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# siRNA knockdown of ADAM-10, but not ADAM-17, significantly reduces fractalkine shedding following pro-inflammatory cytokine treatment in a human adult brain endothelial cell line

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## HIGHLIGHTS

- ADAM-10 siRNA treated cells shed significantly less fractalkine under TNF treatment.
- ADAM-17 siRNA treated cells shed control levels of fractalkine under TNF treatment.
- ADAM-10 is responsible for TNF-induced shedding of fractalkine in hCMEC/D3 cells.

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## ABSTRACT

Fractalkine shedding is believed to occur constitutively and following induction via the activity of two membrane-bound enzymes, ADAM-10 and ADAM-17. However, our previous work suggested that ADAM-17 is not involved in the proteolytic release of fractalkine under TNF treatment of a human adult brain endothelial cell line, hCMEC/D3. The pro-inflammatory cytokine, TNF, has previously been shown to be expressed in the perivascular cuffs in multiple sclerosis. Here we sought to identify, using siRNAs to silence the expression of ADAM-10 and ADAM-17, whether ADAM-10 is responsible for TNF-induced shedding of fractalkine from the cell membrane in hCMEC/D3. Our findings suggest that ADAM-10, and not ADAM-17, is the major protease involved in fractalkine release under pro-inflammatory conditions in this human adult brain endothelial cell model.



## 1. Introduction

Fractalkine (CX3CL1) is the only member of the CX3C class of chemokines and possesses an unusual property in that in its membrane-bound form it acts as an adhesion molecule and when it is shed from the cell membrane it acts as a chemokine [2]. Fractalkine is expressed in various tissues of the body and within the central nervous system (CNS) it has been found to be localised to astrocytes, neurons and endothelial cells [2,15,26]. The chemotactic and adhesive properties of fractalkine are mediated through fractalkine's receptor, CX3CR1, which is expressed on T cells, NK cells, macrophages and monocytes [2,12,19], strongly suggesting fractalkine plays a significant role in neuroimmune cross talk, particularly in CNS disorders [21]. Indeed, fractalkine shedding is thought to help mediate the migration of microglia and monocytes to sites of neuronal injury contributing to neuronal cell death [6]. Conversely, CX3CR1 knockout mice experience more extensive neuronal cell loss and microglial activation than their littermate controls in various neurodegenerative paradigms, suggesting fractalkine signalling is an essential regulator of inflammatory neurotoxicity [5]. Increased intrathecal and serum levels of fractalkine have been described in various inflammatory neurological disorders including HIV and multiple sclerosis (MS) [20,31], and fractalkine expression and shedding in endothelial cells has been shown to be induced in vitro by various inflammatory cytokines [2,9,18,24].

Ectodomain shedding of membrane proteins is involved in a number of pathophysiological processes, including inflammation, cell degeneration, apoptosis and oncogenesis (for review see [7]). It provides a post-translational mechanism by which the surface expression of a membrane protein can be down-regulated and its function modulated. Shedding can be induced by a number of factors including phorbol 12-myristate 13-acetate (PMA), ionophores, growth factors, cholesterol depletion and cytokine treatment [8,23,27,30]. In the case of fractalkine, ectodomain release determines whether the molecule acts as an adhesion

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molecule or as a chemokine. Cytokine-induced ectodomain shedding has not been investigated extensively, which with regard to MS is surprising given that a number of pro-inflammatory cytokines have been found to be expressed within the perivascular cuffs [29,36] and therefore can directly activate the CNS endothelium, inducing the shedding of a number of membrane-bound molecules, which are reported to be increased in MS patient serum, e.g. vascular cell adhesion molecule-1 (VCAM-1) [4], intercellular adhesion molecule-1 (ICAM-1) [32] and fractalkine [20].

Fractalkine has been found to be proteolytically cleaved by ADAM-17 under PMA-induction in transfected human umbilical vein endothelial cells (HUVECs) and murine fibroblast cells [10,34], whereas ADAM-10 is responsible for its constitutive shedding in transfected HUVECs, and murine and primate fibroblast cells [16]. The physiological counterpart of PMA induction is unknown however, and previous work using a human adult brain endothelial cell line, hCMEC/D3, has shown that following TNF administration, an increase in fractalkine shedding is not associated with an increase in ADAM-17 expression or activity. Thus, suggesting that under TNF treatment, ADAM-17's role as a fractalkine sheddase is limited [18].

Here we sought to determine whether ADAM-10 is the major protease responsible for fractalkine shedding following TNF treatment in hCMEC/D3 cells using siRNA to independently and in combination knockdown ADAM-10 and ADAM-17 expression. This study revealed that under TNF treatment ADAM-10, and not ADAM-17, silencing significantly affected fractalkine shedding, suggesting ADAM-10 activity is responsible for the pathophysiological release of this chemokine under inflammatory conditions.

## 2. Materials and methods

The human adult brain endothelial cell line, hCMEC/D3 (a kind gift from Dr. P-O Couraud [35]), was cultured as previously described [18]. During siRNA application, antibiotics were removed from the cell culture medium to reduce cell death (manufacturers' recommendations).

siRNA oligonucleotides were supplied from the ON-TARGET*plus* collection (Dharmacon, Lafayette, USA) and designed to target either ADAM-10 (SMARTpool; accession number NM_001110), ADAM-17 (SMARTpool; accession number NM_003183) or GAPDH (GAPD control pool; accession number NM_002046) mRNA sequences. The latter was used as a positive control. As a negative control, a non-targeting siRNA pool (catalogue number D-001810-10-05) was selected. DharmaFECT 1 (Dharmacon) was selected as the transfection agent as it had previously been used for gene silencing in HUVECs (manufacturers' brochure). siRNA and transfection reagent concentrations were optimised individually by monitoring the mRNA levels of GAPDH during knockdown and following treatment with non-targeting siRNA. The level of knockdown was compared to the level of cell proliferation to find the optimal balance between cell survival and gene silencing (data not shown).

To knockdown ADAM-10 and/or ADAM-17 expression at the mRNA level, $5 \times 10^4$ cells/500 μl/well of a 24 well plate were seeded in complete medium without antibiotics. After 24 h in culture, the medium was aspirated and antibiotic-free medium with 20 nM of siRNA and 1.0 μl/well of DharmaFECT 1 were added. The non-target control and GAPDH siRNA were used as controls. Where both ADAM-10 and -17 were knocked down jointly, 20 nM of each siRNA was applied. After 24 h in culture, the medium was aspirated and replaced with antibiotic-free complete medium so that the cells could be cultured for a further 24 h (this limits cell death due to exposure to the siRNA reagents; manufacturers' guidelines). Following this, the cells were rinsed twice with PBS and the RNA extracted using TRI Reagent (Sigma–Aldrich, U.K.) according to the manufacturers' guidelines. Each experiment was repeated three times.

qRT-PCR was carried out as previously described [18]. Briefly, 1 μl of total RNA was reverse transcribed to cDNA using superscript II reverse transcriptase (Invitrogen, U.K.); negative controls were run in parallel. Using the fluorescent TaqMan 5′ nuclease assay (Applied Biosystems, U.K.) cDNA samples were analysed for the expression of ADAM-10, ADAM-17, GAPDH and cyclophilin A (housekeeping gene) by qRT-PCR. Each 10 μl reaction (consisting of 2× TaqMan Universal PCR Master Mix (Applied Biosystems, U.K.), 3.6 μM of each primer, 1 μM of the FAM labelled probe and 1 μl of template cDNA) was carried out in a 384-well reaction plate (Greiner Bio-One, U.K.) using the Applied Biosystems 7900HT fast real-time PCR system (Applied Biosystems, U.K.). The thermal profile of the reaction was as follows: 50 °C for 2 min, 95 °C for 2 min, and 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Emitted fluorescence was measured at each stage of the process to construct an amplification plot using ABI Prism 7900HT Sequence Detection System software version 2.2.1. To determine the relative mRNA levels of the above genes the cycle threshold ($C_T$) and the $2^{-\Delta\Delta C_T}$ method [22] was used.

To determine the effects of ADAM-10 and/or ADAM-17 knockdown on fractalkine shedding in hCMEC/D3 cells, $2.5 \times 10^5$ cells/2 ml/well of a 6-well plate were seeded in complete medium without antibiotics. Each experiment was performed three times. After 24 h, siRNA was added in the same manner as described above using 4 μl of DharmaFECT 1/2 ml/well. After 24 h, the medium was aspirated and replaced with antibiotic-free complete medium and the cells cultured for a further 48 h, at which point the cells were washed twice with PBS, and FCS-/antibiotic-free medium with 100 ng/ml TNF was added. Following 24 h treatment with TNF, the medium was aspirated, centrifuged at 300 × *g*, the supernatant collected and stored at −20 °C until analysis by enzyme-linked immunosorbent assay (ELISA).

Fractalkine in cell supernatants from siRNA and TNF-treated cells was measured using a fractalkine DuoSet ELISA assay (R&D Systems, U.K.) designed to detect the shed chemokine domain of human fractalkine (N terminus). The assay was performed as recommended by the manufacturer. Absorbances were read at 450 nm in a Wallac Victor$^{2TM}$ 1420 Multilabel Counter using Wallac 1420 software version 2.00 release 8. Optical imperfections were corrected for by subtracting absorbance readings at 570 nm. To determine the mean values each standard and sample was assayed in duplicate. Using a seven point standard curve of 2-fold serial dilutions and a minimum standard of 0.625 ng/ml fractalkine, the concentrations of fractalkine were then determined. Results were normalised to MTS proliferation assay data (data not shown) to take into account any changes in cell density due to the different treatments.

qRT-PCR and ELISA data were analysed using the non-parametric Kruskal–Wallis test followed either by a Conover–Inman post hoc test (multiple data sets) or the non-parametric Mann–Whitney *U*-test (two data sets). For the qRT-PCR data $2^{-\Delta C_T}$ was calculated for each sample to determine the relative gene expression and then the values for each treatment condition were compared to the non-target control siRNA treated values. Significance was set at $p \leq 0.05$ and the data are presented as mean ± SEM for the indicated number of experiments.

## 3. Results and discussion

Much of the work on shedding has been carried out with cells stimulated by agents which do not necessarily reflect the in vivo physiological process, e.g. PMA. In addition the endothelial cell