Short communication

THE FUNCTIONAL ANTAGONIST MET-RANTES: A MODIFIED AGONIST THAT INDUCES DIFFERENTIAL CCR5 TRAFFICKING

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Abstract: CC chemokine receptor 5 (CCR5) is a pro-inflammatory chemokine receptor that is expressed on cells of the immune system, and specializes in cell migration in response to inflammation and tissue damage. Due to its key role in cell communication and migration, this receptor is involved in various inflammatory and autoimmune diseases, in addition to HIV infection. Met-RANTES is a modified CCR5 ligand that has previously been shown to antagonize CCR5 activation and function in response to its natural ligands in vitro. In vivo, Met-RANTES is able to reduce inflammation in models of induced inflammatory and autoimmune diseases. However, due to the fact that Met-RANTES is also capable of partial agonist activity regarding receptor signaling and internalization, it is clear that Met-RANTES does not function as a conventional receptor antagonist. To further elucidate the effect of Met-RANTES on CCR5, receptor trafficking was investigated in a CHO-CCR5-GFP cell line using the Opera confocal plate reader. The internalization response of CCR5 was quantified, and showed that Met-RANTES internalized CCR5 in a slower, less potent manner than the agonists CCL3 and CCL5. Fluorescent organelle labeling and live cell imaging showed CCL3 and CCL5 caused CCR5

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Abbreviations used: CCR5 – CC chemokine receptor 5; GFP – green fluorescent protein; CHO – Chinese hamster ovary; GPCR – G-protein coupled receptor; HIV – human immunodeficiency virus; MIP-1α or CCL3 – macrophage inflammatory protein-1 alpha; RANTES or CCL5 – regulated on activation normal T cell expressed and secreted
to traffic through sorting endosomes, recycling endosomes and the Golgi apparatus. In contrast, Met-RANTES caused CCR5 to traffic through sorting endosomes and the Golgi apparatus in a manner that was independent of recycling endosomes. As receptor trafficking impacts on cell surface expression and the ability of the receptor to respond to more ligand, this information may indicate an alternative regulation of CCR5 by Met-RANTES that allows the modified ligand to reduce inflammation through stimulation of a pro-inflammatory receptor.

Key words: Met-RANTES, CCR5 trafficking, CCR5 internalization

INTRODUCTION

CC chemokine receptor 5 (CCR5) is a seven-transmembrane G protein-coupled receptor (GPCR) expressed on various immune cell types (including T cells, monocytes, and dendritic cells) in addition to cells of the central nervous system, connective and muscle tissue [1, 2]. It is activated by chemotactic cytokines (chemokines), including the pro-inflammatory CC chemokines Macrophage Inflammatory Protein 1-alpha (MIP-1α, or CCL3), Regulated on Activation Normal T Cell Expressed and Secreted (RANTES, or CCL5) and the Monocyte Chemoattractant Proteins [3, 4]. CCR5 and its ligands have been shown to play an important role in the co-ordination of inflammatory responses regarding the chemotaxis of immune cells to areas of tissue damage, and the expression of this receptor has been shown to contribute towards the development and severity of diseases such as Human Immunodeficiency Virus (HIV) infection, autoimmune and inflammatory disorders as well as allograft rejection [1, 5, 6]. It has been shown that modifications to CCR5 ligands can profoundly affect events that lie downstream of receptor activation, such as in the case of Amino-oxyepentane (AOP)-RANTES and Met-RANTES [7-9]. AOP-RANTES has been shown to cause internalization of CCR5 whilst inhibiting the recycling of the receptor, causing a downregulation of CCR5 on the cell surface when compared to RANTES-treated cells [8]. Met-RANTES, formed by retention of the initiating methionine residue on the amino terminus of the peptide, is capable of binding CCR5, CCR1 and CCR3 [7, 10]. Met-RANTES was initially shown to be a CCR5 antagonist regarding ligand-induced calcium signaling and chemotaxis in vitro, and was unable to cause measurable CCR5 phosphorylation upon binding [7, 11]. This modified ligand appeared to have anti-inflammatory activity regarding its ability to inhibit eosinophil effector functions and monocyte recruitment [10, 12]. When used in vivo, Met-RANTES was able to block CCR5-mediated inflammatory processes in induced arthritis, colitis, airway inflammation and allograft rejection [12-15]. Further studies have shown, however, that Met-RANTES was capable of partial agonist activity through CCR5 in vitro, whereby it produced tyrosine kinase phosphorylation, a small but measurable calcium flux, and a slow internalization
of the CCR5 receptor [16, 17]. Thus, it is apparent that Met-RANTES is a functional antagonist of CCR5 with partial agonist activity. Here we have investigated the trafficking of CCR5 tagged with green fluorescent protein (GFP) in a Chinese hamster ovary (CHO) cell line following stimulation with the natural agonists, CCL3 and CCL5, and Met-RANTES. We have used fluorescently tagged markers of the endocytic recycling pathway to perform colocalization studies with CCR5-GFP. The results show that there are variations in the localization of the receptor in the cytoplasm following stimulation with the different ligands, which may suggest differential regulation of CCR5 activity by ligands.

**MATERIALS AND METHODS**

**Cell culture**

CHO-K1 cells stably expressing human CCR5 tagged with GFP on the C-terminus (CHO-CCR5-GFP) had been constructed previously [17]. CHO-CCR5-GFP cells were maintained in Hams F-12 nutrient mix with 10% (v/v) fetal bovine serum and 200 µg/ml hygromycin B. For assays, cells were plated onto 96 well tissue culture-treated plates (Matrical) at a density of 1x10⁴ cells per well for 24 hours. Cells were grown in Hams F-12 nutrient mix and L-glutamine without serum for 24 hours prior to experiments to minimize non-specific activation of the receptor.

**Receptor internalization assays**

CHO-CCR5-GFP cells treated with 10 nM concentrations of CCL3 (isoform LD78β), CCL5 or Met-RANTES (R&D Systems) for the indicated times. The cells were then fixed with 2% paraformaldehyde (Sigma-Aldrich) for 30 minutes and stained with 5µm DRAQ5 (Biostatus Limited) in Hanks balanced salt solution (HBSS; Invitrogen) for 1 hour. DRAQ5 weakly stains RNA in the cell cytoplasm and densely stains DNA in the nuclear region. The cells were then imaged on the Opera™ confocal plate reader (PerkinElmer), and images were analyzed as described in spot detection.

**Spot detection: image analysis**

The Acapella™ analysis software (Perkin Elmer) was used to apply a spot detection algorithm to quantify receptor internalization into intracellular ‘spots’. Firstly, the nuclei were detected on the basis of dense DRAQ5 nuclear staining, whilst the cytoplasm was detected by extending the nuclear border to encompass DRAQ5 cytoplasmic staining. Spots were then detected within the cytoplasmic border. Spots were selected by configuring the following algorithm parameters. The minimum distance allowed between spots ensured that one spot was not detected as two (spot minimum distance, set to 2 pixels), the size of the brightest portion of the spot (peak radius, set to 1 pixel), the reference radius to which the peak radius was compared (reference radius, set to 6 pixels), the minimum contrast of the peak radius to the reference radius (spot minimum contrast, set to
a factor of 0.1), and the minimum spot intensity over the basal cell intensity (spot to cell intensity, set to a factor of 2). The extent of receptor internalization was expressed as the number of spots per cell.

Colocalization assays
CHO-CCR5-GFP cells were stained with 2.5 mg/ml Transferrin-Alexa Fluor 633 or 2.5 µM borate-dipyrrromethene (BODIPY)-TR Ceramide (Invitrogen) fluorescent complexes for 30 minutes at 4°C to allow accumulation of fluorescent labels at the cell surface. The cells were then washed twice with ice-cold HBSS, before being left untreated or treated with 100 nM CCL3, CCL5 or Met-RANTES. Cells were then incubated in the Opera™ climate control chamber at 37°C with 5% CO₂ and 75% humidity. Images were taken before ligand application, at the time of ligand application and at 10 minute intervals for 90 minutes using x40 water immersion objective on the Opera™.

Fluorescent imaging
The wavelength excitation and emission spectra were obtained from the manufacturer for all fluorescent labels, and the Opera™ excitation and emission settings were adjusted accordingly. GFP was excited at 488 nm (detected with 510-610 nm band pass filter), Transferrin-Alexa fluor 633 and DRAQ5 at 633 nm (detected with 665-715 nm band pass filter), BODIPY–TR ceramide at 532 nm (detected with 580-620 nm band pass filter). Multiple images of the same well were taken as separate exposures to minimize signal bleed-through. The focal plane on which images were taken was also adjusted between labels to provide optimal detection of signal. A height of 3 μm was used for detection of transferrin labeling, and 7 μm was used for ceramide.

Graphical and statistical analysis
GraphPad Prism version 4.03 was used to generate graphs. Statistical significance was calculated using a two-tailed paired t test with 99% confidence interval.

RESULTS AND DISCUSSION

Internationalization of CCR5-GFP in response to treatment
The amount and rate of the internalization of CCR5-GFP was evaluated by treating the cells with 10 nM doses of each ligand for 90 minutes. CCL3 and CCL5 produced a significantly more potent and rapid internalization response than Met-RANTES (Fig. 1). The times taken to internalize 50% of CCR5-GFP were 14 and 19 minutes for CCL3 and CCL5, respectively, and 38 minutes for Met-RANTES.
Fig. 1. CCR5 agonists, CCL3 and CCL5 and Met-RANTES cause a time-dependent internalization of the receptor into intracellular spots. CHO-CCR5-GFP cells were treated with 10nM concentrations of ligand for the indicated times, before being fixed and stained with DRAQ5. CCR5-GFP internalization was quantified and expressed as a percentage of the maximum response (90 minute treatment with CCL3). An asterisk (*) or double asterisk (**) indicates statistically significant differences (P < 0.0001) between the data for Met-RANTES and CCL3, or Met-RANTES and CCL5 respectively. Results are the mean and standard error of three separate experiments.

Colocalization of CCR5-GFP with transferrin labeled recycling endosomes
The subcellular location of internalized receptor was then investigated by fluorescently labeling recycling endosomes with transferrin and the Golgi apparatus with ceramide. Transferrin has previously been described to move from the cell surface through early endosomes to recycling endosomes within 20 minutes, and ceramide has been shown to move from the cell surface and accumulate at the Golgi apparatus within 30 minutes [18-20]. Both CCL3 and CCL5 caused substantial internalization of CCR5-GFP into transferrin-containing vesicles (Fig. 2). The initial colocalization observed after 5-20 minutes of ligand treatment represents the presence of CCR5-GFP in sorting endosomes. This colocalization of GFP and transferrin was seen to increase throughout the assay, and at later time points represents the arrival of the receptor at recycling endosomes [18, 19] (Fig. 3). These results concur with those of Signoret et al, which were performed in CHO and mink lung endothelial cell lines [19, 21]. In contrast to this data, other groups have presented evidence for clathrin-independent internalization of CCR5 in HeLa cells, indicating a possible cell type dependent difference [22, 23].

Met-RANTES treated cells (Fig. 3C), however, showed only a low amount of small receptor-containing vesicles colocalising with transferrin over the same time period. Large pools of internalized CCR5-GFP in Met-RANTES treated cells did not colocalize with transferrin labeling. Untreated cells showed no distinct colocalization with transferrin labeling at the cell surface (Fig. 3D).
Fig. 2. Internalized CCR5 colocalizes with transferrin in sorting endosomes. The green channel represents CCR5-GFP, red represents transferrin-Alexa Fluor 633, and the third image is an overlay of the two channels where yellow illustrates colocalization. These areas are also indicated by white arrows. A – CHO-CCR5-GFP cells stimulated with 100 nM doses of CCL3, B – CCL5 or C – Met-RANTES, for 20 minutes. Colocalization was evident after 5 minutes of ligand treatment. D – Unstimulated cells. Images are representative of three separate experiments.

Colocalization of CCR5-GFP with ceramide labeling at the Golgi apparatus

CHO-CCR5-GFP cells were labeled with ceramide in order to determine whether the Golgi apparatus was involved in the trafficking of CCR5. Treatment with ligands caused an accumulation of CCR5-GFP at the perinuclear area colocalising with bright ceramide staining at the Golgi apparatus (Fig. 4). The involvement of the Golgi apparatus in CCR5 trafficking was consistent with a previous study in this CHO cell line [17]. The observed CCR5-GFP localization to the Golgi apparatus and recycling endosomes after stimulation with CCL3 or CCL5 implies that both organelles are being utilized simultaneously, with a possible exchange of receptor via the recycling
endosome-Golgi trafficking route [24]. Interestingly, CCR5-GFP treated with Met-RANTES did not appear to cause any substantial accumulation in recycling endosomes whilst distinct localization to the Golgi apparatus was observed. This suggests that the Golgi apparatus serves as the primary trafficking organelle following Met-RANTES treatment.

The process of receptor trafficking impacts upon the receptor’s ability to recycle to the cell surface, as well as its rate of turnover, and the rate at which it is to be replaced by newly synthesized receptor on the cell surface [17]. Variations in receptor trafficking in response to a modified chemokine have been previously reported by another RANTES derivative, Amino-oxypentane(AOP)-RANTES.

Fig. 3. Colocalization of CCR5-GFP with transferrin in recycling endosomes. The green channel represents CCR5-GFP, red represents transferrin-Alexa Fluor 633, and the third image is an overlay of the two channels where yellow illustrates colocalization. These areas are indicated by the white arrows. A – CHO-CCR5-GFP cells stimulated with 100 nM CCL3, B – CCL5 or C – Met-RANTES for 30 minutes. D – Unstimulated cells. Images are representative of three separate experiments.
This modified ligand was able to internalize CCR5, but in contrast to CCL5 treatment, the receptor was prevented from recycling to the cell surface and the major pool of receptor was relocated to recycling endosomes [8]. Here we have observed a different response to treatment with Met-RANTES producing accumulation of CCR5 at the Golgi apparatus without any apparent trafficking through the recycling endosomes. This may be due to altered structural and binding properties of this modified chemokine, as there is evidence to suggest that the amino-terminal domain of chemokines can have an effect on the ligand-receptor interaction, resulting in altered activation of the receptor [25, 26]. The fate of the CCR5 receptor subsequent to accumulation at the Golgi apparatus following Met-RANTES treatment requires further investigation. Also, how
applicable these results are with regards to the various immune cell types and how much this alternative trafficking contributes to the effect of Met-RANTES on CCR5 in vivo needs to be determined.

The evidence presented here for differential trafficking of CCR5 in response to Met-RANTES may suggest an alternative mechanism for regulating CCR5 cell surface expression and function. This information may contribute to establishing how a functional antagonist with partial agonist activity can function through a pro-inflammatory receptor to reduce inflammation.

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