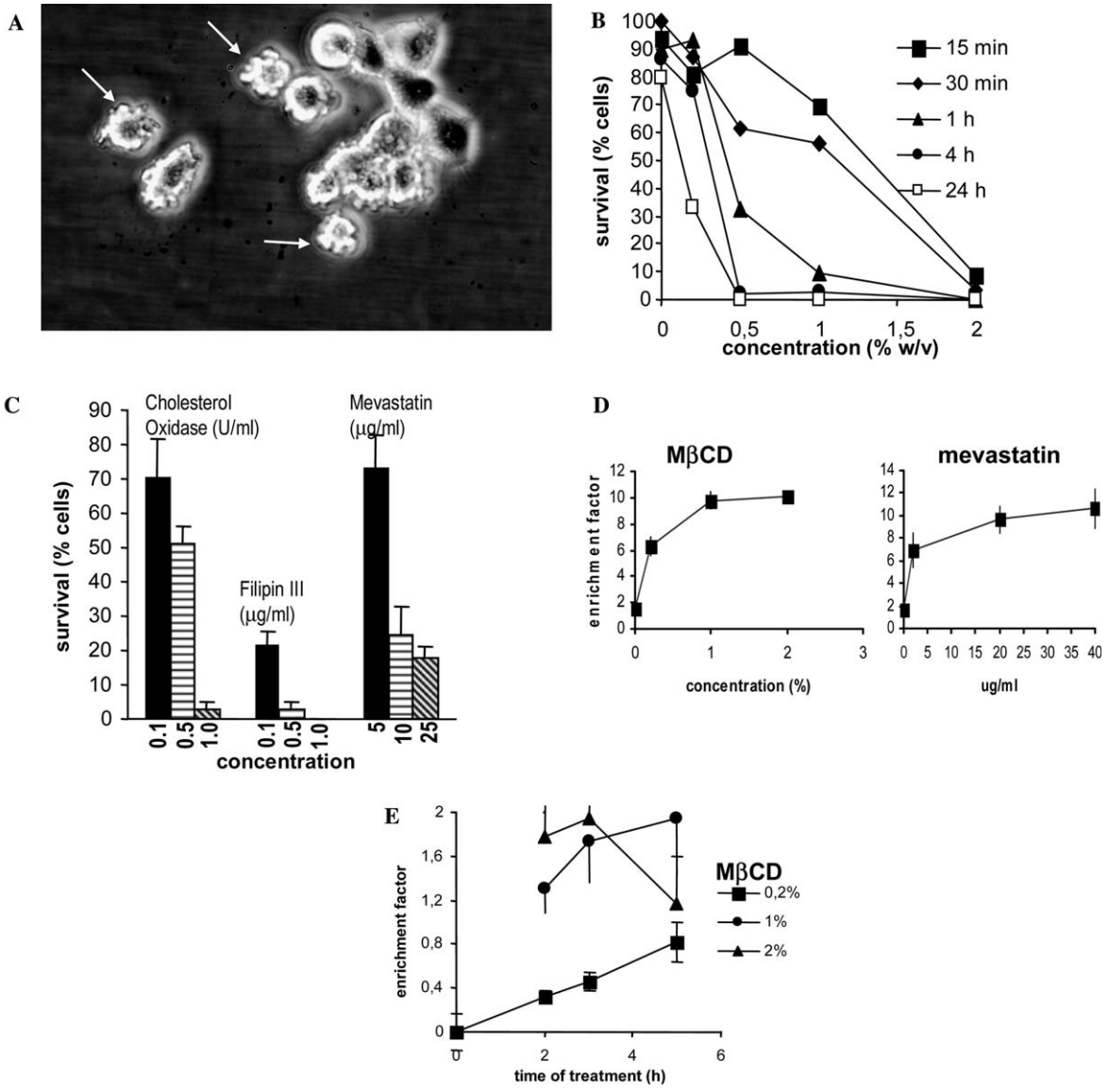

Fig. 1. Cholesterol depletion causes apoptosis of cultured HaCaT keratinocytes. (A) Apoptotic morphology comprising cell rounding, pyknosis, detachment, and cytoplasmic budding (arrows) in response to MβCD, filipin III, cholesterol oxidase, or mevastatin. This figure shows the effect of 1% MβCD for 6 h. (B,C) Reduction in cell survival measured by clonogenic assay after treatment with the cholesterol-modifying agents: MβCD 0.2–2% for 15 min, 24 h (B) or cholesterol oxidase (0.1–1 U/ml), filipin III (0.1–1 μg/ml), and mevastatin (1–25 μg/ml) for 6 h (C). Data show the proportion of control, $n = 3$ (independent experiments) ± SD. (D) Oligonucleosome DNA fragmentation measured by the cell death detection ELISA 20 h after the 6 h treatment with MβCD or mevastatin. Mean values ($n = 3$) ± SD. (E) Induction of caspase-8 activity after incubation with MβCD at concentrations 0.2–2% for 2–6 h. Mean ($n = 3$) ± SD.

resembles that of EGFR that also becomes spontaneously activated by MβCD [7,27–29]. Release of EGFR from lipid rafts is believed to relieve the functional inhibition of this receptor. We speculated whether the same mechanism operates in case of Fas. A helpful clue was the fact of the increased total immunoreactivity of Fas after MβCD treatment (compare Fas-specific red fluorescence intensities in Figs. 2A and E). A similar phenomenon has previously been noted in relation to EGF expression in MβCD-treated cells and occurs due to an unmasking of receptor molecules by cholesterol-depleting agents [7,28]. Another helpful observation was an apparently independent distribution of Fas aggregates and membrane ganglioside GM1. The latter was detected with the use of the FITC-labelled cholera toxin B subunit (CTx–FITC) that is a useful, specific probe of raft-associated ganglioside ([3,30] and references therein). To test this hypothesis we have separated the membrane fractions into lipid raft and non-raft fractions by discontinuous density gradient ultracentrifugation [9] and immunoprecipitated Fas with specific antibodies (Fig. 3B). In untreated, control keratinocytes the membrane-bound Fas is found predominantly in buoyant, lipid-enriched, raft

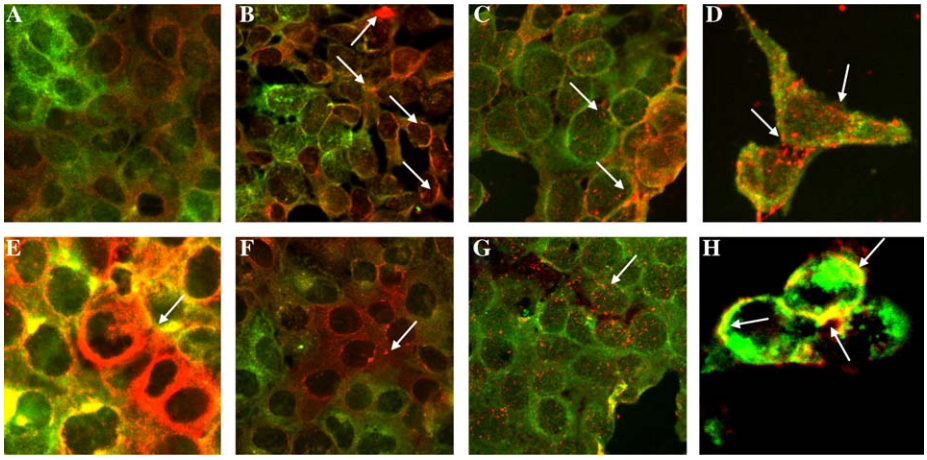

Fig. 2. Induction of Fas clustering in HaCaT cells after cholesterol depletion. Cells were treated with cholesterol-depleting agents. (A) Control; (B,H) 1% MβCD 10 min; (C) 1% MβCD 30 min; (D) 1% MβCD 120 min; (E) filipin III 0.5 μg/ml, 5 min; (F) cholesterol oxidase 1 U/ml, 15 min; and (G) mevastatin 5 (g/ml, 3 h). Arrows in (B–G) mark Fas aggregates (red). Note lack of correlation between raft-enriched regions (CTx–FITC^{bright}, green) and Fas aggregates. (H) An overlap between Fas (green) and FADD (red) immunoreactivities suggesting formation of active Fas–FADD complexes (arrows). Image dimensions are 100 × 100 μm (A–C,E–G) and 25 × 25 μm (D,H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

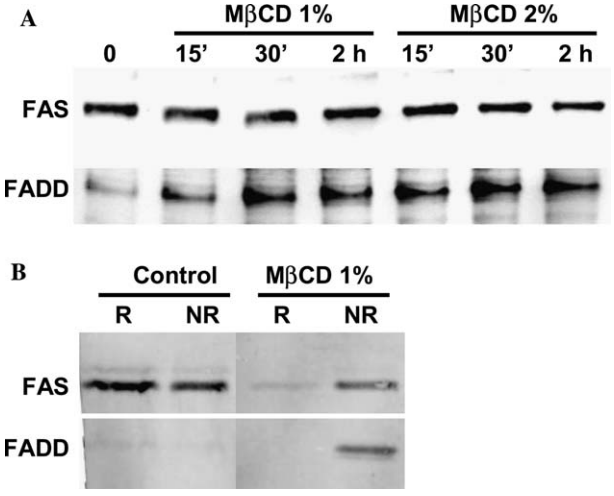

Fig. 3. Activation of Fas and formation of Fas–FADD complexes in HaCaT cells after cholesterol disruption with MβCD. (A) Fas immunocomplexes were precipitated from homogenates prepared from the cells treated with 1–2% MβCD for indicated periods of time. (B) Dissociation of activated Fas from lipid rafts upon cholesterol depletion. Cells were treated for 1 h with 1% MβCD, lysed, and separated by sucrose gradient ultracentrifugation. Light fractions containing rafts (R) and heavy non-raft (NR) fractions containing other membrane fragments and soluble proteins were immunoprecipitated with anti-Fas antibody, resolved by SDS–PAGE, and blotted with anti-Fas and anti-FADD antibodies. Treatment with MβCD resulted in the translocation of a portion of Fas to the non-raft fractions and recruitment of FADD. Experiments were repeated twice with similar results.

compartment. Treatment with MβCD causes a redistribution of a portion of Fas to the non-raft compartment. FADD was also present in this fraction supporting the hypothesis that dissociation of Fas from rafts is associated with receptor activation and DISC formation (Fig. 3B).

This study shows that unlike in lymphoid cells, cholesterol depletion from the membranes causes Fas activation and apoptosis in keratinocytes. The reason for this cell type specificity remains unknown at present. One explanation could be different composition and structure of raft and non-raft domains of plasma membranes. In lymphocytes lipid rafts have submicron dimensions whereas in keratinocytes the rafts form very large aggregates ("flotillas") which may comprise the majority of plasma membrane [30]. It is possible that very large amounts of cholesterol in keratinocyte membrane alter Fas function by changing the biophysical properties of the membrane [29] or inducing conformational changes in the receptor. It is also conceivable that yet unidentified lipid or protein component of the rafts inhibits Fas function in the cell type specific mode.

Over the past decade several in vitro studies have shown that cholesterol synthesis blockade by statins have antiproliferative, proapoptotic, and radiosensitizing, properties in many cancer cell types [31–33]. Several mechanisms have been proposed to account for this effect, including proteasome inhibition [34] and direct cholesterol depletion [33,35]. Several statins are currently in clinical trials for the therapy of cancer. Current data demonstrating the ligand-independent activation of Fas (and probably other death receptors) in response to cholesterol depletion may provide further rationale to the use of cholesterol modulating compounds in cancer therapy.

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