Effects of Pro-Inflammatory Cytokines on the Production of Soluble Fractalkine and ADAM17 by HepG2 Cells

Sharon L. Turner¹, David Mangnall², Nigel C. Bird², Maria E. Blair-Zajdel¹, Rowena A.D. Bunning¹

¹) Biomedical Research Centre, Sheffield Hallam University, Sheffield; 2) Liver Research Group, Royal Hallamshire Hospital, University of Sheffield, Sheffield, UK

Abstract

Background & Aims: Soluble fractalkine is increased in the liver during times of injury; however the effect of pro-inflammatory cytokines in this process is currently unknown. The aim of this study was to determine whether pro-inflammatory cytokines elevated in patients with hepatocellular carcinoma influence fractalkine shedding from HepG2 cells and whether ADAM17 was involved in this process. Methods: In vitro experiments were performed in the human hepatocellular carcinoma cell line HepG2. Soluble fractalkine was detected using an ELISA. ADAM17 expression was investigated using quantitative real time (reverse transcription)-polymerase chain reaction and flow cytometry. Short interfering RNA transfection was used to down-regulate ADAM17 expression. Results: Soluble fractalkine was present in supernatants of HepG2 cells, and was significantly increased by interleukin-1β (p≤0.005) and tumour necrosis factor-α (p≤0.043), but not by interleukin-6 (p≥0.316). This corresponded to minor increases in ADAM17 protein, but not ADAM17 mRNA, following the same treatments. However, the down-regulation of ADAM17 protein did not affect fractalkine shedding. Conclusions: This study showed that soluble fractalkine is up-regulated under inflammatory conditions associated with hepatocellular carcinoma development, but ADAM17 does not appear to be responsible for regulating this process.

Keywords

Introduction

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed cancer worldwide (626,000 cases per annum; 5.6% of all cancers), and the third most common cause of cancer death (598,000 deaths per annum) [1]. It is most prevalent in Southeast Asia and sub-Saharan Africa [2], and although uncommon in developed countries, trends over the last two decades have indicated an increase in HCC incidence in Central Europe, the United Kingdom, North America and Japan [3]. Furthermore, the only curative treatments for HCC are liver transplantation and resection, which are only available to the minority of patients [4].

There is growing evidence that inflammation is a factor in the development of cancers, including HCC [5], consequently many cytokines are being implicated in malignancies. Fractalkine, a chemotactic cytokine (chemokine) also known as CX3CL1, is an important inflammatory mediator, but unlike conventional chemokines exists in two forms. Membrane-bound fractalkine located on the surface of cells acts as an adhesion molecule, facilitating the adhesion of fractalkine expressing cells and leucocytes expressing its receptor, CX3CR1 [6]. Whereas, soluble fractalkine, generated from the cleavage of membrane-bound fractalkine, can induce the chemotactic migration of leucocytes expressing CX3CR1.

Fractalkine is linked to a number of inflammatory diseases, including rheumatoid arthritis [7], atherosclerosis [8], acute hepatitis [9] and cancer [10, 11], where it has either a protective or deleterious effect. For example, the up-regulation of fractalkine in colorectal carcinoma and gastric adenocarcinoma can invoke anti-tumour responses leading to a better prognosis of disease-free survival [10, 11]. Conversely, fractalkine present in the synovial fluid of rheumatoid arthritis patients can act as a mediator of pathogenic angiogenesis in vitro [7].

One of the principal fractalkine sheddases is proteolytically active a disintegrin and metalloproteinase 17 (ADAM17) [12, 13], a multi-domain, type I transmembrane protein belonging to the adalysin subfamily of the metzincin superfamily [14]. ADAM17 is necessary in many physiological
processes, but has been implicated in pathological conditions including multiple sclerosis [15], Alzheimer’s disease [16] and numerous cancer types, such as breast [17], prostate [18], kidney [19], ovary [20] and liver cancers [21].

Although fractalkine and ADAM17 are known to be up-regulated in the human liver during times of injury [9, 21], it is unknown whether there is a correlation between the two factors during HCC development. This study aims to determine whether pro-inflammatory cytokines elevated in patients with HCC influence fractalkine shedding in a HCC cell line, HepG2, and whether ADAM17 is involved in this process.

Materials and Methods

Cell culture

The human HCC cell line, HepG2 (Sigma-Aldrich, Gillingham, UK), was cultured in EMEM supplemented with 10% heat-inactivated foetal calf serum, 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine (Gibco Invitrogen, Paisley, UK) and 1% non-essential amino acids (Sigma-Aldrich). Cells were maintained in a humidified atmosphere of 95% v/v air and 5% v/v carbon dioxide at 37°C.

Cytokine treatment of cells

For enzyme-linked immunosorbent assay (ELISA) sample collection, cells were seeded at 5.4 x 10^4 cells/well of a 6-well plate in 3 mL complete culture medium for 24 hours. Cells were treated with 1, 10 or 100 ng/mL interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α; reconstituted in water) or interleukin-6 (IL-6; reconstituted in 5 mM acetic acid; PeproTech, London, UK) in 3 mL of serum-free medium for 24 or 48 hours. Combinations of 1 and/or 10 ng/mL IL-1β and TNF-α were also used. Serum-free medium was used as a control for IL-1β and TNF-α treatments and serum-free medium containing 1 μL/mL 5 mM acetic acid for IL-6 treatments. For quantitative real time (reverse transcription)-polymerase chain reaction (qRT-PCR), cells were seeded at 1 x 10^5 cells/well of a 24-well plate in 1 mL complete culture medium, then treated in triplicate in 1 mL of serum-free medium for 24 hours. For flow cytometric analysis, cells were seeded at 1.4 x 10^6 cells/T25 flask in 3 mL complete culture medium, then treated in 3 mL of serum-free medium for 24 hours (single treatments only).

ELISA for fractalkine detection

The DuoSet human CX3CL1/Fractalkine ELISA kit (R&D Systems Europe Ltd, Abingdon, UK) was used to quantify the amount of soluble fractalkine in HepG2 culture medium after 24 and 48 hours of cytokine treatment. To allow the concentration of soluble fractalkine to be expressed per mg of protein, protein lysates were prepared from treated HepG2 cells by the application of CellLytic-M supplemented with 10% protease inhibitor cocktail and 10 mM 1,10-phenanthroline (Sigma-Aldrich). Protein concentrations were determined by bichinonic acid (BCA) assay (Sigma-Aldrich).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from HepG2 cells using Tri-Reagent (Sigma-Aldrich) and quantified using a Nanodrop ND-1000 spectrophotometer (Labtech International, East Sussex, UK). cDNA was synthesised from 1 μg total RNA per 20 μL reaction using the iScript™ cDNA synthesis kit (Bio-Rad, Hemel Hempstead, UK). PCR primers for ADAM17 were designed using Primer Express software (Applied Biosystems, Warrington, UK): sense, 5’ACGAAAGCCGACTACCTGCAA-3’; anti-sense, 5’ATCATCATCTCTTTATGGCTAGAA-3’.

PCR primers sequences for the reference genes β-actin and HPRT1 were as stated by Vandesompele et al [22]. All PCR primers were obtained from Invitrogen. Reactions were performed in duplicate with an iCycler multicolour real-time PCR detection system (Bio-Rad) using 2X ABsolute QPCR SYBR green fluorescein mix (ABgene Ltd, Epsom, UK) and the thermal profile: 2 minutes at 50°C, 15 minutes at 95°C, then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Melt curve data was collected using the profile: 30 seconds at 95°C, 30 seconds at 50°C, then 45 cycles of 10 seconds starting at 50°C with an increase of 1°C per cycle. Data are expressed relative to the reference genes using the Pfaffl method [23] and all treatments were compared to an appropriate control.

Flow cytometry

Cells were harvested using non-enzymatic cell dissociation solution (Sigma-Aldrich), and cell surface antigens were labelled with carboxyfluorescein (CFS)-conjugated mouse anti-human TACE monoclonal antibody or CFS-conjugated isotype-matched mouse IgG1 (R&D Systems Europe Ltd). For intracellular antigen labelling, the IntraSure kit (Becton Dickinson UK Ltd, Cowley, UK) was used together with the TACE antibody and isotype-matched IgG1 detailed above. Samples were analysed on a FACScalibur flow cytometer using CellQuest software (Becton Dickinson UK Ltd) with a total of 1.0 x 10^6 events acquired for each sample. The mean fluorescence index (MFI) for each set of samples was calculated using the equation: MFI = Mean Antibody Fluorescence / Mean Isotype Control Fluorescence.

Ribonucleic acid interference (RNAi)

Post-transcriptional gene silencing of ADAM17 in HepG2 cells was performed using lipid-mediated short interfering RNA (siRNA) transfection (Dharmacon at PerbioScience UK Ltd, Cramlington, UK). Cells were seeded at 1.0 x 10^4 cells/well of a 96-well plate in 100 μL complete culture medium without antibiotics for 24 hours. Cells were then transfected in triplicate wells for 48 hours for mRNA analysis or 10-wells for protein analysis for 72 hours, with a positive silencing control siRNA (ON-TARGETplus GAPDH control pool), negative silencing control (ON-TARGETplus non-targeting pool), or target gene siRNA (ON-TARGETplus ADAM17 SMART pool) in 100 μL of antibiotic- and serum-free medium. For ELISA sample
collection, following 72 hours of transfection, 100 µL of antibiotic- and serum-free medium or 100 µL antibiotic- and serum-free medium containing 100 ng/mL IL-1β or TNF-α were applied to the cells for 24 or 48 hours. Antibiotic- and serum-free medium was used as a control for IL-1β and TNF-α treatments.

**Western blotting**

Protein lysates (6 µg/lane) were fractionated under reducing conditions on 10% Bis-Tris gels (NuPage Invitrogen) using the Laemmli system [24], then transferred onto Hybond-C Extra nitrocellulose membrane (Amersham Biosciences, Amersham, UK). Following membrane blocking with 5% blocking buffer (5% milk powder in Tris-buffered saline containing 0.05% Tween-20 (TBS-T); Sigma-Aldrich), primary antibody was applied overnight (4°C), followed by an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 2 hours. Luminography was performed using ECL Plus Chemiluminescence (Amersham Biosciences), and data captured and analysed on a UVP Bio-Imager using Labworks 4 software (Ultra Violet Products Ltd, Cambridge, UK). Data were then quantified using integrated optical density analysis, with normalisation against the internal control protein actin.

The following primary and secondary antibody combinations were used consecutively on a single blot: rabbit anti-human TACE polyclonal antibody (Santa-Cruz, Heidelberg, Germany; 1:300 in TBS-T), followed by HRP-conjugated goat anti-rabbit Ig (Sigma-Aldrich; 1:80000 in 2.5% blocking buffer); rabbit anti-human actin polyclonal antibody (Sigma-Aldrich; 1:1000 in 5% blocking buffer), followed by HRP-conjugated goat anti-rabbit secondary antibody (Sigma-Aldrich; 1:80000 in 5% blocking buffer); mouse anti-human GAPDH monoclonal antibody (Abcam, Cambridge, UK; 1:5000 in 5% blocking buffer), followed by HRP-conjugated rabbit anti-mouse Ig (Dako UK Ltd, Ely, UK; 1:1000 in 5% blocking buffer). Membranes were stripped after ADAM17 and actin data capture using Restore Plus Western Blot Stripping Buffer (Perbio Science UK Ltd, Cramlington, UK).

**Statistical analysis**

SPSS was used to perform all statistical analyses. Data were normalised by logarithmic, square root or negative reciprocal transformations as necessary, prior to use of the parametric one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test to determine differences between cytokine treated and control groups. Data are presented as mean ± standard error of the mean (SEM), with p≤0.05 considered significant.

**Results**

**IL-1β and TNF-α, but not IL-6, increased soluble fractalkine production by HepG2 cells**

Quantification of fractalkine using ELISA showed that under control conditions, a mean of 35.7 ng fractalkine/mg protein was cleaved from HepG2 cells after 24 hours (Fig. 1a). IL-1β and TNF-α treatments increased the amount of soluble fractalkine with significance reached following 10 and 100 ng/mL of treatments. Mean values of 145.9 (p<0.001) and 142.9 (p<0.001) ng fractalkine/mg protein for IL-1β treatments and 82.4 (p<0.001) & 113.4 (p<0.001) ng fractalkine/mg protein for TNF-α treatments were obtained. Dual treatments performed with different combinations of sub-maximal concentrations of IL-1β and TNF-α also resulted in significant, but not additive, increases in soluble fractalkine compared to the control (p<0.001) (data not shown).

These trends were paralleled following 48 hours of treatment with IL-1β and TNF-α (Fig. 1b), albeit with higher levels of soluble fractalkine detected. HepG2 cells shed a mean of 90.9 ng fractalkine/mg protein under control conditions, which was significantly increased in a concentration dependent manner following 1, 10 and 100 ng/mL of IL-1β and TNF-α, with mean values of 171.8 (p=0.005), 203.6 (p=0.001) & 212.7 (p≤0.001) ng fractalkine/mg protein following IL-1β treatments, and 124.8 (p=0.043), 172.8 (p=0.004) & 205.6 (p<0.001) ng fractalkine/mg protein after TNF-α treatments. Dual treatments performed with IL-1β and TNF-α for 48 hours also resulted in significant, but not additive, increases in soluble fractalkine compared to the control (p=0.05 to p<0.001) (data not shown).

IL-6 treatment did not affect fractalkine shedding relative to the vehicle control at the 24 hours time point (Fig. 1a; p>0.705). However, fractalkine was shed at a significantly
higher level in the vehicle control compared to the untreated control with a mean of 99.3 ng fractalkine/mg protein detected (p≤0.001). Similarly, no significant changes in soluble fractalkine were observed following 48 hours of IL-6 treatment compared to the vehicle control (Figure 1b; p≥0.316); however the amount of soluble fractalkine detected in the untreated and vehicle controls were comparable.

**IL-1β, and TNF-α, but not IL-6, caused minor modulations of ADAM17 protein in HepG2 cells**

ADAM17 is known to be expressed by HepG2 cells [21]; this was confirmed by qRT-PCR and flow cytometry and extended upon by determining that IL-1β, TNF-α and IL-6 treatments did not modulate the expression of ADAM17 mRNA (Fig. 2a) or protein (Figs. 2 - b c) to a statistically significant level (mRNA p≥0.311; cell surface p≥0.372; intracellular p≥0.204). However, minor increases observed in cell surface and intracellular ADAM17 protein following IL-1β and TNF-α treatments corresponded to modulations in soluble fractalkine following the same treatments.

**Down-regulation of ADAM17 did not affect soluble fractalkine production by HepG2 cells**

qRT-PCR performed 48 hours after ADAM17 siRNA transfection showed that ADAM17 gene expression was down-regulated by 78.3% (p=0.031) (Fig. 3a). Small comparable decreases in ADAM17 expression also were observed with GAPDH and non-targeting siRNAs (17.2% (p=0.900) & 19.1% (p=0.534) gene silencing respectively), highlighting the off-target effects of RNAi pathway activation in HepG2 cells. GAPDH mRNA levels were decreased by 71.3% (p=0.033) following GAPDH siRNA transfection.

Western blot analysis performed after 72 hours of siRNA transfection confirmed these findings translated from the mRNA level to the protein level. ADAM17 protein (Fig. 3b) was present on the blots as two predominant bands representing processed forms (66 and 58 kDa) in the control and GAPDH siRNA transfected samples. The 66 kDa band was reduced or absent from the non-targeting and ADAM17 siRNAs transfected samples. Total band intensity analysis of ADAM17 protein (Fig. 3c) showed that ADAM17 was decreased in ADAM17 siRNA transfected samples by 76.8% (p=0.026). Non-targeting and GAPDH siRNAs transfected samples also had decreased ADAM17 protein expression (p=0.175 & p=0.236 respectively), representing the off-target effects of RNAi pathway activation. Analysis of GAPDH protein by western blot illustrated its presence in all samples as a 37 kDa band (Fig. 3b), this was decreased in the GAPDH siRNA transfected samples by 66.1% (p=0.036); an observation confirmed by densitometry (Fig. 3c).

The down-regulation of ADAM17 by siRNA transfection for 72 hours, significantly altered the constitutive shedding of fractalkine from HepG2 cells following 24 (Fig. 3d; mean of 86.0 ng fractalkine/mg protein; p=0.020) and 48 hours (Fig. 3e; mean of 211.8 ng fractalkine/mg protein; p=0.573) of concomitant treatment with antibiotic- and serum-free medium compared to an untreated control (mean of 148.1 and 350.7 ng fractalkine/mg protein respectively), as determined by ELISA. However, the amount of fractalkine shed by ADAM17 siRNA transfected HepG2 cells was comparable to GAPDH (mean of 88.4 ng fractalkine/mg protein at 24 hours; mean of 217.5 ng fractalkine/mg protein at 48 hours) and non-targeting (mean of 75.1 ng fractalkine/mg protein at 24 hours; mean of 179.2 ng fractalkine/mg protein at 48 hours) siRNAs transfected samples.

Additionally, IL-1β and TNF-α (100 ng/mL) induced fractalkine shedding was observed following 72 hours of ADAM17 siRNA transfection with 24 (mean of 163.4 & 150.2 ng fractalkine/mg protein respectively; p=0.005 &
Fractalkine and ADAM17 in HepG2 Cells

Discussion

The opposing functions of fractalkine in conditions such as gastric and colorectal cancers (protective) [10, 11] and rheumatoid arthritis (delerious) [7] have resulted in much interest in this unique chemokine. Fractalkine and its receptor, CX3CR1, are up-regulated in patients with cirrhosis associated with chronic hepatitis C virus infection and patients with acute hepatitis B virus infection [9], both conditions associated with the development of HCC, but its expression in HCC has not been investigated.

In this study, we show that fractalkine is released from the human well-differentiated HCC cell line HepG2 generating soluble fractalkine, confirming data reported by Efsen et al [9], and demonstrate for the first time that this release is increased upon treatment with the pro-inflammatory cytokines IL-1β and TNF-α, but not IL-6. Other researchers have reported similar increases in soluble fractalkine levels in response to IL-1β in the human intestinal epithelial cell line T-84 [25] and in response to TNF-α in the human umbilical vein endothelial cell line HUVEC [26] and human brain endothelial cell line hCMEC/D3 [27].

These data may indicate that liver tumours, in particular well-differentiated liver tumours, up-regulate soluble fractalkine in response to the pro-inflammatory cytokines that are elevated during times of liver injury and liver tumour development [28, 29]. Furthermore, HepG2-conditioned medium is chemotactic for cells over-expressing CX3CR1 [9], suggesting that soluble fractalkine could recruit CX3CR1 expressing inflammatory cells and activated hepatic stellate cells (HSCs), which also express CX3CR1 [30], to the tumour site. The combination of recruited...
lymphocytes, which are important in the inflammatory phase of the fibrogenic response, and activated HSCs, which are important in the later stages of liver fibrosis, may facilitate the formation of the fibrotic capsule (desmoplastic reaction) surrounding well-differentiated primary liver tumours.

When fractalkine is up-regulated in certain cancers, e.g. colorectal carcinoma and gastric adenocarcinomas, it can invoke anti-tumour responses by the recruitment of tumour infiltrating lymphocytes (TILs), comprising mainly NK cells and cytotoxic T-cells [10, 11]. Generally patients with colorectal carcinoma and gastric adenocarcinoma have impaired local and systemic immune responses, with few TILs. However, when fractalkine is up-regulated in these tumour types, they contain a large fraction of TILs, which correlates with a better prognosis of disease-free survival [10, 11].

Conversely, soluble fractalkine is an angiogenic mediator, inducing the chemotaxis and chemokinesis of endothelial cells both in vitro and in vivo by the binding of fractalkine to its receptor on the surface of endothelial cells [7]. This same study also demonstrated that soluble fractalkine can induce endothelial cells to form tubes in vitro and functional blood vessels in vivo, establishing its angiogenic properties. As fractalkine is elevated by cytokines present during the initial stages of liver tumour formation, it is possible that soluble fractalkine could attract endothelial cells to the tumour and induce the formation of a neovascularature capable of supplying blood to the developing tumour, although this remains to be determined.

Soluble fractalkine can be generated by the actions of four proteinases, ADAM17 [12, 13], ADAM10 [31], cathepsin S [32] and MMP-2 [33]. We investigated the relationship between fractalkine shedding and ADAM17 expression, as ADAM17 mRNA is known to be up-regulated in HCCs [34]. This study demonstrated the expression of ADAM17 mRNA and protein (cell surface and intracellular) by HepG2 cells, and determined that it was not significantly modulated by the cytokines investigated at either the mRNA or protein level. However, increases in fractalkine shedding may be a result of increased ADAM17 activity, which may not be reflected by increased mRNA or protein expression. Interestingly though, flow cytometric analysis did demonstrate minor increases in cell surface and intracellular ADAM17 in response to IL-1β, and TNF-α (significance not reached), and the vehicle control compared to the untreated control, trends that were also observed in fractalkine shedding under the same conditions. Therefore, it would be possible to postulate that minor modulations of the proteolytically active membrane-bound form of ADAM17 can potentially control the amount of fractalkine cleaved from the surface of HepG2 cells.

However, the post-transcriptional silencing of ADAM17 protein by 76.8% did not significantly reduce constitutive fractalkine shedding when compared to the silencing controls, as would be expected if this hypothesis were true, indicating that ADAM17 is not involved in the constitutive shedding of fractalkine by HepG2 cells. A similar conclusion was also reached by Hurst et al [27] in relation to constitutive fractalkine shedding from hCMEC/D3 cells. Furthermore, the constitutive shedding of fractalkine has been attributed to the proteolytic action of ADAM10 and not ADAM17 in human bladder carcinoma cells (ECV304), primate (COS-7) and murine fibroblast cells at least [31].

ADAM17 has, however, been recognised as the proteinase responsible for the inducible shedding of fractalkine from phorbol 12-myristate 13-acetate (PMA) treated ECV304 and murine fibroblast cells [12, 13]. However, the down-regulation of ADAM17 in HepG2 cells by siRNA transfection did not negate IL-1β or TNF-α inducible fractalkine shedding from these cells. Similarly, Hurst et al [27] determined that TNF inducible fractalkine shedding from hCMEC/D3 cells was not regulated by ADAM17. Furthermore, Bourd-Boittoin et al [35] demonstrated that fractalkine was mainly processed by MMP-2 in interferon-γ treated human hepatic stellate cells isolated from patients with liver metastases, with ADAM17 and ADAM10 only partially contributing to fractalkine shedding in this system [35].

In conclusion, the up-regulation of soluble fractalkine under specific inflammatory conditions, namely IL-1β and TNF-α treatments, as demonstrated here in the human HCC cell line HepG2, could regulate the attraction of CX3CR1 bearing cells to the tumour site. However, it remains to be determined whether this provides protective (anti-tumour responses) or deleterious (pathogenic angiogenesis) effects in patients with HCC.

Acknowledgements

Particular thanks to Dr. Sarah Haywood-Small and Dr. Christine Le Maitre for their invaluable guidance on flow cytometry.

Conflict of interest statement

We have no conflict of interest to declare.

Financial disclosure

This project was funded by the Biomedical Research Centre, Sheffield Hallam University, Howard Street, Sheffield, S1 1WB, UK & Yorkshire Cancer Research, East Parade, Harrogate, HG1 5LQ, UK.

References

5. Jung Y, Isaacs JS, Lee S, Trepel J, Neckers L. IL-1β-mediated up-regulation of HIF-1α via an NFKappaB/COX-2 pathway identifies...
HIF-1 as a critical link between inflammation and oncogenesis. FASEB J 2003; 17: 2115-2117.