Secretion of RANTES and Interleukin-8 by the Human Gastric Epithelial Cell Line (MKN45) through Modulation with Cytokines

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ABSTRACT

In this in vitro study, the secretion of interleukin-8 (IL-8) and RANTES proteins by the MKN45 gastric epithelial cell line was examined using the ELISA technique after the cells had been stimulated with pro-inflammatory cytokines, i.e. IL-1α, TNF-α and IFN-γ. The chemokines demonstrated a time-dependent increase in concentration after stimulation with the various cytokines, alone or in combination. IL-1α was the weakest stimuli while a combination of IL-1α, TNF-α and IFN-γ provided the strongest stimulation.

The immunomodulatory effects of IL-4 and IL-13 on the chemokine protein
secretion were also studied. Stimulation of the cells with IL-4 and IL-13 for an hour at relatively high concentrations (10 and 30 ng/ml) prior to stimulation with the combination of IL-1α, TNF-α and IFN-γ inhibited the secretion of RANTES protein but did not inhibit the secretion of IL-8 mRNA or protein. This implied that the chemokines might have different inhibitory pathways. IL-4 and IL-13 played an important role in decreasing certain inflammatory chemokines and this might have an important effect in decreasing gastric inflammation.

Key words: chemokines, interleukin-8, RANTES, cytokines, IL-4, IL-13, MKN45 gastric epithelial cell

INTRODUCTION

Interleukin-8 (IL-8) belongs to the ‘CXC’ chemokine family while the ‘regulated on activation, normal T cell expressed and secreted’ (RANTES) belongs to the ‘CC’ chemokine family. The distribution of the chemokines into the different families is based upon the 4 cysteines (represented by the letter ‘C’ ) in the terminal amino section of the chemokine family which are linked by disulphide bonds to the more structured core of the molecule while the ‘X’ refers to an amino acid separating the cysteine molecules. In the ‘CC’ chemokine family, two of the cysteines are adjacent. ‘CXC’ chemokines attract mainly neutrophils while ‘CC’ chemokines attract monocytes, basophils, eosinophils, dendritic cells and T cells (Bliss et al. 1998; Mantovani 1999; Rollins 1997).

It has been shown that in the stomach the pro-inflammatory cytokines such as interleukin-1α (IL-1α) and tumour necrosis factor-α (TNF-α) were induced by infection with the Helicobacter pylori bacteria (Yamaoka et al. 1995; Crabtree 1998; Zarrilli et al 1999). Studies had also shown that H. pylori infection induce the expression of IL-8, growth-related oncosine (GRO-α), macrophage inflammatory protein-1α (MIP-1α) and 78-amino acid epithelial-cell-derived neutrophil activator (ENA-78) (Yamaoka 1995; Ando et al. 1996; Ando et al. 1998; Rieder et al. 2001). Cytokines and chemokines play a major role in recruiting many types of leucocytes including neutrophils, T cells, monocytes, eosinophils to sites of inflammation and infection. This is achieved by the activation of cell adhesion molecules which regulate the movement of the leucocytes (Zack Howard et al. 1999). The migration of leucocytes is important in the immunosurveillance of the tissues in the body. Recently, chemokines have also been demonstrated to have angiogenic and angiostatic properties (Strieter et al. 1995).

In this study, T-cell-derived cytokines, IL-4 and IL-13 were studied in the regulation of IL-8 and RANTES production as these cytokines have been found to be potent in the modulation of immune function (Minty et al. 1993).
IL-4 is produced by T lymphocytes, mast cells and basophils and it regulates the Immunoglobulin E (IgE) and mast cell/eosinophil-mediated immune reactions. IL-13 is distantly related to IL-4 and both share some similar structural characteristics (Brombacher 2000). IL-13 is produced by activated T lymphocytes, mainly Th2 cells, mast cells, basophils, dendritic cells and natural killer cells (Zurawski & de Vries 1994). IL-13 has both immunosuppressive and anti-inflammatory properties and has been shown to suppress some chemokines such as IL-8, monocyte chemotactic protein-1 (MCP-1), MIP-1α, RANTES, monocyte chemotactic protein-3 (MCP-3) and eotaxin in response to interferon-γ (IFN-γ) or bacterial lipopolysaccharides (Minty et al. 1993). IL-13 had also been shown to inhibit the secretion of IL-8 and MIP-1α by monocytes and this led IL-13 to be termed as an anti-inflammatory cytokine (De Waal Malefyt et al. 1993).

The immunomodulatory properties of IL-13 and IL-4 on the chemokines expressed by the gastric epithelial cells have not been studied. In this study, the gastric epithelial cell line, MKN45, stimulated with a combination of the pro-inflammatory cytokines, IL-1α, TNF-α and IFN-γ was used as a model of the inflamed gastric mucosa. The MKN45 cell line had been used as a model of the gastric epithelial mucosa in previous studies (Rieder et al. 2001; Mori et al. 2001). The effects of IL-4 and IL-13 on the expression of IL-8 and RANTES were studied for possible anti-inflammatory properties. Due to the immunomodulatory and anti-inflammatory properties of IL-13 and IL-4 demonstrated in other cells, there is a possibility that these cytokines could also be used in the treatment of gastritis or gastric diseases where there is an elevation in the levels of chemokines produced.

MATERIALS AND METHODS

GENERAL

The human gastric epithelial cell line, MKN45, was obtained from the Japanese Collection of Research Bioresources (JCRB) and had been isolated from a poorly differentiated adenocarcinoma in the stomach of a 62 year old female. The materials used were obtained as follows:

IL-1α and IL-4 from PeproTech (London, UK), TNF-α from Bayer (UK), IFN-γ, blocking agent, CSFD, Digoxigenin chemiluminescent detection kit and positively charged nylon membranes from Roche (Lewes, UK), IL-13 from Sanofi (France), anti-IL-8 mouse anti-human monoclonal antibody, anti-IL-8 biotinylated mouse anti-human monoclonal antibody, anti-RANTES mouse anti-human monoclonal antibody, anti-RANTES biotinylated goat anti-human polyclonal antibody and 5’ Digoxigenin labeled probes for IL-8, RANTES and β-actin from R & D Systems (Abingdon, UK), RPMI 1640
medium, agarose, amphotericin, streptomycin, penicillin and phosphate buffered saline without calcium and magnesium from Gibco BRL (Paisley, UK), bovine serum albumin, citric acid monohydrate, diethyl pyrocarbonate (DEPC), dimethyl sulphoxide, ethylenediaminetetraacetic acid (EDTA), ethidium bromide, Ficoll, hydrogen peroxide, maleic acid, 3-N-morpholino-propane sulfonic acid (MOPS), o-phenylene-diamine dihydrochloride (OPD) tablets, sodium acetate, sodium azide, sodium dodecyl sulfate (SDS), sodium hydroxide, sodium sulphite, streptavidin peroxidase, polyoxyethylene 20 sorbitan monolaurate (Tween 20), maleic acid and Trizma base from Sigma (Poole, UK), bromophenol blue, formaldehyde, formamide, sarcosyl, sodium chloride, sucrose from BDH (Poole, UK), cell culture plastics from Nunc (Paisley, UK), chloroform, propan-2-ol and glacial acetic acid from Fisons (Loughborough, UK), filter tips from Greiner (Gloucestershire, UK), ethanol from Hayman Ltd. (Witham, UK), hydrochloric acid, sulphuric acid and glycerol from Fisher Scientific (Loughborough, UK), trisodium citrate dehydrate from Aldrich (Gillingham, UK), RNAZOL B® from Tel-test (Texas, USA) and X-Omat film from Amersham International (Little Chalfont, UK).

CELL CULTURE

MKN45 adherent gastric epithelial cells were cultured in 80 cm² tissue culture flasks in RPMI 1640 medium respectively for the Northern analysis experiments and in 96 well plates for the enzyme-linked immunoassay (ELISA) experiments. The medium contained 2 mM glutamine and were supplemented with penicillin (10 U/ml), streptomycin (10 µg/ml), amphotericin (0.5 µg/ml) and 10% foetal bovine serum. These cell cultures were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO₂ and 95% air. The medium was changed every 2-3 days.

EXPERIMENTAL PROTOCOL

Prior to the cells being stimulated with pro-inflammatory cytokines IL-1α, TNF-α and IFN-γ alone or in combination (cytomix), confluent cells were serum-starved for 24 hrs to retard cell growth. These growth-arrested cells were then treated with fresh serum-free medium and specific doses of cytokines or vehicle controls for the stated times described in the results section. For the experiments in which the immunomodulatory effects of IL-4 or IL-13 were studied, the 'serum starved' cells were stimulated with varying concentrations of IL-4 or IL-13 from 0 ng/ml (control) up to 30 ng/ml for an hour before these cells were stimulated with a combination of IL-1α, TNF-α and IFN-γ (cytomix) for 24 or 48 hrs depending on the chemokine studied, IL-8 or RANTES, respectively. After 24 or 48 hrs, the supernatants were analysed according to the ELISA method. The experiments were conducted in triplicate.
RNA ISOLATION AND DETECTION OF mRNA

For the Northern analysis experiments, total cellular RNA was extracted from the MKN45 cells by adding RNAzol B® and the cells removed with a plastic cell scraper. To extract total RNA, 100 ml of chloroform was added to each 1 ml of homogenate. The homogenates were centrifuged at 12000 g for 15 min at 4°C. The upper, clear layer was mixed with 0.5 ml of propan-2-ol. The mixture was left on ice for 15 min before it was centrifuged again at 12000 g for 15 min. After centrifugation, the supernatant was discarded and the white RNA pellet at the bottom of the tube was washed with 1 ml 75% cold ethanol and centrifuged again at 12000 g for 8 min at 4°C. The contents of the centrifuge tube was frozen at -70°C for use in the subsequent Northern analysis experiments.

NORTHERN ANALYSIS

After overnight storage at -70°C, the RNA pellet was rewashed in 1 ml of fresh 75% cold ethanol and centrifuged at 12000 g for 8 min. Then the ethanol was removed and the RNA pellet was air dried in a fume cupboard for an hour. When the pellet was dry, it was solubilised in 20 - 40 µl of DEPC-treated water and kept on ice. Total RNA was measured using a Gene Quant spectrophotometer. Two ml of RNA was diluted into 100 µl of DEPC-treated water in a cuvette and its absorbance was read at 260 nm.

For Northern analysis, 30 µl of sample buffer containing ethidium bromide was added to 5-7 µg of sample and the samples vortexed and heated for 15-30 min at 80°C. The cooled samples were stained with 2.5 µl bromophenol blue solution. The samples were centrifuged and loaded onto 1% agarose gels submerged with cold 1X MOPS running buffer. RNA (5 - 7 µg calculated from the spectrophotometer readings) was loaded per lane and the gel was run at a current of 100 mA. The gel was placed on an ultraviolet transilluminator and the ethidium bromide-stained 18S and 28S ribosomal RNA bands were examined for equal loading and photographed. The gel was rinsed in sterile water prior to transblotting.

The gel was transblotted overnight onto a nylon membrane in a gel blotting tank which was partially filled with 20XSSC buffer. A strip of filter paper was placed in the tank as a wick to draw up the 20XSSC buffer. The RNA was baked in an oven at 120°C for 20 min to allow the RNA to be fixed onto the membrane. The nylon membrane was then hybridized.

Nylon membranes were hybridized with Digoxigenin-labelled oligonucleotide probes for either β-actin, IL-8 or RANTES. The nylon membrane was prehybridised with hybridization solution for an hr at 42°C and then hybridized with 1 ml of 10 ng/ml IL-8 or RANTES RNA probe diluted in hybridization solution at 42°C overnight. The membrane was washed twice at 42°C with 2XSSC containing 0.1% SDS followed by washing buffer,
The membrane was then washed for 30 min with a buffer containing 10% blocking stock solution and incubated for 30 min with a Dig-labeled antibody diluted 1:10000 in a buffer containing 10% blocking stock solution. After this, the membrane was washed twice in a wash buffer. The membrane was then equilibrated with a buffer containing TRIS and NaCl, drained and incubated for 5 min with 1 ml of CSPD substrate liquid diluted 1:100 in the same buffer. After draining, the membrane was incubated in a dark place at 37°C for 10 - 15 mins. The membrane was then exposed to a Kodak X-Omat AR5 X-ray film for about 2 hrs at room temperature in a dark room. The film was then developed using an automatic RGH Fuji X-ray film developer. The nylon membranes which were tested for IL-8 and RANTES mRNA expression were subsequently stripped of the chemokine probe and re-probed for the housekeeping gene β-actin to prove that cellular mRNA was equally loaded in each well. Relative amounts of IL-8 or RANTES mRNA were measured using a densitometer.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

This sandwich ELISA technique was used to quantify antigenic chemokine protein secretion into cell culture media supernatants and all samples were measured in duplicate.

The monoclonal capture anti-IL-8 or anti-RANTES antibodies were diluted in coating buffer to a concentration of 2 μg/ml in PBS (pH 7.4) buffer. Immediately, 50 μl of coating antibody was added to each of the 96 wells in a 96-well microtitre plate and the plate was left covered overnight at room temperature. The plate was washed with wash buffer and 100 μl of blocking buffer was added to each well and the plate incubated for an hour at room temperature. Following this, the plate was washed with wash buffer and the IL-8 or RANTES standards and culture supernatants which were diluted in dilution buffer were loaded into the wells in duplicates and incubated at room temperature for 2 hrs. The same standard concentration range of 0.02 – 2 ng/ml was used for both the IL-8 and RANTES standard curves. The plate was washed and 100 μl of 20 ng/ml of biotinylated anti-human IL-8 detecting antibody or 5 ng/ml of biotinylated anti-human RANTES detecting antibody was added onto each well and the plate incubated at 37°C for an hour.

The plate was then washed with wash buffer and 50 μl/well of streptavidin peroxidase (0.2 μg/ml) was added to each well on the plate and incubated at 37°C for 30 min. The plate was washed with wash buffer and 50 ml of substrate solution containing hydrogen peroxide and a 10 mg OPD tablet was added to each well at a volume of 100 μl. The plate was incubated in the dark for 30 min for IL-8 and 45 min for RANTES. Then 150 μl of 1 M H₂SO₄ was added to each well and the optical density of the plate was determined at 490 nm on Dynatech MR500.
RESULTS AND DISCUSSION

ELISA analysis of the MKN45 cell supernatants indicated that unstimulated cells did not secrete IL-8 or RANTES proteins. Stimulation of the cells with 10 ng/ml of IL-1\(\alpha\) resulted in low levels of IL-8 protein being secreted in a time-dependent manner, i.e. 1.38 ng/ml at 1 hr and 3.64 ng/ml at 72 hrs (Fig.1). This suggests that IL-1\(\alpha\) is a fairly weak stimulant as it was also shown to induce very short-lived IL-8 mRNA expression. Secreted IL-8 protein levels were higher when the MKN45 cells were stimulated with TNF-\(\alpha\), indicating that it was a more potent inducer (Fig.1). The levels of IL-8 did not increase after 48 hrs stimulation with TNF-\(\alpha\) since the MKN45 cells were starting to undergo apoptosis due to foetal bovine serum depletion. When the IL-1\(\alpha\), TNF-\(\alpha\) and IFN-\(\gamma\) combination was used to stimulate the MKN45 cells, high IL-8 protein levels were secreted similar to levels seen when TNF-\(\alpha\) was added alone (Fig.1). These high concentrations of cytokines had previously been used on the HT-29 colonic epithelial cells to induce maximum expression of IL-8 and RANTES protein (Kolios et al. 1996; Kolios et al. 1999).

Cytokines added singly to the MKN45 cells did not induce RANTES protein. Stimulation with TNF-\(\alpha\) and IFN-\(\gamma\) or a combination of IL-1\(\alpha\), TNF-\(\alpha\) and IFN-\(\gamma\) induced a similar time-dependent secretion of RANTES (Fig. 2). This indicated that IL-1\(\alpha\) was not important in inducing RANTES expression. The concentration of RANTES protein secreted in response to stimulation with TNF-\(\alpha\) and IFN-\(\gamma\) was 0.05 ng/ml at 1 hr and 2.19 ng/ml at 72 hrs.

![Graph showing IL-8 protein secretion](image)

**FIGURE 1.** A time course of IL-8 protein secretion by the MKN45 cell line.
Stimulation with IL-1α, TNF-α and IFN-γ yielded RANTES protein levels of 0.02 ng/ml at 1 hr, increasing to 3.29 ng/ml at 72 hrs post-stimulation. The kinetics of TNF-α and IFN-γ induced RANTES secretion suggests that this chemokine may be involved in the recruitment and activation of T cells over long periods.

The ELISA results showed that there was no significant effect of IL-4 on IL-8 protein secretion in the MKN45 cell line upon modulation of the cells with IL-4 prior to stimulation with IL-1α, TNF-α and IFN-γ (Fig. 3). Northern analysis experiments carried out indicated that IL-13 also did not modulate cytokine-induced IL-8 mRNA expression in MKN45 cells (Fig. 4). Thus IL-4 and IL-13 did not act as a stimulating or inhibitory cytokine on the production

FIGURE 2. A time course of RANTES protein secretion by the MKN45 cell line

FIGURE 3. The expression of IL-8 protein by MKN45 cells pre-stimulated with IL-4 (1 hour) and stimulated with IL-1α (10ng/ml), TNF-α (100ng/ml) and IFN-γ (300/ml) for 24 hours
FIGURE 4. The expression of IL-8 mRNA by MKN45 cells pre-stimulated with different concentrations of IL-13 (for an hour) and stimulated with IL-1α (10ng/ml), TNF-α (100ng/ml) and IFN-γ (300/ml) for 24 hours of IL-8 protein in MKN45 cells. However, IL-4 and IL-13 were both able to inhibit RANTES protein production in a concentration-dependent way in the MKN45 cell line. Both of these results were similar to the effects in the HT-29 colonic epithelial cell line where the 'CC' chemokines MCP-1 and RANTES were inhibited by IL-4 and IL-13 whereas IL-8 was not affected (Kollios et al. 1996). There was a 31% and 25% decrease in RANTES secretion compared to control when 10 ng/ml and 30 ng/ml of IL-4 were used. Dunnett’s test indicated that these concentrations of IL-4 significantly (P <0.05) inhibited RANTES secretion by MKN45 cells (Fig. 5). IL-13 when applied in the same manner was also found to inhibit the expression of RANTES. The highest concentrations of IL-13 used (10 and 30 ng/ml), together with IL-1α, TNF-α and IFN-γ caused a 33.3% and 60% decrease in RANTES secretion (Fig. 6). Dunnett’s test indicated that these concentrations of IL-13 significantly (p < 0.05) inhibited RANTES secretion. These results indicate that the MKN45 gastric epithelial cells have functional IL-4 and IL-13 receptors which are closely related but are distinct. These receptors are known to have independent biological functions; although
FIGURE 5. The secretion of RANTES/CCL5 protein by MKN45 cells pre-stimulated with IL-4 (1 hour) and stimulated with IL-1α (10ng/ml), TNF-α (100ng/ml) and IFN-γ (300U/ml) for 48 hours * (p < 0.05), ANOVA and Dunnett’s test

FIGURE 6. The secretion of RANTES protein by MKN45 cells pre-stimulated with IL-13 (1 hour) and stimulated with IL-1α (10ng/ml), TNF-α (100ng/ml) and IFN-γ (300U/ml) for 48 hours * (p<0.05), ANOVA and Dunnett’s test

RANTES down-regulation in the MKN45 cells was a similar response to both IL-4 and IL-13.

The ability to inhibit RANTES but not IL-8 production indicates that differential signaling pathways by TNF-α and IFN-γ are used by these two chemokines. IL-4 and IL-13 may be able to decrease inflammation associated with monocyte and macrophage-derived cytokines and chemokine (Minty et al. 1993). The responses measured as a result of IL-4 and IL-13 modulation
are variable depending on the cell type studied, the stimulus used and the chemokine expression investigated. The inability of IL-4 and IL-13 to increase or decrease IL-8 production in IL-1α, TNF-α and IFN-γ stimulated gastric epithelial cells suggests that these Th2 cytokines may not modulate neutrophil infiltration in vivo. However, the ability of IL-4 and IL-13 to decrease RANTES production in the presence of the pro-inflammatory mediators suggests that these Th2 cytokines may possibly modulate the T cells, basophils, monocytes and natural killer cells in vivo.

CONCLUSION

Stimulation of the MKN45 cells with pro-inflammatory mediators IL-1α, TNF-α and IFN-γ alone or in combination, which are normally elevated in inflammatory conditions in the stomach, resulted in the expression of IL-8 protein which recruits neutrophils in acute inflammatory states. The minimal stimulation to express RANTES protein was a combination of TNF-α and IFN-γ which would cause the recruitment of T cells, basophils, monocytes and natural killer cells in an in vivo setting. TNF-α appeared to be a more potent stimuli of RANTES protein secretion compared to IL-1α, and the combination of TNF-α and IFN-γ effects seemed to be comparable to the effects of a combination of IL-1α, TNF-α and IFN-γ. IL-4 and IL-13 did not have any significant effects on the secretion of IL-8, therefore they may not be useful in the modulation of neutrophil recruitment in gastric diseases. IL-4 and IL-13 used at higher concentrations were effective in down-regulating the expression of RANTES protein in MKN45 cells stimulated with a combination of the three pro-inflammatory cytokines. This suggests that IL-4 and IL-13 could possibly play an important role in decreasing the inflammatory component of gastric diseases.

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