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MOLECULAR REGULATION OF INTERLEUKIN-8 IN HUMAN COLONIC EPITHELIAL CELLS

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ABSTRACT

Interleukin-8 is a chemokine which is chemotactic for neutrophils and T-lymphocytes and plays a crucial role in the pathogenesis of inflammatory bowel disease. Intestinal mucosal epithelial cells produce IL-8 in response to pathogens which mediates bidirectional communication between pathogen and host. The objective of this study was to investigate the molecular mechanisms involved in IL-8 gene regulation in T84 human colonic epithelial cells. To determine if IL-8 plays a role in the pathogenesis of intestinal amebiasis, the effect of *Entamoeba histolytica* on IL-8 gene expression was investigated. *E. histolytica* secreted components enhanced IL-8 mRNA expression and protein production in the absence of amebae-enterocyte contact. The proinflammatory cytokines IL-1ß and TNF-α were not involved in IL-8 protein production. As PGE_2 is central in mucosal inflammation, the effect of PGE_2 on IL-8 gene expression was determined. Using purified PGE_2 and PGE_2 receptor agonists, it was shown that PGE_2 coupled to the EP_4 receptor and triggered cAMP-dependent PKA signaling which upregulated IL-8 mRNA expression at the posttranscriptional level. Elevation of $[Ca^{2+}]_i$ from intracellular Ca^{2+} stores by A23187 or thapsigargin stimulated IL-8 mRNA transcription and IL-8 protein production through the activation of calcineurin. Moreover, IL-8 3'-UTR had a strong suppressive effect on CAT reporter gene expression in COS7 cells by reducing its mRNA level. A unique fragment (nt 2387-2743) containing AU rich elements was shown to attenuate CAT mRNA expression by destabilizing the transcripts. Secondary structure but not AU rich elements played a major role in CAT mRNA turnover.

ABRÉGÉ

L'interleukine-8 est une chimiokine qui est chimiotactique pour les neutrophiles et les lymphocytes-T, et qui joue un rôle important dans la pathogenèse des maladies inflammatoires de l'intestin. Lorsqu'exposées aux pathogènes, les cellules épithéliales de la muqueuse intestinale produisent de l'IL-8 qui dirige une communication bidirectionnelle entre les pathogènes et l'hôte. L'objectif de ce travail était d'étudier les mécanismes moléculaires impliqués dans la régulation du gène de l'IL-8 dans les cellules épithéliales coloniques humaines T84. Afin de déterminer si l'IL-8 joue un rôle dans la pathogenèse de l'amibiase intestinale, nous avons étudié les effets qu'*Entamoeba histolytica* a sur l'expression du gène de l'IL-8. Les produits de sécrétion solubles des amibes ont augmenté l'expression d'ARNm d'IL-8 et la production de la protéine, sans qu'il y ait contact entre les amibes et les entérocytes. Les cytokines pro-inflammatoires IL-1ß et TNF-α ne jouaient aucun rôle dans ce phénomène. Puisque la PGE_2 est impliquée dans l'inflammation des muqueuses, ses effets sur l'expression génique de l'IL-8 ont été étudiés. Nous avons démontré, en utilisant de la PGE_2 purifiée et des agonistes des récepteurs de PGE_2, que la PGE_2 se fixe au récepteur EP_4 et déclenche une signalisation PKA dépendante de l'AMPc qui augmente l'expression d'ARNm d'IL-8, agissant au niveau de la post-transcription. L'augmentation du $[Ca^{2+}]_i$ induite par l'A23187 ou la thapsigargine, et provenant des réserves de Ca^{2+} intracellulaires, a stimulé la transcription de l'ARNm d'IL-8 et la production d'IL-8 via l'activation de la calcineurine. De plus, le 3'-UTR de l'ARNm d'IL-8 a eu un effet suppressif marqué sur l'expression du gène témoin CAT chez les cellules COS7, en diminuant la quantité de son ARNm. Un fragment (nt 2387-2743) contenant des éléments riches en AU a attenué

l'expression d'ARNm de CAT en déstabilisant ces derniers. La structure secondaire du fragment, mais non ses éléments riches en AU, a joué un rôle primordial dans le roulement de l'ARNm de CAT.

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TABLE OF CONTENTS

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STATEMENT OF ORIGINALITY

The following elements are considered contribution to original knowledge.

1). The first demonstration that *E. histolytica* secreted soluble components can stimulate IL-8 mRNA expression and protein production in human colonic epithelial cells in the absence of enterocyte-parasite contact. This suggests that *E. histolytica* may initiate host inflammatory responses and tissue injury prior to amebic contact with mucosal epithelial cells via the induction of IL-8.

2). The first demonstration that the lipid mediator of inflammation, PGE_2 can stimulate IL-8 mRNA expression and protein production in human colonic epithelial cells. This event occurred by PGE_2 binding to EP_4 receptors which activated cAMP-dependent PKA signal transduction pathway. PGE_2 responsive elements present in IL-8 3'-UTR contributed to the stabilization of IL-8 mRNA. These data strongly suggest that PGE_2 may exacerbate inflammatory response in the intestinal mucosa via IL-8.

3). The first demonstration that elevation of $[Ca^{2+}]_i$ in response to either A23187 or thapsigargin can up-regulate IL-8 gene expression in human colonic epithelial cells through a Ca^{2+}/calmodulin-dependent calcineurin signal transduction pathway. Newly synthesized protein(s) was not necessary for this event to occur. Both transcriptional and posttranscriptional mechanisms are involved in the up-regulation of IL-8 evoked by A23187 and thapsigargin.

4). The demonstration that IL-8 3'-UTR can suppress reporter gene expression and protein production. We identified a unique 357-base sequence (designated **e**, bp 2387-2743 in genomic DNA) within the 3'-UTR that suppressed reporter gene expression by accelerating mRNA turnover. Secondary structure but not AU rich sequences of **e** played a major role in IL-8 mRNA turnover. This study gives new insights into the molecular mechanism involved in the role of IL-8 3'-UTR in posttranscriptional regulation of the IL-8 gene.

STATEMENT OF AUTHORSHIP

This thesis contains four manuscripts co-authored with my supervisor, Dr. Kris Chadee. I was responsible for data collection, analysis, and manuscript preparation. My supervisor provided financial resource for the laboratory work, guidance on the design of the study, interpretation of the data, and preparation of the manuscripts and thesis. Carla De Waele was a summer student who assisted with some experiments on calcium-dependent IL-8 gene expression in human colonic epithelial cells, and is therefore included as a co-author on the manuscript III.

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACT D	Actinomycin D
AU	Adenosine/uridine
bp	Base pair
°C	degree Celsius
CaM	Calmodulin
CAT	Chloramphenicol acetyltransferase
COX	Cyclooxygenase
CHX	Cycloheximide
DMEM	Dulbecco's modified Eagle's medium
dNTP	Deoxynucleoside
DTT	Dithiothreitol
E.h P	Amebic protein
E.h SC	Amebic secretory components
ELISA	Enzyme-linked Immunosorbent assay
ERK	Extracellular signal regulated kinase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Guanine/cystine
IL	Interleukin
IP3	Inositol-1,4,5-triphosphate
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activation protein kinase
nt	Nucleotide
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PGE_2	Prostaglandin E_2
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol myristate acetate
3'-RACE	3' rapid amplification of cDNA ends
RT	Reverse transcription
TNF	Tumor necrosis factor
UTR	Untranslated region

SECTION I: LITERATURE REVIEW

INTRODUCTION

Interleukin-8 (IL-8) is a chemokine of the CXC family which causes recruitment and infiltration of neutrophils into local inflammatory sites, resulting in non-specific tissue damage (1). IL-8 has been associated with various inflammatory conditions, such as psoriasis, arthritis, respiratory diseases, inflammatory bowel diseases (IBD) and infectious diseases (1, 2).

IL-8 gene expression is regulated transcriptionally and/or posttranscriptionally by various stimuli from bacterial substances to proinflammatory cytokines through various signal transduction pathways (1, 3). Although transcriptional regulation of IL-8 gene expression is well defined (4-9), there is limited information with regards to posttranscriptional regulation of IL-8 gene expression (10, 11). Adenosine/uridine (AU) rich sequences such as AUUUA pentamers in the IL-8 3'-UTR were suggested to be involved in posttranscriptional regulation (12-14) but these elements are not defined. Clearly, more studies need to be done to further understand the molecular mechanisms involved in the posttranscriptional regulation of the IL-8 gene.

Mucosal epithelial cells in the respiratory and gastrointestinal tracts are considered the first line of host defence against infectious pathogens and also produce IL-8 (15). In bacterial infections, IL-8 stimulated by soluble bacterial components or bacterial entry into target cells contributed to mucosal inflammation (16-20). Similarly, up-regulation of IL-8 in human colonic epithelial cells by *E. histolytica* trophozoites led to intestinal inflammation in both *in vitro* and *in vivo* models. This event was dependent on contact between amebae and enterocyte and subsequently, cytolysis of enterocytes which released preformed IL-1α leading to up-regulation of IL-8 from the remaining intact epithelial cells (21-23). However, the molecular mechanisms involved in the induction of IL-8 by amebae is not known. There is strong evidence supporting the involvement of IL-8 in

IBD such as Crohn's disease and ulcerative colitis (24, 25). Although IL-8 are produced mainly from infiltrated macrophages and neutrophils in IBD (26), activated intestinal epithelial cells may contribute to IL-8 production in the inflamed mucosa. The amount of IL-8 produced was correlated with the histologic severity (grade) of disease activity (27, 28). While it is certain that a variety of proinflammatory cytokines can stimulate IL-8 during inflammatory states, little is known on the role of lipid mediators, especially PGE_2 in mediating IL-8 expression. PGE_2 has regulatory functions on IL-8 gene expression in various cell types in response to certain stimuli, however, it is not able to regulate IL-8 gene expression by itself (29-31). It is not known if PGE_2 stimulates IL-8 gene expression in mucosal epithelial cells.

The objective of this study was to investigate the molecular mechanisms involved in IL-8 gene regulation in human colonic epithelial cells. The specific aims of the study were:

1). To determine the mechanisms involved in *E. histolytica*-induced IL-8 gene expression in human colonic epithelial cells,

2). To elucidate the effect of PGE_2 on IL-8 gene expression in T84 human colonic epithelial cells,

3). To characterize the mechanisms involved in Ca^{2+}-dependent IL-8 gene expression,

4). To examine the role of 3'-untranslated region (UTR) of IL-8 in posttranscriptional regulation of the IL-8 gene.

The results of this study will be very helpful in unraveling the molecular regulation of IL-8 gene expression and protein production in human colonic epithelial cells and its crucial role in intestinal inflammation in amebiasis and in inflammatory bowel disease.

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Chapter 1. IL-8: structure and regulation

IL-8, previously called monocyte-derived neutrophil-activating peptide (MONAP) (1, 2), neutrophil activating factor (NAF) (3, 4), monocyte-derived neutrophil chemotactic factor (MDNCF)(5), lymphocyte derived neutrophil activating peptide (LYNAP)(6), or neutrophil activating peptide-1 (NAP-1) (7), belongs to the CXC subfamily of chemokines. It is produced by various cell types, including monocytes, macrophages, T-lymphocytes, epithelial and endothelial cells, hepatocytes, gastric and intestinal carcinoma cells, and neutrophils and is a potent chemoattractant for neutrophils, T-lymphocytes, basophils, and eosinophils (8). IL-8 has been shown to be involved in inflammation during inflammatory and infectious diseases (8-11).

Chemokines are 70 to 100 amino acid peptides classified into four subfamilies according to the position and the number of their cysteine residues (Cys or C) in the N-terminus (Table 1). Three subfamilies have been named according to the position of the first two cysteines, CXC (the first two of four cysteines are separated by one amino acid, X), CC (the first two cysteine residues are adjacent), and CXXXC (the first two cysteines are separated by three amino acids). The fourth chemokine subfamily has been discovered recently and was called C chemokine because its members contain only one cysteine at the N-terminus instead of two. CXC chemokines are divided into two groups, those that contain the ELR motif (glutamic acid-leucine-arginine) near the N-terminus attract neutrophils, and those that lack this motif act on T lymphocytes. The members of the human chemokine superfamily are shown in the phylogenic tree in Figure 1. Chemokines exert their functions through coupling to their receptor(s) on target cells (Tables 2 and 3) (12-23). IL-8 activates neutrophils through its two high affinity receptors CXCR1 and CXCR2, leading to chemotaxis and activation of neutrophils

Table 1. The Chemokine superfamily

CXC

Interleukin-8 (IL-8)
Growth-related oncogene α (GRO-α)
Growth-related oncogene β (GRO-β)
Growth-related oncogene γ (GRO-γ)
Granulocyte-related chemotactic protein-2 (GCP-2)
Epithelial neutrophil activating protein-78 (ENA-78)
Neutrophil activating protein-2 (NAP-2)
Interferon-γ-inducible protein (IP-10)
Monokine inducible protein (MIG)
Stromal cell derived factor-1 (SDF-1)
Platelet factor 4 (PF-4)
  Platelet basic protein (PBP)
  Connective tissue activating protein-III (CTAP-III)
  β-thromboglobulin (β-TG)
B cell-attracting chemokine-1 (BCA-1)

CC

Monocyte chemoattractant protein-1 (MCP-1)
Monocyte chemoattractant protein-2 (MCP-2)
Monocyte chemoattractant protein-3 (MCP-3)
Monocyte chemoattractant protein-4 (MCP-4)
Macrophage inflammatory protein-1α (MIP-1α)
Macrophage inflammatory protein-1β (MIP-1β)
Regulated on activation normal T-cell expression and secreted (RANTES)
Eotaxin
Eotaxin-2
I-309
6Ckine
Macrophage-derived chemokine (MDC)
Thymus and activation regulated chemokine (TARC)
Hemofiltrate-derived CC chemokine I (HCC-1)
Stimulated T cell chemotactic protein (STCP-1)
Dendritic-cell-derived chemokine (DC-CK-1)
Liver and activation-regulated chemokine (LARC)
Epstein-Barr virus (EBV)-induced gene 1 (EBI 1)
Secondary lymphocyte chemokine (SLC)

CXXXC

Neurotactin/Fractalkine

C

Lymphotactin (LTN)

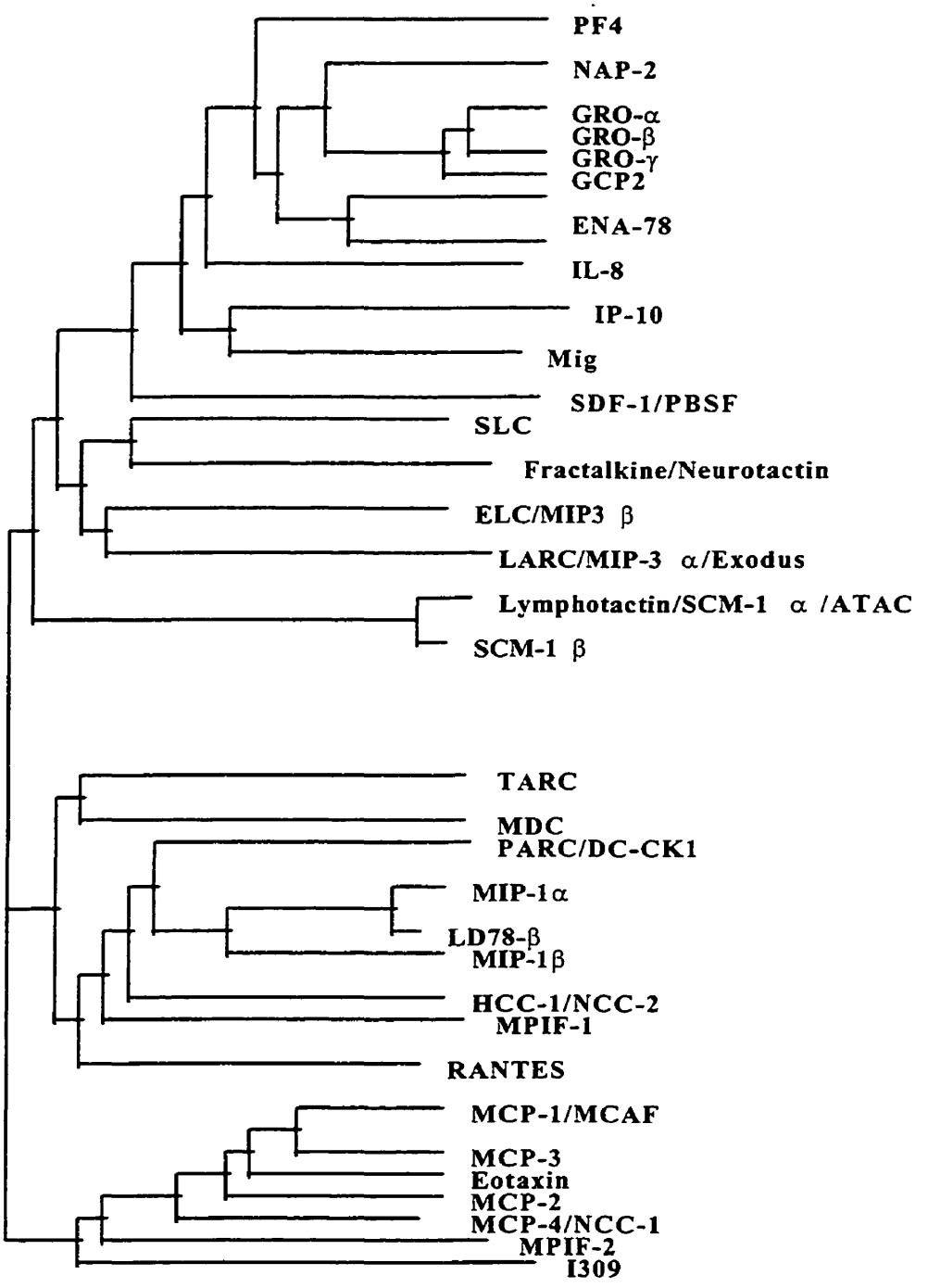

Figure 1. Phylogenic tree of the human chemokine superfamily. Mature forms of the members of the human chemokine superfamily were aligned using the Clustal W program with a BLOSUM residues weight table and default settings of gap penalties for pairwise and multiple alignments. Adapted from Yoshie et al (12).

characterized by neutrophil shape change, exocytosis, up-regulation of receptors and adhesion molecules, formation of bioactive lipids, and initiation of respiratory burst to release oxygen intermediates (3, 5).

Table 2. Receptors for Chemokines

Chemokines	Receptors
IL-8, GCP2	CXCR1
IL-8, GRO α,β,γ, ENA-78,NAP2, PF-1	CXCR2
IP-10, MIG, 6Ckine	CXCR3
SDF-1	CXCR4
BCA-1	CXCR5
MIP-1α, RANTES, MCP-2, MCP-3	CCR1
MCP-1, MCP-3, MCP-4	CCR2
Eotaxin, Eotaxin-2, RANTES, MCP-2, MCP-3, MCP-4	CCR3
TARC, MDC	CCR4
RANTES, MIP-1α, MIP-1β	CCR5
LARC, MIP-3α, Exodus	CCR6
EBI1, SLC	CCR7
I-309	CCR8
Neurotactin/Fractalkine	CX_3CR1

Table 3. Target cells of chemokines

Chemokines	Target cells
IL-8, MIP-1α, MCP-1, MCP-3, RANTES, Eotaxin-2, GRO α, GRO β , GRO γ	Basophils
IL-8, MIP-1α, MCP-2, MCP-3, RANTES, Eotaxin, Eotaxin-2	Eosinophils
IL-8 GRO α, GRO β, GRO γ, ENA-78, NAP-2	Neutrophils
MIP-1 α, MIP-1β, MCP-1, MCP-2, MCP-3, Neurotactin, MDC	Monocytes
MCP-1, MCP-3, MIP-1, MIP-1, RANTES, I-309, IL-8, LTN, Neurotactin, 6Ckine, TARC, BCA-1, TARC, ELC, LARC, Exodus, SLC, DC-CK-1	Lymphocytes

1.1 IL-8 gene structure

The IL-8 gene is located on human chromosome 4, q 12-21, and contains 4 exons and 3 introns (Figure. 2) (24, 25). A number of regulatory sequences have been identified in the IL-8 5'-UTR, including a TATA box (bp - 20 to -13), AP-1 binding element (bp -126 to -120), AP-2 (bp -946 to -937), AP-3 (bp -82 to -77), hepatocyte nuclear factor-1 binding site (bp -381 to -376), interferon-γ responsive element (IRF-1) (bp -1299 to -1294; -576 to -571; -425 to -420), enhancer core sequence (bp -1382 to -1375; -851 to -844), heat shock element (bp -1175 to -1166), glucocorticoid responsive element (bp -330 to -325), cAMP responsive element (bp +828 to +835), nuclear factor(NF)-κB (bp -80 to -70), NF-IL-6/C/EBP (bp -94 to -82), and Oct-1(bp -82 to -77) (25-28). Mature IL-8 mRNA is about 1.8 kb with a 1.2 kb 3'-UTR that contains nine AUUUA elements and a polyadenylation site (AAUAUA) located at bp 3132-3137 (29,

30). These AU rich motifs have been suggested to contribute to the turnover of IL-8 mRNA but the molecular mechanisms involved is still unclear.

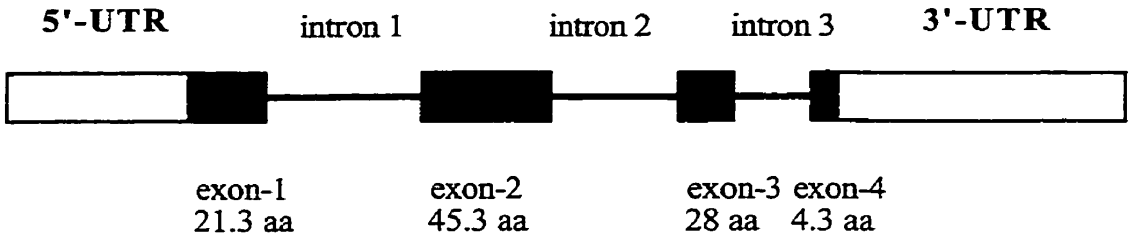

Figure 2. Diagramatic representation of IL-8 genomic DNA. Note the 4 exons (■), 3 introns (▬), and 5'- and 3'-UTR (□).

1.2 Regulation of IL-8 gene expression

There are numerous studies demonstrating that IL-8 gene expression can be modulated in various cells by cytokines, bacterial components, viral products, or parasites through invasion or adherence. However, the mechanisms involved and the signal transduction pathways that lead to transcriptional or posttranscriptional expression of IL-8 are still not clear. This review will summarize the salient features of IL-8 gene regulation.

1.2.1 Transcriptional regulation

Transcriptional regulation of IL-8 gene expression has been extensively studied (25-28, 31-39). Reporter gene transfection techniques and gel shift assay analyses have been used as the major approaches. IL-1, TNF-α or LPS stimulates the transcription of IL-8. The transcription factors recruited during the initiation of IL-8 gene expression vary according to cell types used and experimental procedures. Using chloramphenicol

acetyltransferase (CAT) reporter gene transfection techniques with differentially deleted IL-8 promoter sequences in the human fibrosarcoma cells, Mukaida et al. (26) demonstrated that the nucleotides between -94 to -71 bp from the start of the first exon were crucial and sufficient for IL-8 gene expression in response to IL-1, TNF-α, and PMA, accompanied with the activation of NF-κB and C/EBP(NF-IL-6). Mutations of either the NF-κB or C/EBP binding sites abolished the effect of IL-1 and TNF-α. However, in the human bronchial epithelial cell HS-24, -130 to -112 bp of the IL-8 promoter contributed to TNF-α stimulation (31). TNF-α-induced IL-8 transcription apparently underwent a promoter recruitment mechanism. In a ligation-mediated polymerase chain reaction (LMPCR), G residues at -97 to -80 bp in the IL-8 5'-UTR were protected, which presumably led to IL-8 gene transcription induced by TNF-α in A549 human alveolar cells. There was an abundance of NF-κB subunits (Rel A, NF-κB α, and c-Rel) that had translocated into the nuclei and bound to the TNF-α responsive elements (32). However, only Rel A (p65), but not NF-κB1(p50), NF-κB2(p50B) or Rel B, was involved in IL-8 gene expression through a NF-κB dependent mechanism in Jurkat T cells in response to PMA (33). Accumulated studies have shown a synergism in transcriptional activation of the IL-8 gene, especially the cooperativity of NF-κB and C/EBP (34-37). In human gastric cell lines (MKN 45 and KATO), for example, IFN-γ enhanced TNF-α-induced IL-8 gene transcription. Mutation of either the AP-1 or NF-κB binding elements attenuated the synergistic effect of IFN-γ (37). In contrast, IFN-α, ß and γ inhibited TNF-α induced IL-8 gene expression in human diploid F54 fibroblasts (38). The inhibitory effects of IFN-ß was always markedly higher than IFN-α or IFN-γ. Surprisingly, IFN-ß treatment did not block the activation of NF-κB proteins or their ability to bind to the NF-κB site. It seems that the inhibitory effect of IFN-ß on TNF-α-

induced IL-8 gene expression occurred through an unknown mechanism (38). IFN-ß probably inhibited the binding of C/EBP to its site since C/EBP-α has the ability of activating some κB elements, such as p50. Co-transfection of C/EBP-α and a reporter gene with the NF-κB binding element increased reporter gene expression (39). Wu et al. (28) have shown a negative transcription factor Oct-1 that strongly repressed transcriptional activity of the IL-8 promoter by binding independently to an element overlapping the C/EBP binding site.

1.2.2 Posttranscriptional regulation

There is limited information about the posttranscriptional regulation of the IL-8 gene, although its transcriptional regulation has been extensively studied. An increase in IL-8 gene expression through the stabilization of its transcripts has been reported. Treatment of human diploid fibroblasts with IL-1ß caused an accumulation of IL-8 mRNA through the delay in the degradation of IL-8 transcripts (40). Bosco et al. (41) reported that IFN-γ augmented IL-8 mRNA level in U937 cells by a posttranscriptional mechanism. Moreover, in human endometrial stromal cells pretreated with transforming growth factor (TGF)-ß and stimulated with IL-1α, the half life of IL-8 mRNA increased from 40 min to 150 min. Inhibition of protein synthesis by cycloheximide (CHX) prevented the effect of TGF-ß suggesting a role for *de novo* proteins (42). In whole human blood cells, LPS caused IL-8 mRNA accumulation by stabilizing IL-8 transcripts. The IL-8 messenger, after treatment with actinomycin D (Act D) following 23 hour LPS stimulation had a greater half life (>10 hours) than that after treatment with Act D following 2 hour LPS stimulation (4.6 hrs). However, the mechanism involved in this event has not been fully addressed (43). In the human lung-derived mucoepidermoid adenocarcinoma cell H292, CHX alone or CHX plus TNF-α increased the half life of IL-

8 mRNA (>4 hours) in comparison to that in unstimulated cells or cells treated with TNF-α alone (about 40 min) (44). Although AU rich elements have been suggested to be involved in the posttranscriptional regulation of the IL-8 gene (29, 30, 44), the molecular mechanisms involved in contribution of the 3'-UTR to mRNA stability still need to be elucidated.

1.3 Effect of glucocorticoids

Glucocorticoids are potent immunosuppressive agents which suppress inflammation and immune responses (45) and the most effective inhibitor of IL-8 gene expression (46, 47). The molecular mechanism involved in the regulatory effect of glucocorticoids on IL-8 gene expression is not well established due to the complexity of the involvement of various signal transduction pathways and trans-acting factors. The inhibitory effect of dexamethasone (Dex), a synthetic glucocorticoid, on IL-8 production was observed in fibroblasts, synoviocytes, chondrocytes, mesengial cells, monocytes, and macrophages (8). However, Dex can not inhibit IL-8 expression in peripheral blood monocytes in response to PMA (48), fibroblasts stimulated with leukoregulin (49), and airway epithelial cells stimulated with elastase (50). A glucocorticoid receptor element (GRE) was identified at -1175 to -1166 bp in the IL-8 5'-UTR (25). Deletion of the GRE element from the IL-8 promoter region abolished Dex-mediated repression of reporter gene expression; however, Dex had no effect on reporter gene expression in constructs lacking GRE sites but retaining NF-κB and AP-1 binding sites (51). These data suggest that GRE element is necessary for the action of Dex and there is no interference with the DNA binding of NF-κB and AP-1. However, GRE is not always the responsive element to Dex treatment. In the human glioblastoma cell line T98G, GRE did not contribute to Dex-induced suppression of IL-8 gene expression induced by IL-1α (52). Reporter gene

studies revealed that NF-κB was an exclusive cause. Mutation of either the AP-1 or C/EBP sites had no effect on IL-8 gene suppression by Dex although C/EBP and AP-1 were required for IL-1α in up-regulation of IL-8. Surprisingly, Dex only dimished IL-1α-induced NF-κB (p65 and p50) complex formation but could not alter the amount of NF-κB translocation into nuclei (52). It has also been shown that Dex could inhibit IL-8 gene expression through a posttranscriptional mechanism. Dex inhibited IL-ß-induced IL-8 gene expression in human bone marrow cells by destabilizing IL-8 mRNA and the inhibitory effect of Dex was dependent on newly synthesized protein(s). The stability of IL-8 mRNA could be influenced by the RNA destabilizing element AUUUA in the 3'-UTR which may contribute to the function of Dex (53), but more evidence is required to support this presumption.

1.4 Effect of prostaglandins

Low mol. wt. arachidonic acid metabolites, particularly PGs, are primarily involved in inflammatory reactions (54). PGs are synthesized from arachidonic acid by cyclooxygenase (COX). COX is the rate-limiting enzyme in the biosynthesis of PGs. Two isoforms of COX have been described to date: COX-1, which is constitutively expressed and distributed in most cell types and COX-2, which is induced by proinflammatory cytokines, LPS, growth factors and mitogens in cells that actively participate in inflammation such as macrophages and fibroblasts (55). Among the PGs, PGE_2 is considered to be the most potent mediator of inflammation. PGE_2 exerts its effects by interacting with specific receptors, which in turn activate diffferent signal transduction pathways (56, 57). It is clear that PGE_2 plays a very important role in inflammation and the pathology caused by infectious diseases. However, the mechanism involved in the action of PGE_2 is still not known. Regardless of the etiologic causes that

stimulate the production of PGs in local inflammatory sites, it is crucial to understand the role PGE_2 play in modulating inflammatory processes. Some studies have shown the regulatory effect of PGE_2 on IL-8 gene expression in various cell types (58-61). In human synovial fibroblasts, for example, exogenous PGE_2 enhanced IL-8 production in response to IL-1 stimulation but PGE_2 alone did not have any stimulatory effect. Interestingly, inhibition of PGE_2 synthesis by the COX inhibitor indomethacin, increased IL-8 production (58). It is unclear why the effect of exogenous PGE_2 on IL-8 production is different from that of endogenous PGE_2. However, PGE_2 had no effect on neutrophil- or macrophage-derived IL-8 gene expression evoked by LPS (59, 60), but down-regulated IL-8 production in human alveolar macrophages and blood monocytes in response to LPS (61). More recently (62), it has been shown that bradykinin, a potent inflammatory mediator in patients with bronchial asthma, enhanced IL-8 production in human airway smooth muscle cells through a COX-2-dependent mechanism. Inhibition of COX-1&2 activity with indomethacin, or COX-2 specific activity with NS-398, abolished the effect of bradykinin, suggesting the involvement of endogenous PGE_2 in IL-8 gene expression (62). These findings were confirmed by the observation of at least a six-fold increase in IL-8 protein production in response to exogenous PGE_2 (62). Thus, PGE_2 released from local inflammatory sites may promote or maintain a chronic airway inflammation.

1.5 Signal transduction of IL-8 gene expression

Differential activation of signal transduction pathways such as protein kinase C (PKC), protein kinase A (PKA), protein tyrosine kinase (PTK), or elevation of

intracellular Ca^{2+} concentrations cause IL-8 gene expression (transcription or posttranscription). However, the linkage between signal transduction pathways and the activation of transcription factors is still poorly understood. LPS, IL-1ß, TNF-α, PMA, and agents which increase cAMP or intracellular Ca^{2+} may enhance IL-8 gene expression in various cell types. Gross et al. (63) defined the role of protein kinases in IL-8 gene expression in a human colonic epithelial cell line (HT-29). IL-1ß and TNF-α stimulated IL-8 gene expression through a tyrosine kinase-dependent pathway. Staurosporine, a selective PKC inhibitor, additively increased the stimulatory effect of both IL-1ß and TNF-α, but attenuated the effect of PMA, implicating the possible role of PKC in the activation of tyrosine kinases. Depletion of PMA-dependent PKCs could not impair IL-1ß and TNF-α-induced IL-8. Interestingly, forskolin, a PKA activator, caused only a weak enhancement of IL-8 gene expression which was insensitive to PKA inhibitors. In other studies, ozone increased activities of both PTK and PKA in the human alveolar epithelial cell line A549, leading to the up-regulation of IL-8 (64). In a cell free system it was shown that LPS-dependent NF-κB activation was inhibited by protein kinase inhibitors, such as staurosporine, herbimycin A, tyrophostin, and genistein, but not mitogen-activation protein kinase substrate, cGMP-dependent protein kinase, cAMP-dependent protein kinase, PKC, or Ca^{2+}/calmodulin (CaM)-dependent protein kinase II inhibitory peptides (65) suggesting the involvement of both staurosporine-sensitive kinase(s) and tyrosine kinases. In human alveolar macrophages, LPS-induced IL-8 gene expression was tyrosine kinase and phosphatidylcholine specific phospholipase C (PLC)-dependent because the tyrosine kinase inhibitor genistein and PLC inhibitor D609, but not the PKC inhibitor bisindomaleamide, inhibited the binding capability of NF-κB evoked

by LPS (66). Activation of mitogen-activated protein kinase in human promyelocyte cells (HL60) also contributed to IL-8 gene expression by recruiting NF-κB (67).

In other studies, Okamoto et al. (68) demonstrated that the elevation of intracellular Ca^{2+} led to IL-8 gene transcription in Jurkat T cells. Both AP1 and NF-κB are involved in PMA- and ionomycin (Ca^{2+}-dependent signaling) -stimulated IL-8 gene expression. FK 506 is an immunosuppressive drug which suppresses the transcription of IL-2, IL-3, IL-4, and IFN-γ (69). It inhibits IL-2 gene expression by blocking the binding of NF-AT and NF-IL-2A to their binding elements in the IL-2 promoter through inhibition of Ca^{2+}/calmodulin-dependent phosphatase 2B (calcineurin) (70). The IL-8 gene does not contain NF-AT or NF-IL-2A binding elements in its promoter region. Suprisingly however, IL-8 was up-regulated in Jurkat T cells following stimulation with PMA plus ionomycin or thapsigargin through the activation of CaM- dependent calcineurin which was inhibited by FK506. Gel shift assays revealed that FK506 had no effect on AP-1 complex formation whereas it strongly disrupted NF-κB complex formation. On the other hand, no effect was observed in response to PMA or forskolin alone, which were Ca^{2+}-independent stimuli (68).

1.6 IL-8 protein structure

IL-8 protein isolated from the culture supernatant of LPS-stimulated human blood monocytes has been identified as a peptide with 72 a (aa) and a mol. wt. of about 8.4 kDa. The cDNA open reading frame encodes a 99 aa protein which is released after the cleavage of a 20 aa signal sequence (29, 70, 71). Further truncation of the N-terminus yields mature 69, 70, 72 and 77 aa proteins. Among them, the 72 aa and 77 aa proteins are the major forms (73). The 77 aa IL-8 is predominant in endothelial cells, synovial cells, and fibroblasts while the 72 aa IL-8 is largely present in monocytes, macrophages,

and epithelial cells (74).

IL-8 protein exists naturally as a homodimer but the monomeric form has also been shown to be functional on neutrophils (75). Nuclear magnetic resonance spectroscopy and X-ray crystallography analyses of recombinant 77 aa IL-8 purified from *E. coli* confirmed that IL-8 is present in a homodimer form. Four cysteines are located at amino acids 7, 9, 34, and 50 in IL-8. Among them, Cys-7/Cys-34 and Cys-9/Cys-50 form two disulfide bonds (Figure. 3). The N-terminus of the monomer is followed by three antiparallel ß-strands connected by the loops and an α-helix extending from residue 57 to the C-terminus. The dimer is stabilized by six hydrogen bonds between residues 23-29 of the first ß-strand and its side chain interactions. Two antiparallel α-helices are located on the top of a six-stranded antiparallel ß-sheet. The first and the second ß-strands are connected by a disulfide bridge between Cys-7 and Cys-34, forming a contiguous surface projecting from the edge of the ß-sheet (76-77). The N-terminal residues, ELR motif [Glu-4(E), Leu-5(L) and Arg-6(R)], plays a critical role in receptor binding and neutrophil activation (78, 79). Through scanning mutagenesis of single amino acids with alanine (A), it was confirmed that the ELR was required for receptor binding (80). In contrast, the C-terminal α-helix, which has been previously reported to bind to heparan sulfate or heparin to enhance the activity of IL-8 on neutrophils (81), was not essential because deletions within that region could not result in the complete loss of activity (80). Introduction of an ELR motif into PF-4, which lacks an ELR motif and has no effect on neutrophils, caused PF-4 to bind to the IL-8 receptor and activate neutrophils. However, insertion of an ELR motif into MCP-1 and IP-10 failed to activate neutrophils (82). Thus, ELR motif alone is not sufficient to activate neutrophils. Other structural residues rather than the ELR motif within the IL-8 protein are also important in the facilitation of IL-8 binding to its receptors. Substitution of Ile-10 with A caused 29-fold less binding capacity and 22-fold less neutrophil activity (82). Cys-34, which is linked to Cys-7

77 aa AVLPRSAKELRCQCIKTYSKPFKPKFIKELRVIESGPHCANTEIIVKLSDGRELCLDPKENWVQRVVEKFLKRAENS

72 aa SAKELRCQCIKTYSKPFKPKFIKELRVIESGPHCANTEIIVKLSDGRELCLDPKENWVQRVVEKFLKRAENS

70 aa KELRCQCIKTYSKPFKPKFIKELRVIESGPHCANTEIIVKLSDGRELCLDPKENWVQRVVEKFLKRAENS

69 aa ELRCQCIKTYSKPFKPKFIKELRVIESGPHCANTEIIVKLSDGRELCLDPKENWVQRVVEKFLKRAENS

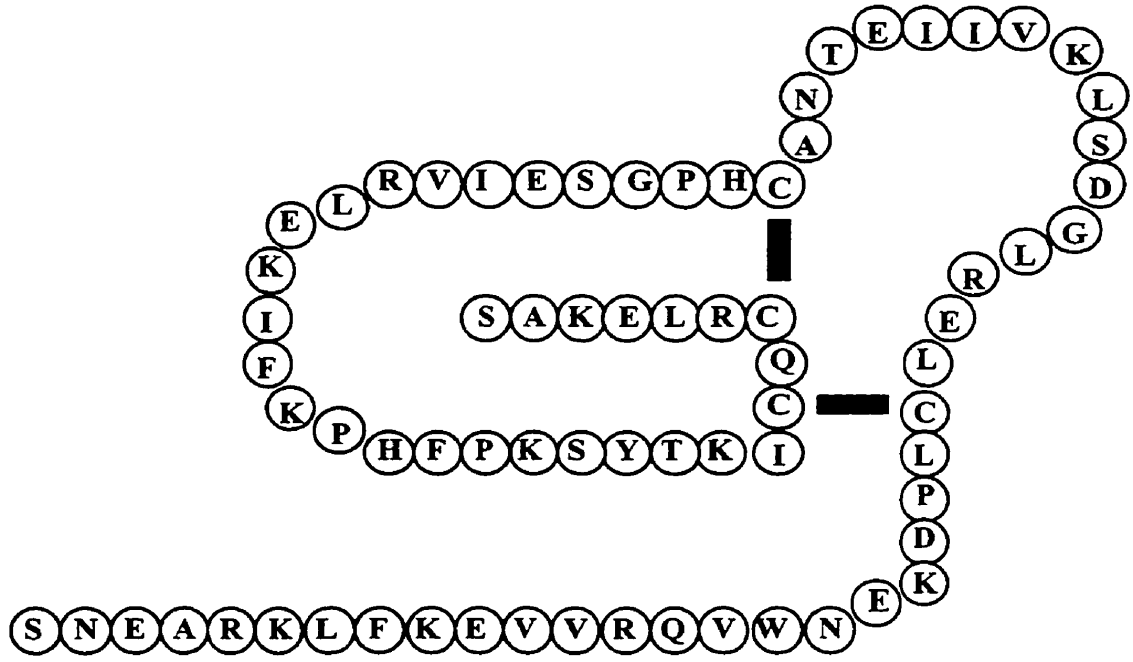

Figure 3. Amino acid sequences of human IL-8 gene (72 amino acid form). Two disulfide bonds are indicated in black bars (▬). Amino acid in one-letter code are: A. Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr, V; W, Trp; and Y, Tyr.

through a disufide bond, has been suggested to contribute to the correct fold of IL-8 protein, enabling it to bind to the receptors (83). As well, the disulfide bridges and the 30-35 aa turn provide structural scaffold for the N-terminal region (84). Moreover, using GROα substitution of IL-8, it was shown that the N-terminus loop (amino acids 1-18) or the first ß–sheet (amino acids 18-32) binds to both CXCR1 and CXCR2. However, the third ß-sheet of IL-8 (amino acids 46-53) was required for interaction with CXCR1 but not CXCR2. The second ß-sheet (amino acids 32-46) and the C-terminal α-helix (amino

acids 53-72) did not seem to have a significant effect (85). Another epitope that is different from the ELR motif has been recently identified to comprise a contiguous group of non-sequential, solvent-exposed, hydrophobic residues (Phe-17, Phe-21, Ile-22, and Leu-43) separated from the ELR motif by about 20 Å. Individual amino acid substitutions showed that residues Tyr-13, Ser-14, Phe-21, and Lys-49, which are in or around this unique hydrophobic pocket on the surface of IL-8, were also key residues for CXCR1 binding (86). Moreover, mutating either Leu-25, Val-27, or both into Tyr led to the impairment of IL-8 binding activity to both CXCR1 or CXCR2 (87) whereas, mutation of Tyr-13 and Lys-15 attenuated IL-8 binding to CXCR1 only (88).

1.7 Properties of IL-8 protein

The IL-8 protein has unique properties. It is resistant to most treatments which can inactivate protein functions, such as heat, low and high pH, detergents, and organic solvents. It is also insensitive to proteolytic processing, such as exposure to plasma peptidase, proteinase 3, elastase, or cathepsin G (8). In contrast, other CXC chemokines including GRO α and NAP-2 are very sensitive to these treatments. Cleavage of the 77 aa IL-8 by proteinase 3 into the 70 aa IL-8 generates a 10-fold increase in activity. A comparison of different IL-8 forms has revealed that the conversion of the 77 aa IL-8 into the 72 aa IL-8 by thrombin and plasmin results in enhanced biological activity (89, 90).

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Chapter 2. Role of IL-8 in disease

2.1 Function of IL-8 in neutrophils

2.1.1 IL-8 receptors

IL-8 exerts its function on target cells through a receptor coupling-dependent event. Two IL-8 receptors have been identified, named CXCR1 (IL-8 R1 or RA/α) and CXCR2 (IL-8R2 or RB/ß). CXCR1 was initially cloned from a human neutrophil cDNA library. Its cDNA encodes for a 350 amino acid protein (1) while CXCR2 cDNA derived from dibutryryl cAMP differentiated HL-60 cDNA library encodes for a 360 amino acid protein (2). The entire CXCR1 genomic DNA sequence cloned from a human genomic library consists of two exons which are interrupted by a intron of 1.7 kb. The entire 1.05 kb ORF of CXCR1 is encoded in the entire second exon together with a 834 bp 3'-UTR. CXCR2 has 3 exons separated by two introns of 3 and 5.4 kb, respectively and the 1,065 bp ORF is encoded in the third exon with a 1.4 kb 3'-UTR. A GC rich 5'-UTR in both CXCR1 and CXCR2 constitutively promotes gene transcription (3, 4). The translated polypeptides of IL-8 receptors are approximately 40 kDa and 77% identical. Both IL-8 receptors have typical seven transmembrane domains, separated by three extracellular and three intracellular loops. There is a N-linked glycosylation site near the N-terminus, which presumably couples to G-proteins for intracellular signal transduction. Competition assays have revealed that CXCR1 has high affinity (kd=0.1-0.4 nM) for IL-8 but very low affinity (kd>100nM) for NAP-2 and GRO-α, whereas, CXCR2 has high affinity not only for IL-8 but also for NAP-2 and GRO-α (5). Granulocyte chemotactic protein-2 binds to both CXCR1 and CXCR2 at nanomolar concentration leading to the activation of neutrophils (6).

The interaction between IL-8 and its receptors is a complicated event. Distinct functions of CXCR1 and CXCR2 which lead to intracellular signals and cell responses, have been gradually unveiled. CXCR1 and CXCR2 were found to equivalently mediate intracellular Ca^{2+} mobilization, chemotaxis (7), and mitogen-activated protein kinases ($p^{44/42}$ MAPKs) (8). In contrast, CXCR2 is responsible for neutrophil chemotaxis and migration induced by IL-8 as inhibition of CXCR2 by a selective CXCR2 inhibitor (SB225002) prevented neutrophil migration (9). In another report (10), using anti-CXCR1 and -CXCR2 monoclonal antibodies, it was found that CXCR1 but not CXCR2 mediated superoxide generation and release of granule enzymes through the activation of NADPH oxidase and phospholipase D in human neutrophils.

CXCR1 and CXCR2 share a high degree of sequence similarity within the transmembrane domains but differ significantly within the extracellular and intracellular loops, the N-terminus, and the C-terminus. The C-terminus of CXCR2 is pivotal to CXCR2 mediated signal transduction. Deletion of the cytoplasmic part of the C-terminus (amino acids 317-324) abolished signalling mediated by CXCR2 in transfected cells, in which chemotaxis was measured as a marker in transfected cells (11). Two basic residues, Lys158 and Arg159, in the second inner loop of the IL-8 receptors are crucial for $G\alpha16$ coupling and Met241 in the third loop with other several non-charged amino acid residues in the second loop are involved in coupling to $G\alpha2$ (12). Discrimination between CXCR1 and CXCR2 is important in further understanding their distinct functions in neutrophils. Based on the sequence of the secondary binding determinants of GROα and PF4, two CXCR2 antagonists (R)GROα and (R)PF4 were generated (13). Binding assays revealed that (R) GROα and (R) PF4 had 33-60 and 27-67 fold higher affinity to CXCR2 than CXCR1, respectively. These antagonists were able to inhibit CXCR2- but not CXCR1-induced Ca^{2+} mobilization in Jurkat cells in response to IL-8.

Interestingly, granule secretion and superoxide production evoked by IL-8 in human neutrophils were not altered suggesting an exclusive role of CXCR1 in the respiratory burst in neutrophils (13).

Limited studies on the regulation of IL-8 receptor expression have been done to date. In human neutrophils, transcriptional induction of IL-8 receptors was observed after stimulation with granulocyte-colony stimulating factor whereas LPS inhibited their expression through both transcriptional and posttranscriptional mechanisms (14). TNF-α strongly decreased CXCR2 protein expression but not CXCR1, leading to the suppression of Ca^{2+} mobilization and chemotaxis of neutrophils (15). Human T cells contain both CXCR1 and CXCR2, which are responsive to cell chemotaxis. IFN-γ, a Th1 type cytokine, sustained IL-8 binding by stabilizing CXCR1 and CXCR2 on cell surface and maintained the function of these receptors in response to IL-8 (16). Both IL-8 receptors can also be regulated by phosphorylation (17, 18). Phosphorylation of each IL-8 receptor impaired its function with regard to the signal transduction and the stability of IL-8 receptor protein. For example, phosphorylation of CXCR1 stably expressed in RBL-2H3 cells inhibited G-protein turnover, PLC-ß2 activation, and Ca^{2+} mobilization, resulting in a reduction of granule secretion (17). Activation of PKC reduced binding of [^{125}I]-melanoma growth-stimulatory activity (also called Gro) to CXCR2, which was accompanied with a rapid degradation of CXCR2 protein after phosphorylation (18).

2.1.2 Activation of neutrophils by IL-8

IL-8 activates neutrophils following a series of signal transduction events through a receptor-dependent mechanism. The neutrophil response to IL-8 involves intracellular Ca^{2+} mobilization, active motion, exocytosis, up-regulation of receptors and adhesion molecules, and the respiratory burst.

2.1.2.1 Migration of neutrophils towards IL-8

The directional motion or polarization of neutrophils in response to IL-8 is the first cellular step in neutrophil migration. The shape change after encountering IL-8 reflects the activation of the contractile cytoskeleton. During this step, cells protrude large, thin lamellae that point to the source of the chemoattractant. A rapid polymerization of actin occurs in activated neutrophils. The under-agarose assay has been widely used to study chemotaxis or chemokinesis of neutrophils by measuring the distance neutrophils migrating towards IL-8. Neutrophils migrated towards IL-8 in a step by step fashion and the navigation was combinatorially controlled (19). Two important parameters are involved in controlling neutrophil orientation in a gradient of chemoattractant: gradient steepness and mean agonist concentration. Neutrophils are able to migrate down a physiological concentration gradient of IL-8, whereas a high dose beyond the threshold arrests neutrophils, which may be due to receptor saturation or desensitization (19). Moreover, neutrophils are capable of reversing their morphological polarity or changing their direction when exposed to a temporal decrease in the concentration of chemoattractant by remembering their ligand exposure history and relative direction. During their change in orientation, neutrophils undergo a dissolution of both lamellipodium and uropodia, and the reformation of them in different direction (20). In the presence of more than one chemoattractant, the migration of neutrophils is dependent on both the chemoattractants and the receptors involved (19).

2.1.2.2 Exocytosis and upregulation of receptors and adhesion molecules

IL-8-activated neutrophils release a variety of enzymes or other proteins from intracellular storage organelles, which include myeloperoxidase, vitamin B12-binding protein, elastase, ß-glucuronidase and gelatinase (21, 22). The release of certain

proteases from activated neutrophils is involved in the degradation of host proteins leading to non-specific tissue damage. IL-8 also activates neutrophil 5-lipoxygenase to produce large amounts of LTB4, 5-HETE, and thrombin. During neutrophil exocytosis, IL-8 increases the expression of two integrins, complement receptor type3 (CR3, CD11b/CD18) and p150,95 (CD11c/CD18) which mediates adhesion of neutrophils to endothelial cells and increases the expression of complement receptor type 1 (CR1), respectively (12).

2.1.2.3 Respiratory burst

The respiratory burst is a unique property of neutrophil phagocytosis. The activation of the nicotinamide-adenine dinucleotide phosphate (NADPH)-oxidase, a membrane-bound electron transporter that oxidizes cytosolic NADPH and reduces extracellular oxygen into superoxide (eg. O_2^-), can kill pathogens and also causes non-specific tissue damage (23). IL-8 can stimulate phospholipase-dependent O_2^- and H_2O_2 production from freshly isolated human neutrophils *in vitro* (24). Neutrophil phospholipase A2 has been shown to increase the production of arachidonic acid and other unsaturated fatty acids from activated neutrophils, which are very important in the activation of NADPH oxidase (25, 26).

2.2 Role of mucosal epithelial cell-derived IL-8

Mucosal epithelial cells are the first line of host defence against pathogens. They are crucial in mediating host responses through bidirectional communication between immune and inflammatory cells and pathogens in infectious diseases (27-29). It is becoming clear that IL-8 derived from mucosal epithelial cells plays an important role in initiation or exacerbation of inflammatory responses in various diseases (including IBD)

by recruiting neutrophils into local sites (29-31). Th1 cytokines (IL-2, IL-15, IL-18, IFN-γ), Th2 cytokines (IL-4, IL-5 or IL-10), chemotactic cytokines (IL-8, MCPs, MIPs, ENA-78, or RANTES), and other mediators (arachidonic acid metabolites, reactive oxygen intermediates, and growth factors), participate in regulatory and effector activities in mucosal inflammation (29-34). However, the precise mechanisms involved in IL-8 gene regulation and protein function in concert with these cytokines and mediators in the initiation and/or the development of mucosal inflammation are not known. This review will briefly discuss mucosal epithelial cell-derived IL-8 gene regulation and its role in the pathogenesis of infectious (bacterial or parasitic) and inflammatory bowel diseases.

2.2.1 Bacterial infection

Extensive studies (35-54) have shown a crucial role for IL-8 derived from mucosal epithelial cells in the pathogenesis of bacterial infection. Bacterial adherence or secretory components are able to evoke induction of IL-8 in target cells. However, the molecular basis and the signal transduction pathways involved in IL-8 gene regulation are poorly understood. Table 4 summarizes IL-8 gene expression in airway, gastric, intestinal, and cervical epithelial cells induced by bacterial infections.

2.2.1.1 *Pseudomonas aeruginosa*

There are two major characteristics of respiratory diseases: infiltration of neutrophils and chronic bacterial infections (55, 56). In patients with idiopathic pulmonary fibrosis high levels of IL-8 mRNA expression was concomitant with the severity of disease with an increased number of infiltrated neutrophils in the bronchoalveolar lavage, suggesting that IL-8 is one of the causative agents in neutrophil alveolitis (57, 58). High levels of IL-8 protein were also found in acute inflammatory sites of adult respiratory distress syndrome, contributing to the mortality and empyema

Table 4. Induction of interleukin-8 in mucosal epithelial cells in response to bacterial infections

	Bacteria	Cell types	Requirement	Stimuli	Transcription factor	References
Airway epithelial cells	*Pseudomonas aeruginosa* *Burkholderia cepacia* *Mycobacterium tuberculosis*	16-HBS BET-1A IHAEo- A549 CFTEo-	With or without adherence	pilin flagelin elastase nitrate-reductase	NF-κB	35-42
Gastric epithelial cells	*Helicobacter pylori*	Kato-III ST42 AGS MKN28 MKN45	Adherence	cag A vacA	NF-κB AP-1	43-48
Intestinal epithelial cells	*Salmonella dublin* *S. dysenteriae* *S.typhimurium* *Escherichia coli 0159* *Listeria monocytogenes* *Yersinia enterocolitica* *Chlamydia trachomatis* *C. psittaci*	T84 Caco 2 HT-29 SW620 Henle-407	Invasion	*Yop*B *Yop*D	NF-κB AP-1	49-54
Cervical epithelial cells	*S.dublin* *Escherichia coli 0159* *L.monocytogenes* *S.dysenteriae* *Y.enterocolitica* *C.trachomatis* *C.psittaci*	Hela	Invasion	LPS *Yop*B *Yop*D	NF-κB AP-1	49-53

(59, 60). In this system, mucosal epithelial cells played a crucial role in mediating respiratory inflammation (61, 62), although alveolar macrophages were also suggested to be a major cause (57, 58).

P. aeruginonas infection is predominent in patients with cystic fibrosis and plays a major role in morbidity and mortality. From *in vitro* and *in vivo* studies (63, 64), it is apparant that *P. aeruginosa* stimulates high output IL-8 protein from airway epithelial

cells. Using primary cultured 16-HBE bronchial epithelial cells as an *in vitro* model, PA103 (a highly toxic *P. aeruginosa* strain) supernatants markedly increased IL-8 mRNA expression and protein production whereas, *P. aeruginosa* LPS had no effect. Filtration of PA supernatants with different mol. wt. size cut-off membranes revealed that a 1-kD filtrate maintained the ability to induce IL-8 production from airway epithelial cells. In an *in situ* hybridization study, increased IL-8 mRNA expression was observed in the epithelia of human and dog bronchi following incubation with the 1-kD filtrate (63). The 1-kD filtrate also induced IL-8 mRNA expression in primary cultures of human tracheal epithelial cells from different donors. Several soluble substances secreted from *P. aeruginosa* that enhanced IL-8 production in airway epithelial cells have been identified. These include *P. aeruginosa* elastase, nitrate reductase, pilin, and flagelin (35, 37, 38, 40). In *P. aeruginosa* infection, infiltration of neutrophils are presumably recruited by the release of IL-8 derived from airway epithelial cells and other cells such as macrophages and neutrophils. As a result, neutrophils are activated to release large amounts of elastase which cause cellular detachment and deformation and production of more IL-8 which exacerbates inflammation at local sites (36, 40, 65). Thus, it is important to determine the mechanisms involved in IL-8 gene expression in airway epithelial cells in response to *P. aeruginosa* products. Adherence of *P. aeruginosa* to epithelial cells is a prerequisite for the induction of IL-8. *P. aeruginosa* produces two unique molecules, pilin and flagelin, which act as ligands for asialylated glycolipid receptors on the surface of target cells (66). Both nonpiliated and nonflagelated strains evoked much less IL-8 production from airway epithelial cells as compared to wild type *in vitro*. Isogenic, nonadherent *rpoN* mutants lacking pilin and flagelin genes do not bind to epithelial cells or stimulate IL-8 protein (37). Using a variety of airway epithelial cells, it was shown (39) that the interaction between pilin and target cells triggered ceramide-dependent signal transduction, which led to the induction of IL-8 gene expression accompanied by the activation of NF-κB and

C/EBP. Ligation of epithelial receptors by pilin or antibodies against pilin receptors stimulated the activation of NF-κB. Dex treatment decreased nuclear translocation of NF-κB induced by pilin and thus, reduced IL-8 production. In IB3 cells with mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) a greater amounts of endogenous nuclear NF-κB but not C/EBP was observed than those in CF cells corrected by episomal copies of normal CFTR. Epithelial cells derived from patients with cystic fibrosis expressed a higher concentration of asialylated glycolipid receptors than normal epithelial cells (66). Thus, infection of *P. aeruginosa* induces more IL-8 production from airway epithelial cells in patients with cystic fibrosis than healthy individuals, resulting in more severe tissue damage.

2.2.1.2 *Helicobacter pylori*

H. pylori infection is associated with active gastritis, duodenal, and gastric ulceration, which is accompanied by a massive infiltration of neutrophils in the gastric mucosa (67). Although various components from the surface of *H. pylori* are able to attract and activate neutrophils *in vitro* (68, 69), increased IL-8 production in the gastric mucosa in patients may be the major cause for neutrophil recruitment into the local sites resulting in tissue damage (70). IL-8 released from gastric mucosal epithelial cells play a crucial role in the development of the disease. This notion was confirmed when *H. pylori* isolates from patients enhanced IL-8 production from gastric epithelial cells *in vitro* (44, 70). Several disease-associated virulent molecules from *H. pylori* such as CagA and VacA were shown to be involved in gastric ulceration by binding to target cells (70, 71). Interestingly, LPS from *H. pylori* had no effect on IL-8 production from gastric epithelial cells (46). *H. pylori* products that evoked IL-8 production in various gastric epithelial cells *in vitro* were sensitive to various physical or chemical treatments, high or low temperature, fixation with formaldehyde, or digestion with proteinase K. However,

these treatments could not totally eliminate the effect of *H. pylori* on IL-8 production, suggesting the presence of other effector molecules which have high resistance to harsh treatments (44, 45).

H. pylori-induced IL-8 production seems to be specific to gastric epithelial cells. Crowe et al. (43) showed that *H. pylori* evoked IL-8 production from Kato III, a gastric carcinoma cell line, but not from Caco 2 and T84 colonic adenocarcinoma cell lines. In contrast, *S. typhimurium*, an invasive bacterium, but not *Campylobactor jejuni*, a nongastric pathogen, caused IL-8 production in all the cell lines tested. Adherence of *H. pylori* to target cells is a prerequisite for IL-8 production. CagA and VacA are the key molecules in IL-8 gene expression (72). *H. pylori* mutants lacking CagA induced lower IL-8 production in comparsion to its wild type (70, 71). *H. pylori* adherence to AGS human gastric adenocarcinoma cells triggered the signal transduction cascade leading to IL-8 production through activation of PKG (a Ser/Thr kinase) concomitant with tyrosine phosphorylation of a 145 kDa protein. CagA and VacA were required for this event to occur. Interestingly, inhibition of either PKC or PKA partially attenuated the effect of *H. pylori* on IL-8 protein production (73). Enhanced binding of NF-κB and AP-1, but not C/EBP was observed in various gastric cell lines following adherence of *H. pylori* to target cells, which presumably increased IL-8 gene transcription. Predominent p50/p65 heterodimer and p50 homodimers were translocated into nuclei in response to *H. pylori* (47, 74, 75). Furthermore, the proteins encoded by the *cag* pathogenicity island are required for the translocation of NF-κB leading to the induction of IL-8. Mutation of cag E, F, H, I, L, or M caused attenuation of NF-κB translation into nuclei but did not abolish it (75). In a reporter gene study (76), mutation of either *pic*A1 or *pic*B in the *cag* pathogenicity island abolished IL-8 promoter-driven reporter gene expression whereas, wild type *H. pylori* (cag^+) caused elevation of reporter gene expression. The reduced

expression of reporter gene in response to *H. pylori* with either mutation of *pic*A1 or *pic*B was due to the abolition of NF-κB activation but not C/EBP as compared to the effect of wild type *H. pylori*. Surprisingly, mutation of C/EBP also attenuated wild type *H. pylori*-induced reporter gene activity, suggesting C/EBP may be a co-operator for NF-κB in a synergistic manner (76).

2.2.2 *Entamoeba histolytica*

E. histolytica is an enteric protozoan parasite and the causative agent of amebiasis. There are about 50 million cases of amebic dysentery, amebic colitis, and liver abscesses each year, resulting in 50,000 to 100,000 deaths per year. Amebiasis is the third leading parasitic cause of death in the world (77). *E. histolytica* trophozoites colonize the intestine by adhering to colonic mucins *via* the Gal/GalNAc adherence (Gal-lectin) lectin (78, 79). Following depletion or dissolution of the mucus layer, trophozoites adhere to and lyse mucosal epithelial cells and infiltrating leukocytes, facilitating tissue and organ invasion (80-82).

E. histolytica infection is associated with mucosal inflammation which is characterized by the infiltration of neutrophils in the lamina propria. In animals experimentally infected with *E. histolytica*, a prominent infiltration of neutrophils in the intestinal mucosa was observed early in the infection (81-83). In acute human colitis, neutrophils accumulated in the intestinal mucosal layer (84, 85). Virulent amebae secrete a variety of substances that are able to trigger a signal transduction cascade in various experimental models (86-88). Using quantitative RT-PCR, Eckman et al. (89) observed IL-8 mRNA expression and bioactive IL-8 protein production by epithelial cells when *E. histolytica* trophozoites were co-cultured with various human colonic epithelial cells (T84, HT29, and SW620) and other epithelial cells (Hela). Ratios of trophozoite to cells between 2:1 to 15:1 gave the highest production of IL-8. Increased production of IL-8

protein was noted within 2 hour after addition of *E. histolytica* trophozoites that reached maximum levels at 4-6 hours in HT29 cells, and 8 hours in Hela cells. Using luciferase reporter gene linked with IL-8 promoter, they further demonstrated a 20-fold increased in luciferase transcription rate in Hela cells in response to amebae, whereas there was no change in ß-actin transcription. Preformed IL-1α released from lysed T84, I407, SI38, and Hela cells killed by amebae, acted in a paracrine manner to induce IL-8 gene expression from intact target cells. Addition of antibodies against IL-1α or IL-1 receptor antagonist, but not antibodies against IL-1ß or TNF-α, markedly reduced epithelial cell lysate-induced IL-8 protein production in Hela cells (>50%). However, the lysate from HT29 failed to elevate IL-8 production in Hela cells due to its lack of preformed IL-1α. Interestingly, the addition of galactose and N-acetyl-galactosamine, but not glucose and fructose, inhibited IL-8 gene expression induced from HT29 cells incubated with *E. histolytica* trophozoites, suggesting the involvement of Gal-lectin (89, 90). Gal-lectin is a heterodimer composed of a heavy subunit (170 kD) and a light subunit (31-35 kD), linked together with disulfide bonds. The heavy subunit is involved in amebic adherence to and killing of host cells (91). These observations indicate that contact through the amebic Gal-lectin, rather than cell lysis, caused IL-8 induction from HT29 cells. The effect of Gal-lectin on IL-8 gene expression in HT29 cells probably occured through an increase in intracellular Ca^{2+} level (89).

More recently, Kim et al. (92) observed a synergistic effect of *E. coli* on IL-8 production in human colonic epithelial cells (HT29) incubated with *E. histolytica* trophozoites. There was at least a 3-fold increase in IL-8 protein production from HT29 cells co-cultured with *E. histolytica* trophozoites plus *E. coli* in comparison to that in response to *E. histolytica* trophozoites alone. The induction of IL-8 by *E. histolytica* was only synergistically increased by live *E coli* because heat-killed *E. coli* had only a slight

effect. One hypothesis is that *E. coli* activates amebae and increase their virulence (93) which causes a syngergistc increase in IL-8 production from human colonic epithelial cells. However, the precise mechanism still needs to be elucidated. To confirm that intestinal epithelial cells are the source of IL-8 in *E. histolytica* infection, Seydel et al. (94) used a SCID mouse-human intestinal xenograft model of amebiasis to examine IL-8 production and tissue damage *in vivo*. Significant damage in grafted human intestine was observed microscropically 4 hours after inoculation of amebae. After 24 hours, massive neutrophils infiltrated into local sites. Increased IL-8 mRNA and protein production was mainly from enterocytes. In follow-up studies it was shown that IL-8 production occurred through the activation of NF-κB (95). The molecular mechanisms whereby *E. histolytica* evokes IL-8 mRNA expression and protein production is presented in Manuscript I.

2.2.3 Inflammatory bowel disease

IBD such as Crohn's disease and ulcerative colitis, regardless of the initiative cause, share common inflammatory and immune responses. Chronic inflammation in the intestine shows large numbers of activated neutrophils, macrophages, and lymphocytes infiltrating into local sites (96). There is strong evidence suggesting that a variety of mediators including cytokines, arachidonic acid metabolites, reactive oxygen intermediates and growth factors are involved in intestinal inflammation. However, how cytokines and other mediators in concert contribute to IBD remains to be fully understood. A wide array of cytokines such as IL-1, 2, 4, 5, 6, 8, 10, 15, 18, and IFN-γ are associated with IBD (31-34, 96-98). Intestinal epithelial cells play a very important role in mediating intestinal inflammation through communication with adjacent immune and mesenchymal cells and have been demonstrated to be a source for IL-8 and other

cytokines (31, 99-104). Studies on patients have shown that Crohn's disease is very likely mediated by Th1 cytokines whereas, ulcerative colitis is mediated by Th2 cytokines (31). However, high expression of IL-8 is associated with both Crohn's disease and ulcerative colitis, emphasizing the importance of IL-8 in the pathogenesis of IBD(30, 31).

Although IL-8 is produced largely from infiltrated macrophages and neutrophils in IBD, activated intestinal epithelial cells contribute to IL-8 production in the inflammed mucosa (30, 31). Infiltrating inflammatory cells are the main producer of IL-8 in IBD (105, 106). Using *in situ* hybridization with radiolabeled probes of the IL-8 gene, it was observed that epithelial cells expressed higher levels of IL-8 mRNA adjacent to ulcers although macrophages and neutrophils were the major source of IL-8 after examination of surgically resected intestines from patients with IBD. The degree of IL-8 production was correlated with the histologic severity of disease activity in both the large and small intestinal mucosa (107). Moreover, using freshly isolated epithelial cells and mucosal samples from resected specimens from patients with ulcerative colitis and Crohn's disease there was a marked increase in IL-8 mRNA expression as revealed by RT-PCR (108). These studies strongly indicate an important role for epithelial cell-derived IL-8 in intestinal inflammation in IBD. However, using immuno-histochemical approaches from formalin-fixed, paraffin wax-embedded tissues from patients with IBD, it was shown that macrophages and neutrophils, but not epithelial cells, adjacent to ulcers expressed IL-8 mRNA (109). Even though macrophages and neutrophils are the major source of IL-8 in IBD, intestinal epithelial cells may contribute significantly to the total pool of IL-8 which may play a role in initiation or exacerbation of inflammation.

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SECTION II: MANUSCRIPTS I, II, III, and IV

MANUSCRIPT I

Entamoeba histolytica stimulates interleukin-8 from human colonic epithelial cells without parasite-enterocyte contact*

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ABSTRACT

Background&Aims: The mechanisms involved in the initiation of host mucosal inflammation in amebiasis are not fully understood. This study characterized the effect of *E. histolytica* components on interleukin-8 (IL-8) gene expression in human colonic cells. *Methods*: Colonic cells were stimulated with amebic proteins, secretory components, or live trophozoites (separated with 0.45 μm pores), and the levels of IL-8 mRNA and protein were detected. *Results*: Live amebae or its components enhanced IL-8 mRNA levels in the colonic cells (T84, LS174T and Caco-2). In T84 cells, the accumulation of IL-8 mRNA induced by amebic components occurred in a dose- and time-dependent fashion. Increased secretion of IL-8 was noted after 12-hour stimulation; neutralizing antibodies against IL-1ß or TNF-α did not inhibit IL-8 production. Nuclear run-on assays demonstrated that amebae-induced IL-8 gene occurred by a posttranscriptional mechanism. Cycloheximide treatment resulted in superinduction of IL-8 mRNA; however, dexamethasone inhibited *E. histolytica*-induced IL-8 gene expression. *Conclusions*: *E. histolytica* can directly stimulate the induction of IL-8 by colonic cells in the absence of cell-cell contact or injury.

INTRODUCTION

E. histolytica is an enteric protozoan parasite and the causative agent of amebiasis. There are about 50 million cases of amebic dysentery, amebic colitis, and liver abscesses each year, resulting in 50,000 to 100,000 deaths. Amebiasis is the third leading parasitic cause of death in the world (1). *E. histolytica* trophozoites colonize the intestine by adhering to colonic mucins *via* the Gal/GalNAc adherence lectin (2, 3). After depletion or dissolution of the mucus layer, amebae adhere to and lyse mucosal epithelial cells and infiltrating leukocytes, facilitating tissue and organ invasion (4, 5, 6). *E. histolytica* infection is associated with mucosal inflammation and is characterized by the infiltration of neutrophils in the lamina propria. In animals experimentally infected with *E. histolytica*, a prominent infiltration of neutrophils in the intestinal mucosa was observed early in the infection (5, 7). In acute human colitis, neutrophils accumulate in the intestinal mucosal layer (8, 9).

The intestinal mucosa is the first line of defence against amebic infection, and mucosal epithelial cells are critical in mediating host responses to infectious pathogens. Accumulated evidence indicates that epithelial cells are a potential source of various proinflammatory cytokines including interleukin-1 (IL-1), IL-8 and tumor necrosis factor-α (TNF-α) (10, 11, 12). IL-8, a potent chemokine of the C-X-C family, has the capacity to attract and activate neutrophils. It is produced by various cells, including monocytes, macrophages, T-cells, epithelial cells, endothelial cells, hepatic cells, gastric carcinoma cells, and neutrophils. IL-8 is regulated by various stimuli such as IL-1, TNF-α, IL-4, IL-10, interferon gamma, lipopolysaccharide, phorbol myristate acetate (PMA), and calcium ionophore, and glucocorticoids (13, 14). Recently Eckmann et al. (15) demonstrated that coculture of human epithelial cells with *E. histolytica* trophozoites resulted in the secretion of an array of chemoattractant and proinflammatory cytokines,

including IL-8, growth-related oncogene α, granulocyte-macrophage colony-stimulating factor, IL-1α and IL-6. The mechanism involved in up-regulating IL-8 production required amebae-cell contact and the release of preformed IL-1α from lysed cells, which had a paracrine effect on the remaining intact cells (15). It is well-documented that increased induction of IL-8 contributes to the onset of inflammatory skin and joint diseases, such as psoriasis and rheumatoid arthritis (16, 17). The local acute inflammation evoked by the induction of IL-8 contributes to tissue lesions during infection of invasive or non-invasive bacteria and protozoan parasites by direct cell-cell contact, invasion, or secretion of soluble substances functioning on the targets cells (18-22). Because tissue infiltration by neutrophils during the acute inflammatory response is dependent on the local release of soluble chemoattractants such as IL-8, we hypothesize that *E. histolytica* can cause the induction of IL-8 by colonic epithelial cells in the absence of cell-cell contact. The secretory response (IL-8 secretion) to *E. histolytica* may play a role in initiation of an acute inflammatory response before amebic invasion. Damage to mucosal epithelial cells by the host inflammatory response elicited by *E. histolytica* may then facilitate amebic invasion. During invasion, *E. histolytica* cytolysis of epithelial cells and infiltrating neutrophils can cause the release of preformed proinflammatory cytokines (15), proteolytic enzymes, and reactive oxygen intermediates to further exacerbate host tissue injury (4, 5, 6).

In this study, we observed increased IL-8 mRNA expression from several human colonic cell lines (T84, LS174T and Caco-2) after stimulation with soluble *E. histolytica* proteins, secretory components and live trophozoites. Accumulation of IL-8 mRNA elicited by *E. histolytica* components was dose- and time-dependent and occurred by a posttranscriptional mechanism. Because significant IL-8 protein production was observed following stimulation with soluble amebic components, this may play a crucial role in the initiation of acute inflammatory response in intestinal amebiasis. This is the

first report to document up-regulation of IL-8 gene and protein production in human colonic epithelial cells in response to *E. histolytica* in the absence of cell-cell contact or injury.

MATERIALS AND METHODS

Entamoeba histolytica culture and preparation of soluble amebic proteins and secretory components

Virulent axenic *E. histolytica* trophozoites (strain HM1-IMSS), originally provided by L. Diamond (NIH, Bethesda. MD), were cultured in TYI-S-33 medium in our laboratory as previously described (5). *E. histolytica* trophozoites in mid log-phase (3 days old) were harvested by centrifugation at 300 X *g* for 5 min at 4°C and washed twice with Hanks' balanced salt solution (GIBCO Laboratories, Grand Island, NY). Soluble amebic proteins (E.h P) were prepared by collecting the clear supernatant after the amebae were lysed by three freeze-thawing cycles and centrifuged at 15,000 X *g* at 4°C for 10 minutes. Secretory components (E.h SC) were prepared by collecting secretory/excretory components of live amebae incubated in Hanks' balanced salt solution ($2x10^7$/ml) in the absence of serum at 37°C for 2 hours. The viability of amebic trophozoites after incubation in Hanks' balanced salt solution was greater than 95% as determined by the trypan blue exclusion assay. Protein concentration was determined by the method of Bradford (23) using bovine serum albumin as a standard.

Cell culture

The human colonic adenocarcinoma cells T84, LS174T, and Caco-2 were obtained from the American Type Culture Collection (Rockville, MD). T84 cells were grown in 24-well plates in 1 ml of a 1:1 mixture of Dulbecco's modified Eagles medium

(GIBCO BRL Life Technologies, Burlington, Canada) with 4.5 g of D-glucose/litre and Ham's F12 nutrient mixture (GIBCO) containing 5% fetal bovine serum (Hyclone Laboratories, Logan, UT). LS174T and Caco-2 cells were cultured in Eagle's minimum essential medium (GIBCO) containing 10% fetal bovine serum, 100 U/ml of Penicillin, 100 μg/ml of Streptomycin sulfate and 20 mM HEPES (Sigma Chemical Co., St Louis, MO). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere until the cells reached about 80% confluence. Fresh medium was added to the culture 24 hours before each experiment.

Generation of competitor for competitive PCR

A pair of primers were designed according to the human IL-8 sequence: ATGACTTCCAAGCTGGCCGTGGC (sense) and TCTCAGCCCTCTTCAAAAAC-TTCTC(antisense) (DNA Agency, Aston PA). The prospective PCR product (284 base pair {bp}) was amplified from human IL-8 complementary (cDNA) and was confirmed after restriction enzyme digestion with *Hind*III. The PCR fragment was subcloned into pGEM-T vector (Promega Corp., Madison, WI). After digestion with *Hind*III (GIBCO), the linearized DNA fragment was inserted with an irrelevant fragment (360bp). A 644 bp competitor with inframe sense and anti-sense primers was obtained by PCR using this DNA construct as template. The PCR products amplified from the competitor and target DNA can be distinguished by their size. The proper concentration of competitor was established in our laboratory in a preliminary study. The competitor was added to the PCR reaction mixture to compete with the target DNA for the same primers. 4.8×10^{-6} μg competitor was used for T84 cells and 4×10^{-7} μg for LS174T and Caco-2 cells.

RNA preparation and semiquantitative reverse-transcription PCR.

Total RNA was extracted with Trizol (GIBCO). RNA concentration and purification were determined by measuring the absorbance at 260 and 280 nm. Total

RNA (1 μg) extracted was used for reverse transcription (RT) to make cDNA in a 25-μl reaction mixture containing 4 U of RNasin ribonuclease inhibitor (Promega Corporation, Madison, WI), 1 mM deoxynucleoside triphosphate(dNTP), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.1 μg of oligodeoxythymidine triphosphate$_{12-18}$, 4 mM dithiothreitol(DTT), and 50 U Moloney MuLV reverse transcriptase (GIBCO) at 37°C for 1 hour. Reactions were stopped by heat inactivation at 95°C for 10 minutes. Subsequently, 10 μl of RT reaction product was used for PCR in 100 μl of reaction mixture in the presence of 15 pmoles of both sense and anti-sense primers for the IL-8 gene, 50 μM dNTP, 20 mM Tris-Cl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 5 U *Taq* polymerase and a consistent amount of competitor. PCR was carried out in a Perkin-Elmer Cetus Thermal cycler 9600 and consisted of denaturation at 95°C for 30 seconds, annealing at 56°C for 1 minute and extension at 72°C for 2 minutes for a total of 35 cycles followed by a final cycle with extension at 72°C for 8 minutes. The competitive PCR product (30 μl) was electrophoresed in a 1.5% agarose gel containing ethidium bromide to visualize amplification and photographed with Polaroid film (Polariod Canada Inc., Rexdale, Ontario, Canada). The results were analyzed using NIH Image Program 1.59 (24) . The IL-8/competitor ratios were determined for each sample as relative density. The difference in the ratios among samples represented the changes in IL-8 mRNA levels. Heteroduplex was formed between IL-8 and competitor PCR products during competitive PCR amplification reactions. However, the heteroduplex is different in size when compared with the homodimer and therefore it does not interfere with densitometric scanning analysis of the homodimer products (25). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control where a 358 bp fragment was generated using a pair of primers CTACTGGCGCTGCCAAGGCTG (sense) and GCCATGAGGTCCACCACCCTG (antisense).

Measurement of IL-8 protein

An IL-8 enzyme-linked immunosorbent assay (ELISA) was used to measure IL-8 in the supernatant of T84 cultures incubated with *E. histolytica* components or PMA for various time periods. ELISA plates (96-well; Dynatch Lab ImmunnII, San Luis Obispo, CA) were coated with 200 ng anti-human IL-8 neutralizing antibody (R&D Systems, Minneapolis, MN) in 50 µl of carbonate buffer, pH 9.6, overnight at 4°C. The plates were washed with phosphate-buffered saline (PBS) and blocked with 1% BSA overnight at 4°C. Samples (50 µl) were added to each well and incubated for 1 hour at 37°C, and different concentrations of recombinant human IL-8 protein (R&D System, Inc) was used as the standard. After washing, 50 ng of rabbit anti-human IL-8 polyclonal antibody (Endogen, Boston, MA) was added for a further 1-hour incubation at 37°C. Subsequently, 50 µl goat anti-rabbit immunoglobulin (Ig) IgG antibody horseradish peroxidase-conjugate (Bio-Rad Laboratories, Richmond, CA), at a 1:1500 dilution, was added and incubated for 1 hour at 37°C. The plates were washed and 100 µl of ABTS (Bio-Rad) was added to each well to quantify the results. The optical density was measured 30 minutes later at room temperature with an ELISA reader at 405 nm. IL-8 protein levels are expressed as picogram per milliliter.

Nuclear run-on assay

Run-on assay was performed as described (11). Briefly, T84 cells were collected and homogenized in lysis buffer containing 320 mM sucrose, 10 mM Tris-HCl(pH 7.8), 0.1 mM EDTA, pH 8.0; 2 mM $MgCl_2$, 1mM DTT, and 0.2% Triton X-100(Sigma) and kept on ice for 10 min. After centrifugation at 1500 x g for 15 min at 4 °C, nuclei were resuspended in 100 µl of storage buffer containing 50% glycerol, 50 mM Tris-HCl(pH 8.0), 5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM DTT. The reaction was undertaken in 200 µl of reaction buffer containing 25 % Glycerol, 25 mM Tris-HCl(pH 8.0), 5 mM

$MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 120 mM KCl, 500 μM each of adenosine triphosphate, guaniosine triphosphate, and cytidine triphosphate , and 100 μCi {α-P^{32}} uridine triphosphate (3000 Ci/mM) for 60 min at 26 °C. Subsequently, α-P^{32} labeled nuclear RNA was extracted with Trizol reagent and hybridized onto denatured membrane-bound cDNA probes at 65 °C for 48 hours in hybridization buffer containing 5 x Denhart solution, 6 x SSC(900 mM NaCl and 90 mM sodium citrite; pH, 7.0), 0.05% sodium dodecyl sulfate(SDS), 0.1 mM EDTA, and 100 μg/ml of salmon sperm DNA. 3 micrograms of IL-8 or GAPDH cDNAs (generated by PCR using specific primers as previously described) was immobilized onto nitrocellulose membrane using a Minifold I (Schleicher & Schuell, Keene, NH). pGEM vector was used as a control for nonspecific hybridization. After washing with 6 x SSC and 1% SDS at room temperature for 10 min, and with 1 % SSC at 65 °C, the membrane was exposed for autoradiography. Optical density was measured using NIH Image and the ratio of IL-8/GAPDH represents the transcription rate.

Northern Blot analysis

Total RNA was denatured using glyoxal and electrophoresized in a 1% agarose gel. RNAs were transferred onto a Hybond-N nylon membrane (Amersham, Oakville, Ontario, Canada) by capillary diffusion and UV crosslinked. The membrane was prehybridized for 2 hours at 42 °C in a solution containing 5xSSPE (750 mM NaCl, 50 mM NaH_2PO_4, and 50 mM EDTA; pH, 7.4), 5xDenhardt solution, 50% formamide, 0.25 mg/ml of salmon sperm DNA, and 0.1% SDS and then hybridized with ^{32}P-labeled IL-8 cDNA probe generated by nick translation (Amersham) overnight at 42°C. After one wash with 2xSSC at room temperature for 15 min and two washes with 0.2xSSC/0.1% SDS at 55 °C for 30 min, the membrane was exposed to Kodak XAR-5 film at -70°C.

Optical density of the bands was analysed to determine IL-8 mRNA levels. The IL-8 probe was a 284-bp fragment from pGEM-T. The actin probe was a 1.25 kb *Pst* I fragment from pBA-1.

Statistical analysis

The data were calculated as mean ± standard error of the mean (SEM). The results were analyzed by Student'*t* test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

E. histolytica increases IL-8 mRNA levels in human colonic cells without cell-cell contact or amebic killing

To determine whether *E. histolytica* or its components can increase IL-8 mRNA expression, T84, LS174T and Caco-2 cells were co-cultured with $1x10^6$ live trophozoites (L E.h) separated by a 0.45 μm culture plate insert (Millicell-HA, Millipore Co. Bedford, MA), incubated with 20 μg/ml of amebic secretory components (E.h SC, from $4x10^5$ trophozoites) or 100 μg/ml of amebic proteins (E.h P, from 10^5 trophozoites) for 2 hours. PMA was used as a positive control and untreated cells as the negative control. Total RNA extracted from the cells was used for semi-quantitative RT-PCR in the presence of a consistent amount of competitor to assess levels of IL-8 mRNA in the three cell lines. Relative density (IL-8/competitor) represents the changes in the levels of IL-8 mRNA. T84, LS174T and Caco-2 cells constitutively expressed low levels of IL-8 mRNA; a 284 bp PCR product was observed under unstimulated conditions (data not shown). As shown in Figure 1, there was a marked increase in the expression of IL-8 mRNA in T84, LS174T and Caco-2 cells exposed to L E.h, E.h SC or E.h P in

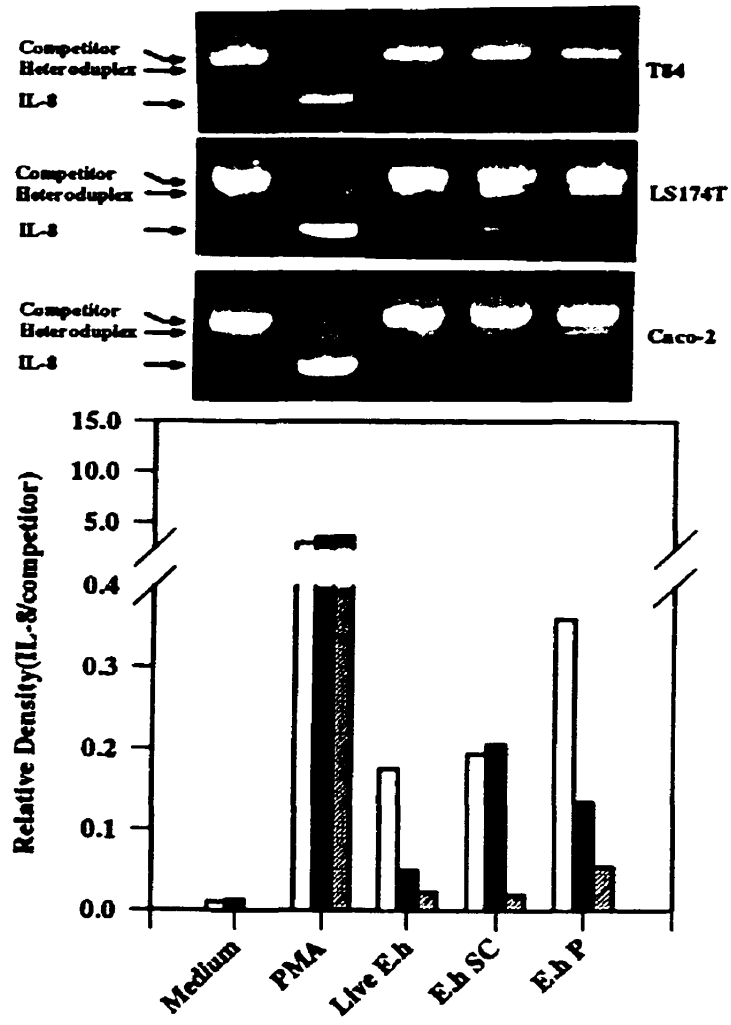


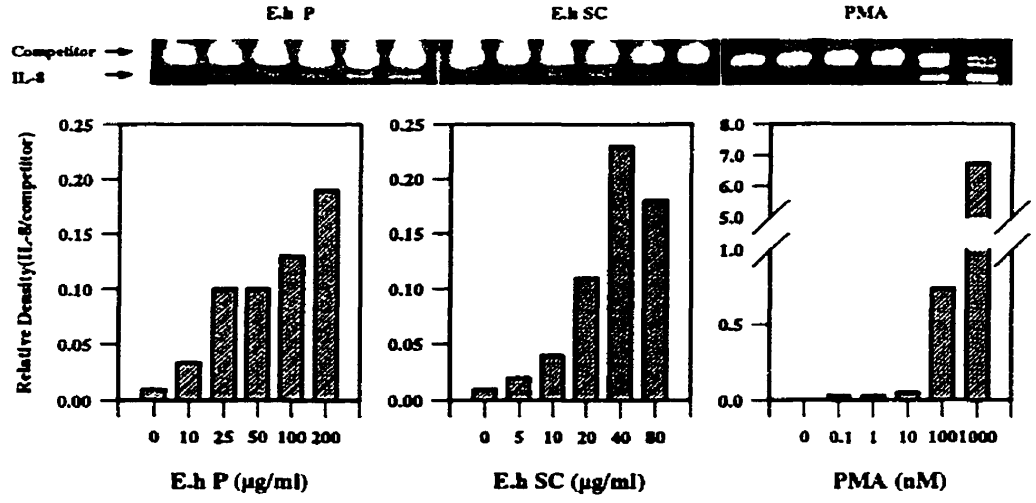
Figure 1.Effect of *E. histolytica* on expression of IL-8 gene in human colonic cells. T84 (☐), LS174T (■) and Caco-2 (▨) cells were incubated with 100 μg/ml of amebic proteins (E.h P), 20 μg/ml of amebic secretory components (E.h SC) or co-cultured with 10^6 live trophozoites (L E.h) in a 0.45 μm culture plate insert (Millicell) for 2 hours. Total RNA (1μg) extracted from each sample was used for semi-quantitative RT-PCR to analyze the changes in IL-8 mRNA levels as described in Materials and Methods. A consistent amount of competitor ($4.8x10^{-6}$ μg for T84 cells and $4.0x10^{-7}$ μg for LS174T and Caco-2) was present during PCR amplification. PMA (1 μM) was used as a positive control. The results were analyzed by NIH Image after PCR products were electrophoresized in a 1.5% agarose gel. Relative density (IL-8/competitor) represents the levels of IL-8 mRNA. Similar results were obtained from three separate experiments.

comparison to untreated cultures. Quantitatively, different levels of IL-8 mRNA were expressed in response to the amebic components. PMA stimulated the highest levels of IL-8 mRNA in the cells. Under the same conditions, *E. histolytica* trophozoites incubated with T84 cells in a cell-cell contact manner caused enhancement of IL-8 mRNA although about 10% of T84 cells were killed by amebae (data not shown). These data indicate that live *E. histolytica* without cell-cell contact, soluble proteins and secretory components can cause the accumulation of IL-8 mRNA in colonic epithelial cells. As the induction of IL-8 mRNA was the highest in T84 cells in response to amebic stimulation, this cell line was used for all subsequent studies.

Dose and time course for accumulation of IL-8 mRNA induced by *E. histolytica* components

To determine whether *E. histolytica*-induced accumulation of IL-8 mRNA was dose-dependent, T84 cells were incubated with various concentrations of E.h P, E.h SC or PMA for 2 hours. Total RNA was extracted and changes in IL-8 mRNA levels were analyzed using semi-quantitative RT-PCR. As shown in Figure 2A, IL-8 mRNA levels increased with increasing concentrations of E.h P, E.h SC or PMA. Under the same conditions, we next determined if *E. histolytica*-induced accumulation of IL-8 mRNA was time-dependent. T84 cells were incubated with 100 μg/ml of E.h P, 20 μg/ml of E.h SC or 1 μM PMA for various time periods. In response to ameba stimulation increased levels of IL-8 mRNA started within 1 hour, peaked at 2 hours and declined rapidly thereafter (Figure 2B). Similarly, stimulation with PMA resulted in increased IL-8 mRNA levels within 1 hour which remained high at 2-6 hours (peaked at 6 hours) and gradually declined to basal levels after 24 hours. Taken together, these data indicate that the accumulation of IL-8 mRNA in response to *E. histolytica* and PMA is regulated in a dose- and time-dependent manner.

A



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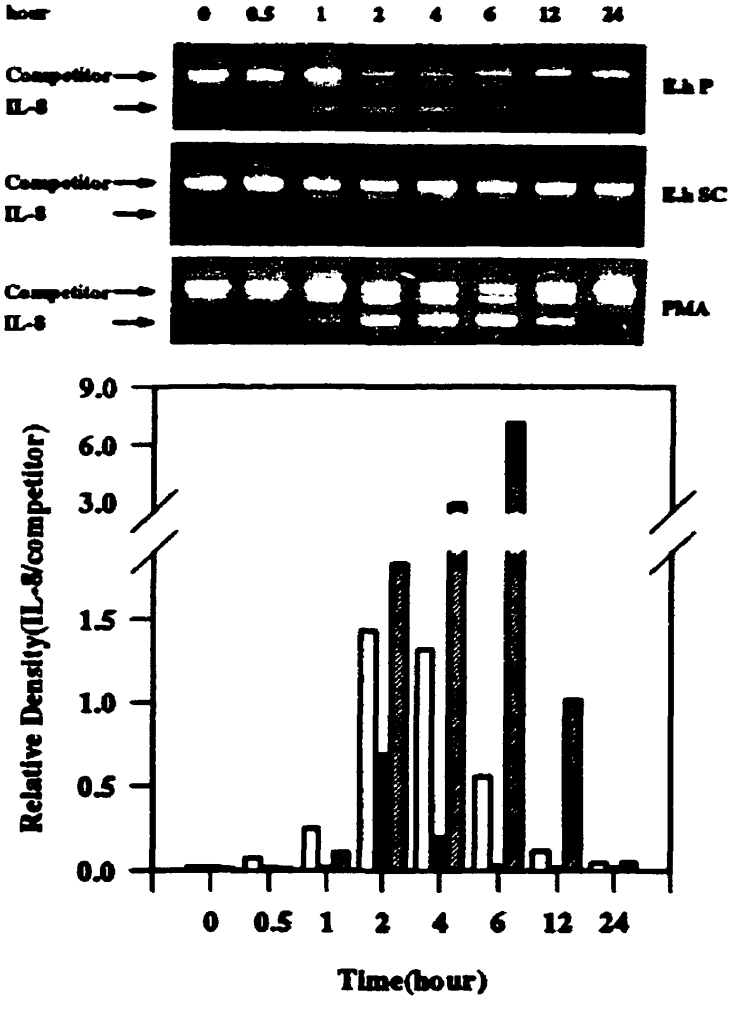

Figure 2A. *E. histolytica*-induced accumulation of IL-8 mRNA is dose-dependent. T84 cells were incubated for 2 hours with various concentrations of amebic proteins (E.h P), amebic secretory components (E.h SC) or PMA. Total RNA (1μg) extracted from each sample was used for semi-quantitative RT-PCR to examine the levels of IL-8 mRNA. Lane designations are identical to the histograms using NIH Image. Similar results were obtained from three separate experiments.

Figure 2B. *E. histolytica*-induced accumulation of IL-8 mRNA is time-dependent. T84 cells were incubated with 100 μg/ml of amebic proteins (E.h P) (□), 20 μg/ml of amebic secretory components (E.h SC) (■) and 1 μM of PMA (▨) for various time periods. Total RNA (1 μg) extracted from each sample was used for semi-quantitative RT-PCR to examine the levels of IL-8 mRNA. Similar results were obtained from three separate experiments.

Secretion of IL-8 protein induced by *E. histolytica*

To demonstrate secretion of IL-8 protein (ELISA) in the culture supernatant, T84 cells were incubated with 100 μg/ml of E.h P or 20 μg/ml of E.h SC for various time periods. PMA (1 μM) was used as a positive control and medium as a blank. As shown in Figure 3, significant production of IL-8 protein occurred after 12 hours post stimulation with E.h P or E.h SC that increased thereafter (up to 1130 ± 264 pg/ml for E.h P and 275 ± 48 pg/ml for E.h SC at 24 hours). In contrast, PMA induced high levels as early as 4 hours that increased thereafter to maximum levels after 24 hours (13,900 ± 669 pg/ml). Unstimulated cells secreted basal levels of IL-8 protein (33 ± 5 pg/ml after 24 hours). Previous studies (15) have failed to detect the secretion of IL-8 protein in T84 cells following stimulation with amebic lysates (50-650 μg/ml)

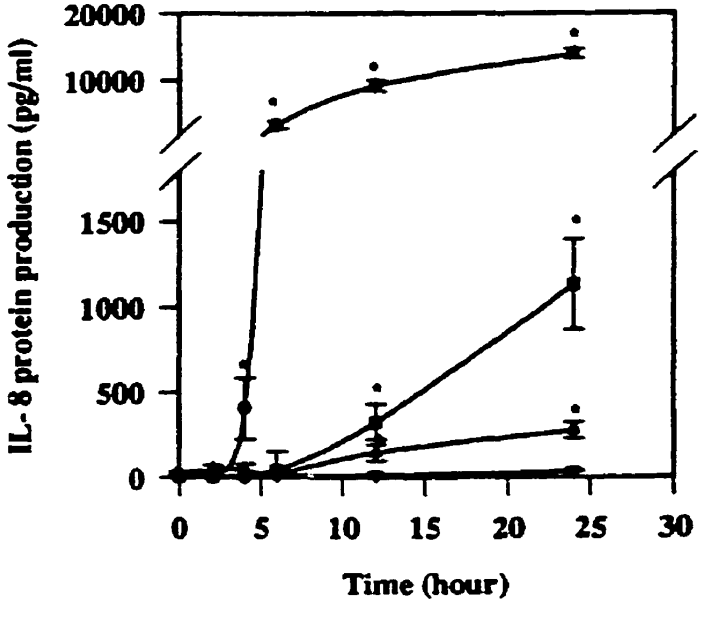

Figure 3. *E. histolytica* proteins and secretory components increase IL-8 protein production from T84 cells. T84 cells were incubated with 100 μg/ml of amebic proteins (E.h P) (■) or 20 μg/ml of amebic secretory components (E.h SC) (▲) for various time periods. The supernatant was collected for each time point and examined for IL-8 protein using a double sandwich ELISA with specific anti-human IL-8 antibodies. PMA (1 μM) (●) was used as a positive control and medium only as a blank (▼) . The data present the mean ± SEM from four separate experiments. *P< 0.05 compared with untreated homologous control .

IL-1 ß and TNF-α are not involved in increased production of IL-8 secretion stimulated by *E. histolytica* components

It is well known that stimulation of IL-8 in response to various pathogens or stimuli is accompanied with the induction of other cytokines, including IL-1 and TNF-α, which may act as intermediatory factors involved in the induction of IL-8. In T84 cells, IL-1 ß, IL-2, IL-4, IL-6, TGF and EGF have no significant effect on the IL-8 secretion while TNF-α enhanced the secretion of IL-8 protein (11). Moreover, T84 cells are not

Table 1. Effect of anti-human IL-1ß and TNF-α antibodies on IL-8 protein production by T84 cells stimulated with *E. histolytica* components and PMA

Inhibitors	IL-8 production (pg/ml)					
	Medium	PMA	E.h SC	E.h P	IL-1ß	TNF-α
No-inhibitor	203±24	4,680±101	349±20	426±47	591±33	628±28
Anti-hIL-1ß	189±17	4,920±438	371±38	513±45	117±21*	667±13
Anti-h TNF-α	212±29	5,040±136	312±37	395±20	518±42	212±15*
Normal Goat IgG	188±24	5,060±185	359±34	356± 7	515±68	592±28

Confluent T84 cells were incubated with PMA (1 µM), Eh. SC (20 µg/ml), E.h P (100 µg/ml) or medium only in the presence of neutralizing goat anti-human IL-1ß or TNF-α antibodies (IgG, 10 µg/ml each), normal goat IgG (10 µg/ml) or no inhibitors respectively. Under the same conditions, IL-1 ß and TNF-α were used as specific controls. After 24 hour incubation, the supernatant was collected from each sample and examined for IL-8 protein by ELISA as described in Materials and Methods. Values are means±SEM of four samples. *$p < 0.05$ compared to no-inhibitor or normal goat IgG controls.

responsive to IL-1α because they lack type-I IL-1 receptors (15). To determine whether ameba was stimulating the production of proinflammatory cytokines which could act in autocrine fashion to upregulate IL-8 mRNA expression the following experiment was performed. T84 cells were stimulated with E.h P (100 μg/ml), E.h SC (20 μg/ml) or PMA (1 μM) in the presence or absence of neutralizing anti human IL-1 ß or TNF-α antibodies (10 μg/ml each), normal goat IgG (10 μg/ml) or medium only. After 24 hour incubation, the supernatant was collected and IL-8 protein was measured by ELISA. As shown in Table 1, cells stimulated with *E. histolytica* or PMA for 24 hours in the presence or absence of anti human IL-1 ß or TNF-α antibodies did not affect IL-8 protein production. However, under the same conditions, anti-human IL-1 ß and TNF-α antibodies significantly inhibited IL-1ß (10 ng/ml) and TNF-α (10 ng/ml) induced IL-8 protein levels. There data suggest that the major proinflammatory cytokines, IL-1 and TNF-α are not involved as co-stimulatory factors for enhanced IL-8 protein production stimulated by amebae.

Effect of cyclohexamide on expression of IL-8 mRNA stimulated by *E. histolytica* components

In this study, we used cycloheximide (CHX), a protein synthesis inhibitor, to assess the regulation of the IL-8 gene in response to *E. histolytica* components and PMA. Cells were stimulated with 100 μg/ml of E.h P, 20 μg/ml of E.h SC or 1 μM PMA in the presence or absence of CHX (10 μg/ml) and total RNA was extracted 2 hours after stimulation. As depicted in Figure 4, whereas IL-8 mRNA were undetectable in unstimulated cultures, significantly increased levels of IL-8 mRNA were induced in the presence of E.h SC, E.h P or PMA as well as CHX. In cells stimulated with E.h SC, E.h P or PMA, CHX treatment resulted in superinduction of IL-8 mRNA. CHX was

specific for IL-8 mRNA induction as the expression of the GAPDH gene was not affected.

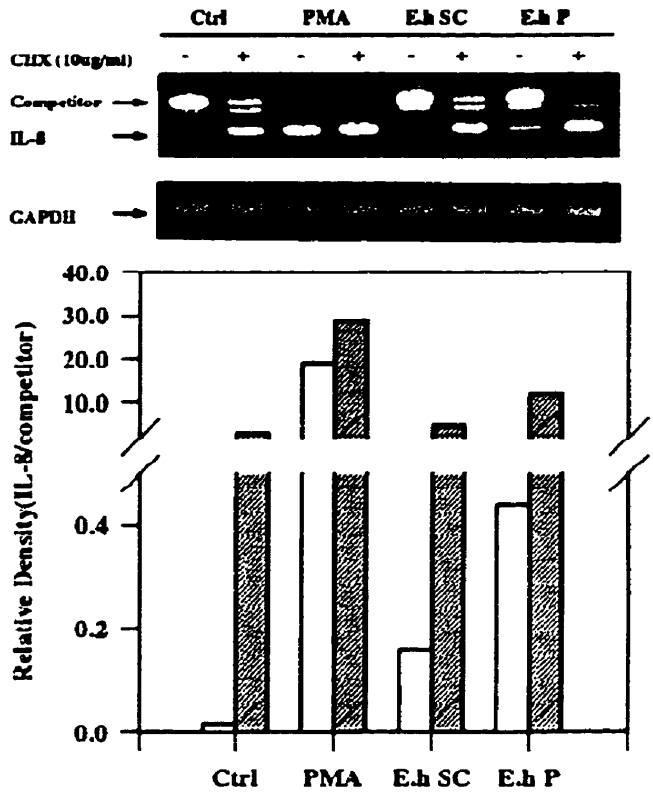

Figure 4. Effect of cycloheximide (CHX) on expression of IL-8 in T84 cells in response to *E. histolytica* components and PMA. T84 cells were incubated with 100 μg/ml of amebic proteins (E.h P), 20 μg/ml of amebic secretory components (E.h SC) or 1 μM PMA in the presence (☐) or absence (☐) of CHX (10 μg/ml) for 2 hours. Total RNA (1 μg) was extracted for semi-quantitative RT-PCR to examine the levels of IL-8 mRNA. GAPDH gene was used as an internal control. Similar results were obtained from two separate experiments.

Upregulation of the IL-8 gene by *E. histolytica* components is not at the transcriptional level

To determine whether enhanced expression of IL-8 gene is regulated transcriptionally or posttranscriptionally by *E. histolytica* components, confluent T84 cells were incubated with E.h SC (40 μg/ml), E.h P (200 μg/ml) or PMA (1 μM) for 2

hours and nuclei were purified from each sample for run-on assay to examine the transcription rate of the IL-8 gene. As shown in Figure 5, E.h P or E.h SC had no detectable effect on the transcription rate of the IL-8 gene whereas PMA caused increased transcription as compared to untreated cells, which showed constitutive transcription activity. These data suggest that *E. histolytica*-induced expression of IL-8 mRNA may occur by a posttranscriptional mechanism.

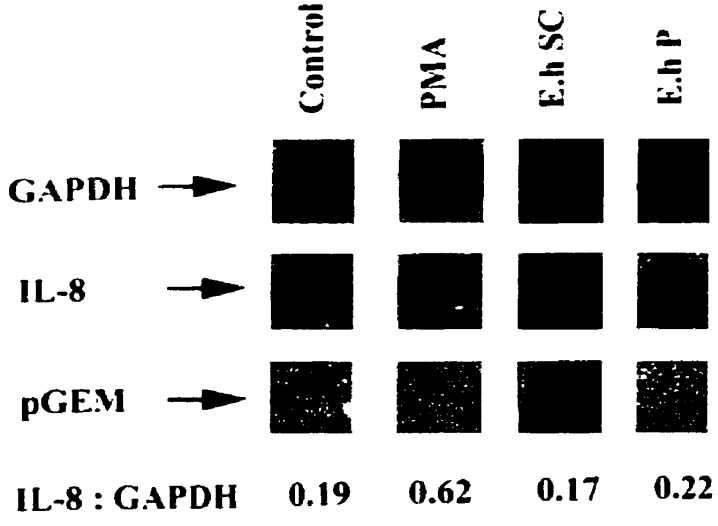

Figure 5. Transcriptional regulation is not involved in accumulation of IL-8 mRNA in T84 cells stimulated with *E. histolytica* components. Confluent T84 cells (70%) cultured in 50 ml culture flask were incubated with 1 μM PMA, 40 μg/ml of amebic secretory components (E.h SC) or 200 μg/ml of amebic proteins (E.h P) for 2 hours and nuclei were purified from each sample. Nuclear run-on assay was performed to examine transcription rate of the IL-8 gene as described in Materials and Methods. Similar results were obtained from two separate experiments.

Stability of IL-8 mRNA in response to *E. histolytica* components and PMA

The stability of the IL-8 mRNA after stimulation with *E. histolytica* was examined in the presence of the transcription inhibitor, actinomycin D. Under the same conditions, the stability of IL-8 mRNA in response to PMA was also examined for comparison. As

shown in Figure 6, IL-8 mRNA, stimulated with E.h SC or E.h P for 2 hours, degraded rapidly and was reduced by about 50% within 3 hours after the addition of actinomycin D (10 μg/ml). In contrast, PMA stimulated higher levels of IL-8 mRNA after 2 hour incubation and was reduced by about 50% after 6 hours. Therefore, the IL-8 mRNA in response to *E. histolytica* components, as determined by the halflife of the percentage of mRNA remaining, was less stable than IL-8 mRNA induced by PMA.

Comparison of the effect of *E. histolytica* and their secretory components on IL-8 mRNA expression

To evaluate the effect of trophozoites and their secretory components on expression of IL-8 mRNA, T84 cells were co-cultured with *E. histolytica* trophozoites (separated with Millicell, 0.45 μm pores) or their secretory components. Secretory components were prepared by collecting the cell-free supernatant after incubation of trophozoites in HBSS at 37°C for 2 hours. After 2 hour incubation, total RNA was extracted from each sample from the two groups in parallel and semi-quantitative RT-PCR was applied to examine IL-8 mRNA levels. As shown in Figure 7, the levels of IL-8 mRNA increased with increasing numbers of trophozoites or concentrations of secretory components. $5x\ 10^5$ trophozoites and secretory components derived from the same number of trophozoites resulted in the highest increase of IL-8 mRNA. Interestingly, live trophozoites elicited lower levels of IL-8 mRNA in comparison to their secretory components. This indicates that the effective components functioning on the target cells are gradually released from the parasites.

Preformed but not newly synthesized soluble amebic components increase IL-8 mRNA expression

To determine if newly synthesized proteins produced by *E. histolytica* are necessary for the enhancement of IL-8 mRNA, amebae were incubated in TYI-S-33

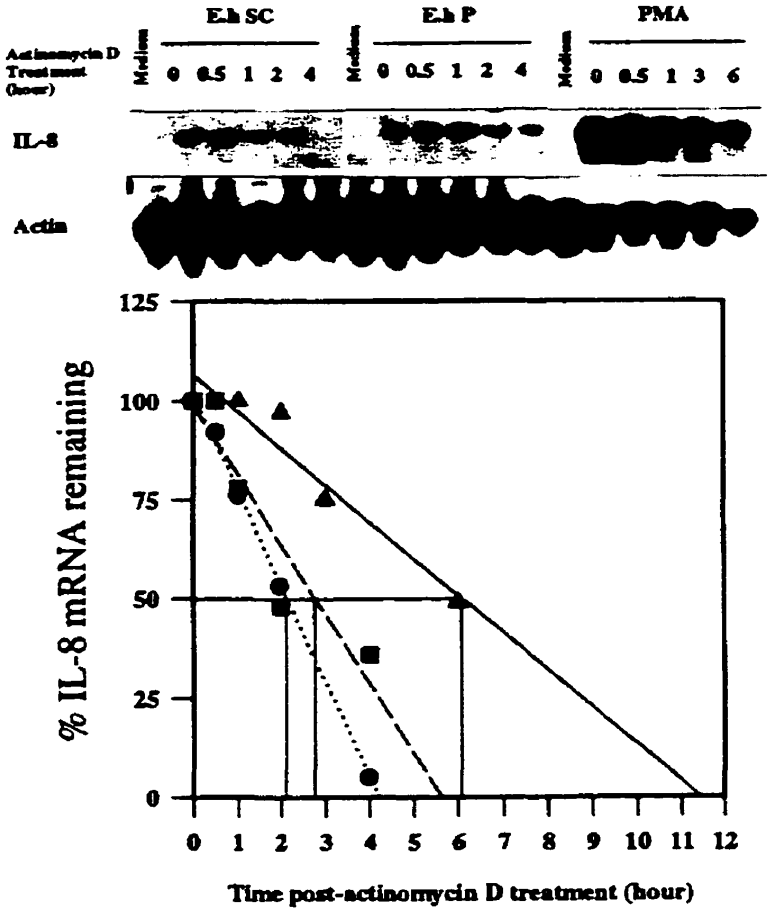

Figure 6. Stability of IL-8 mRNA in T84 cells in response to *E. histolytica*. T84 cells were stimulated with 20 μg/ml of amebic secretory components (E.h SC), 100 μg/ml of amebic proteins (E.h P) or 1 μM PMA for 2 hours and then treated with 10 μg/ml of actinomycin D. Total RNA (30, 20 and 10 μg for E.h SC, E.h P and PMA respectively) was extracted at various time periods following treatment with actinomycin D and Northern blot analysis was performed as described in Materials and Methods. Densitometry of autoradiographs of IL-8 and actin was measured using NIH Image. IL-8 mRNA level is expressed as a percentage of the mRNA level determined before the addition of actinomycin D, which was normalized by actin. 100% of IL-8 mRNA represents the IL-8 mRNA level 2 hour poststimulation with *E. histolytica* or PMA. The $t_{1/2}$ (50% stability) was calculated for E.h SC (●; r=0.998), E.h P (■; r=0.917) and PMA-induced (▲; r=0.926) IL-8 mRNA.

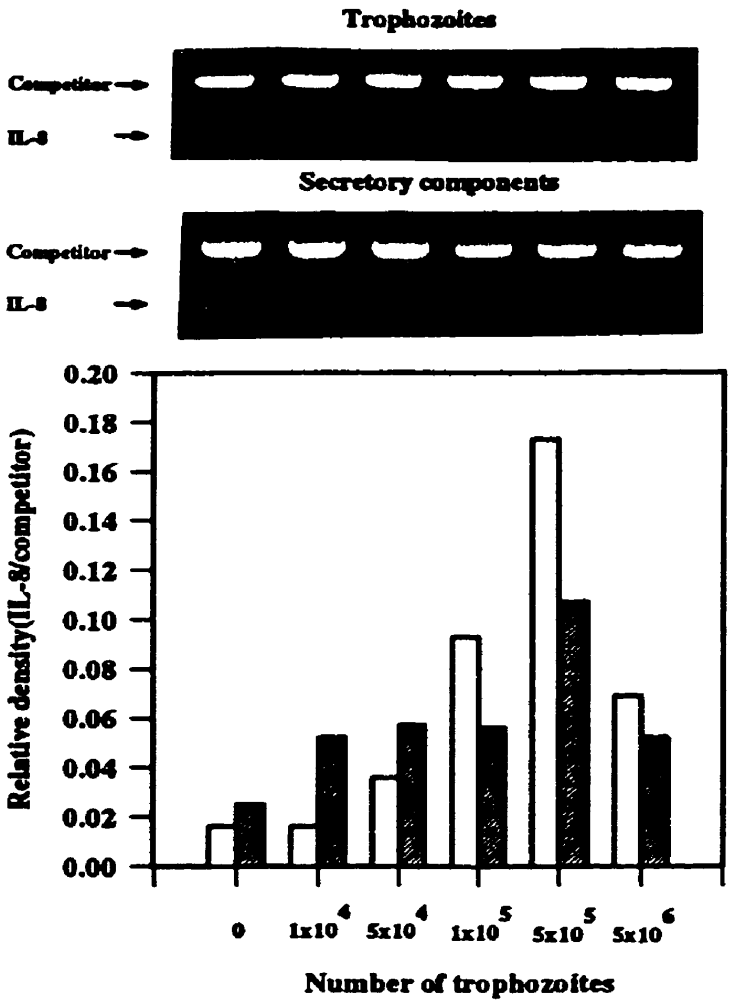

Figure 7. Comparison of *E. histolytica* and its secretory components on IL-8 mRNA expression. T84 cells were incubated with 0, $1x10^4$, $5x10^4$, $1x10^5$, $5x10^5$ and $5x10^6$ live trophozoites (▨) in 0.45 μm culture plate insert (Millicell) or amebic secretory components (□) (0, 1.7, 3.6, 7.1, 15.1 and 145.5 μg respectively in protein concentration), which were prepared by collecting the supernatant of the same number of trophozoites incubated in HBSS at 37°C for 2 hours. After 2 hour incubation, total RNA (1 μg) extracted from each sample was used for semi-quantitative RT-PCR to examine the levels of IL-8 mRNA. Similar results were obtained from two separate experiments.

medium for 2 hours in the presence or absence of CHX (10 μg/ml). 100 μg/ml of soluble amebic proteins or $5x10^4$ trophozoites in Millicell (0.45 μm pores) with or without pretreatment with CHX were added to T84 cell cultures; and after a 2 hour

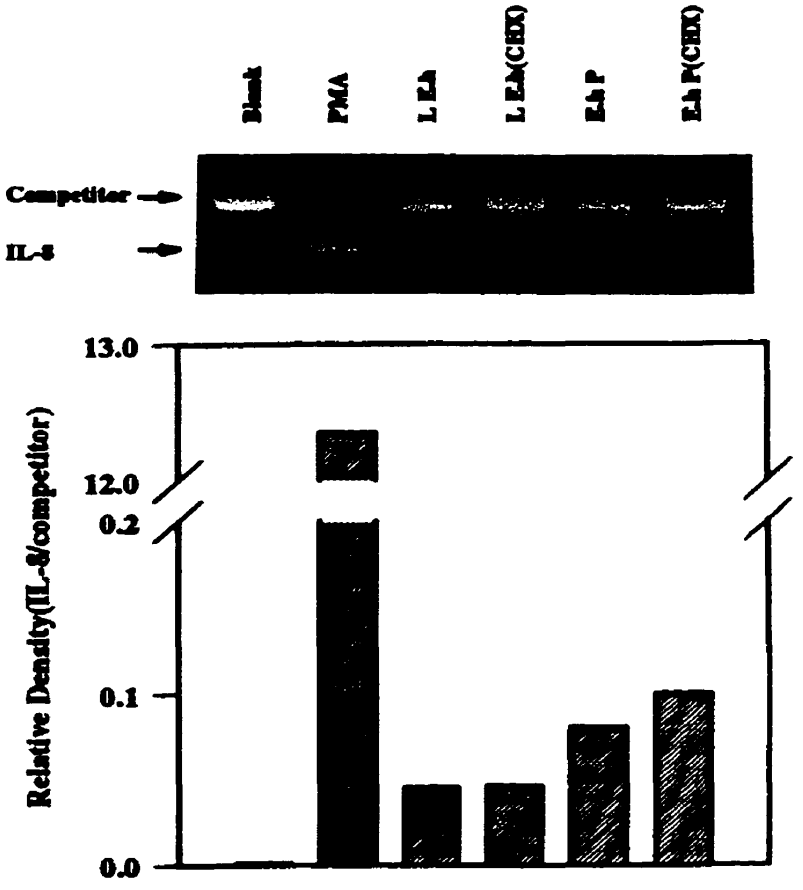

Figure 8. Soluble amebic components that increase IL-8 mRNA expression are preformed and not newly synthesized. Trophozoites were incubated in TYI-S-33 medium for 2 hours in the presence or absence of cycloheximide (CHX, 10 μg/ml). T84 cells were incubated for 2 hours with live amebae ($5x10^4$/ml) or soluble amebic proteins (100 μg/ml) from cells that were treated with or without CHX and total RNA was extracted for semi-quantitative RT-PCR to examine the levels of IL-8 mRNA. Medium was used as a blank control and PMA (1 μM) as a positive control. Similar results were obtained from two separate experiments.

incubation, total RNA was extracted for semi-quantitative RT-PCR to determine the levels of IL-8 mRNA. As shown in Figure 8, pretreatment of amebic trophozoites with CHX for 2 hours did not alter their ability to increase IL-8 mRNA expression in comparison with proteins obtained from untreated culture. These data suggest that newly synthesized proteins in amebae are not necessary for increasing IL-8 mRNA expression in T84 cells. It is likely that the effective soluble amebic components are preformed and released

slowly into the culture medium.

E. histolytica-induced expression of the IL-8 gene is sensitive to dexamethasone

Glucocorticoids (Dexamethasone, Dex) are potent immunosuppressive agents that modulate a variety of inflammatory reactions and immune responses (26) including the induction of IL-8 (15). To determine whether *E. histolytica*-induced IL-8 mRNA expression was sensitive to glucocorticoids, we pretreated the cells with 1 μM Dex for

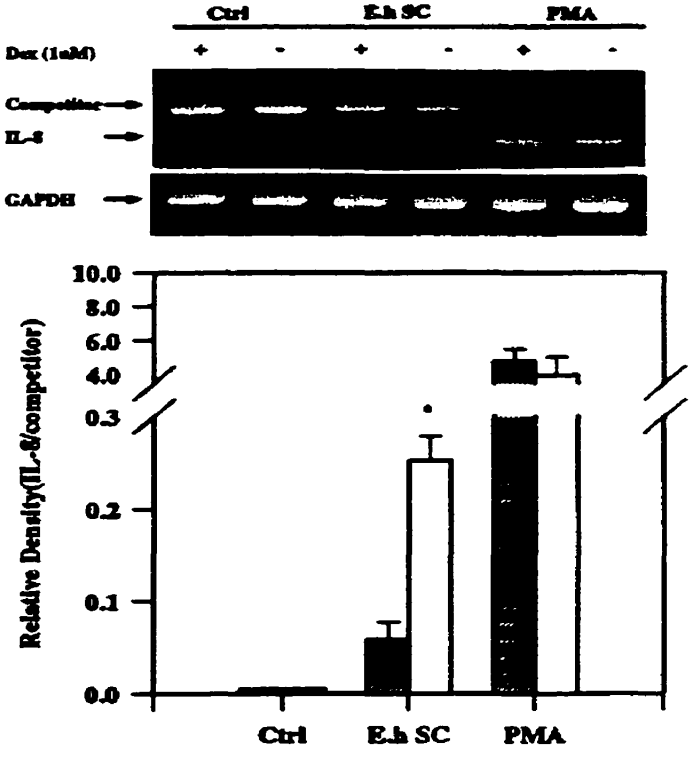

Figure 9. Effect of dexamethasone on expression of IL-8 mRNA in T84 cells stimulated by *E. histolytica* components and PMA. Cells were pretreated with (▨) or without (□) 1 μM dexamethasone (Dex) for 24 hours and then incubated with 20 μg/ml of E.h SC or 1 μM of PMA for 2 hours. Total RNA (1μg) extracted from each sample was used for semi-quantitative RT-PCR to evaluate the levels of IL-8 mRNA. A representative gel electrophoresis of RT-PCR amplification of IL-8 mRNA and internal GAPDH is shown. The results of histogram by NIH Image are from three separate samples and are presented as mean ± SEM. *$P < 0.05$ was considered statistically significant as compared to untreated homologous control.

24 hours and then incubated the cells with 20 μg/ml of E.h SC or 1 μM PMA for 2 hours. Expression of IL-8 mRNA was determined by semi-quantitative RT-PCR. As shown in Figure 9, Dex pretreatment almost completely inhibited the expression of IL-8 mRNA elicited by E.h SC. In contrast, Dex had no effect on expression of IL-8 mRNA induced by PMA. These data suggest that different mechanism are involved in the induction of IL-8 by *E. histolytica* and PMA in response to Dex.

DISCUSSION

E. histolytica infection is associated with an acute inflammatory response (5, 7, 8, 9). However, it still not clear how *E. histolytica* triggers a host inflammatory response and the host and parasite factors which initiate this event have not been clearly identified. The intestinal mucosa is the first line of defence against infectious pathogens and epithelial cells play a role in mediating the host response to pathogens. Accumulated evidence has shown that gastric epithelial cells are a potential source of IL-8 which have biological functions in attraction and activation of neutrophils (10, 11). Infiltration of neutrophils to local sites is the hallmark of acute inflammation, resulting in tissue damage (27). Therefore, the induction of IL-8 by epithelial cells in response the infectious pathogens is critical to host mucosal inflammation. Previous studies (15) have shown that the release of preformed IL-1α from cytolysed epithelial cells with *E. histolytica*(cell-cell contact) contributed to the induction of IL-8 in those cells. In this study, we clearly demonstrate that *E. histolytica* can directly stimulate the accumulation of IL-8 mRNA in human colonic cells in the absence of cell-cell contact or injury. This was supported by the observation of the accumulation of IL-8 mRNA in colonic cells (T84, LS174T and Caco-2) after incubation with soluble amebic proteins (E.h P), amebic secretory components (E.h SC) and in co-culture with live trophozoites (L E.h) with or without

cell-cell contact. We used T84 cells as our experimental model because they are more responsive to *E. histolytica* than the other two cell lines tested (LS174T and Caco-2) and are not affected by stimulation with LPS (11).

IL-8 mRNA accumulation occurred in dose- and time-dependent fashion in response to *E. histolytica* components. The levels of IL-8 mRNA measured by semi-quantitative RT-PCR showed a rapid response to E.h P and E.h SC as well as PMA. Detectable levels of IL-8 mRNA were observed within 1 hour. Peaked mRNA levels occurred within 2 hours in response to *E. histolytica* and 6 hours in response to PMA. Interestingly, delayed production of IL-8 protein was observed in response to E.h P and E.h SC (12 hours after stimulation) in comparison to those in response to PMA (4 hours after stimulation). Increased production of the IL-8 protein was observed after stimulation with E.h P (1130 $\pm$ 264 pg/ml) or E.h SC (275 $\pm$ 48 pg/ml) and increased by ~34 and ~8 fold respectively in comparison with unstimulated cultures (33 $\pm$ 5 pg/ml) after 24 hours. Furthermore, our results showed a similar dose-dependent pattern of IL-8 mRNA accumulation after the cells were stimulated with various concentrations of trophozoites or their secretory components. The secretory components elicited a higher level of IL-8 mRNA, suggesting that the IL-8 eliciting components from amebae are secreted slowly and accumulate in the culture medium. Taken together, these data suggest that soluble amebic components, present in or secreted from trophozoites, are able to not only cause IL-8 mRNA accumulation but also increase IL-8 protein secretion by human colonic cells. Moreover, we demonstrated that soluble amebic components caused accumulation of IL-8 mRNA in T84 cells without the need of newly synthesized proteins as CHX treatment had no inhibitory effects on the amebic components. This suggests that the IL-8 stimulating components by amebae are preformed and not newly synthesized by trophozoites.

Virulent amebae secrete a variety of substances that are able to trigger a signal

transduction cascade in various experimental models (30, 31, 32). Our results clearly demonstrate that amebic soluble components can increase IL-8 mRNA level as well as the secretion of IL-8 protein by human colonic cells and that direct cell-cell contact was not necessary for this to occur. Although direct cell-cell contact and/or adhesion or invasion is a prerequisite for the induction of IL-8 in response to various pathogens, including *Helicobacter pylori* (20, 33, 34, 35), *Salmonella dublin*, *Listeria moncytogenes* (18, 21), *Staphylococci spp* (19) and *Leishmania major* (22), secretory components from certain pathogens resulted in the induction of IL-8 in target cells. Massion et al. (28) observed that soluble products from *Pseudomonas aeruginosa* caused a marked induction of IL-8 gene expression as well as IL-8 protein secretion in human transformed bronchial epithelial cells. Similarly, it was shown that secretory components from *Trichomonas vaginalis* or *Propionibacterium acnes* are responsible for the induction of IL-8 (29, 36). Therefore, we believe that soluble amebic components, which are released in a time-dependent fashion from amebae, directly initiate cell signal transduction cascade (PKA, PKC etc.) leading to the induction of IL-8 from intestinal epithelial cells. As intestinal mucosal epithelial cells are critical mediator between the pathogen and host response, the induction of IL-8 in response to amebic secretory components may play a key role in the initiation of a host acute inflammatory response. Accordingly, we suggest that increased production of IL-8 protein by epithelial cells exposed to *E. histolytica* components elicits a potentially important cell signalling mechanism in the mucosal layer for neutrophil recruitment which contributes to the host inflammatory response. Nonspecific mucosal lesions evoked by infiltrated neutrophils may further facilitate amebic invasion. During amebic invasion, trophozoites kill epithelial cells and neutrophils (4, 5, 6). Reactive oxygen intermediates and lysosomal enzymes from dead neutrophils which are recruited by IL-8 from epithelial cells or even neutrophils can cause tissue injury facilitating amebic invasion into the tissues. The release of preformed IL-1α from lysed epithelial cells could

augment the secretion of IL-8 from epithelial cells exacerbating the host mucosal inflammatory response (15).

It is evident that stimulation of IL-8 in response to various pathogens is accompanied with induction of other cytokines, including IL-1 and TNF-α (15, 18, 29, 36, 37). Lysed colonic epithelial cells by *E. histolytica* trophozoites caused the release of preformed IL-1 α which had a paracrine effect on IL-8 production (15). Similarly, the induction of IL-8 in response to *T. vaginalis* membrane components is partially dependent on TNF-α as indicated by the observation of a decrease of IL-8 production by 25% in the presence of neutralizing monoclonal antibodies against TNF-α (29). *Staphylococci spp* and *L. major* directly induce IL-8 production from monocytes without concomitant increase in IL-1 or TNF-α (19, 22). We demonstrated that neither IL-1 ß nor TNF-α contributed to the production of IL-8 protein in T84 cells after stimulation with *E.histolytica* components or PMA as neutralizing anti-human IL-1ß and TNF-α antibodies had no effect. Furthermore, we examined the effect of CHX, a protein synthesis inhibitor, on expression of IL-8 gene in T84 cells. CHX did not decrease IL-8 mRNA levels in response to either *E. histolytica* components or PMA, indicating that *de novo* or intermediatory protein is not essential for the induction of IL-8 gene expression. CHX treatment caused superinduction of the IL-8 gene by *E. histolytica*-components or PMA-treated cells as well as in unstimulated cultures which suggests the existence of a suppressor protein(s) involved in the degradation of newly synthesized IL-8 mRNA. Under the same conditions, CHX did not alter the expression of the GAPDH gene. As shown in previous studies, *de novo* proteins are essential for the induction of IL-8 in promyelocytic HL60 cells in response to PMA as cycloheximide inhibits PMA-induced expression of the IL-8 gene (38) but superinduction of IL-8 mRNA occurred after

stimulation by IL-1 and TNF-α in the presence of CHX (39). Moreover, we demonstrated that enhancement of IL-8 mRNA in T84 cells elicited by *E. histolytica* components is not regulated at the transcriptional level whereas PMA increased transcription of the IL-8 gene. Therefore, it is very likely that elevated expression of IL-8 mRNA in T84 cells stimulated by *E. histolytica* components occurs through a posttranscriptional mechanism. The stability of the IL-8 mRNA in response to *E. histolytica* components was $t_{1/2} < 3$ hours and for PMA $t_{1/2} > 6$ hours; thus *E. histolytica* components and PMA differently regulate the degradation of newly synthesized IL-8 mRNA. These data suggest that the accumulation of IL-8 mRNA in T84 cells elicited by *E. histolytica* components may be due to the delay in degradation of newly synthesized IL-8 mRNA. Other studies (40) have shown that a posttranscriptional mechanism is involved in the upregulation of the IL-8 gene in human monocytes in response to interferon-γ.

Glucocorticoids, potent immunosuppressive agents, suppress both the process of inflammation and the immune response (26) and it is suggested that they are most effective inhibitors of IL-8 gene expression (41, 42). The inhibitory effect of Dex on IL-8 production was observed in fibroblasts, synoviocytes, chondrocytes, mesengial cells, monocytes and macrophages(13). However, Dex can not inhibit IL-8 expression in peripheral blood monocytes in response to PMA (43), fibroblasts stimulated with leukoregulin (44) and airway epithelial cells stimulated with elastase (45). Our results demonstrate that Dex can inhibit the accumulation of IL-8 mRNA in response to amebic secretory components but has no effect on PMA-induced expression of IL-8 gene. Thus, the signal transduction pathways initiated by soluble amebic components and PMA which lead to the production of IL-8 by T84 cells are differentially regulated by Dex. The complete mechanism of the effect of Dex on the expression of IL-8 gene in response to

E. histolytica or PMA in human colonic cells needs to be further characterized.

In summary, we present evidence that *E. histolytica* components can directly induce IL-8 gene expression in human colonic epithelial cells. This was demonstrated by the observation of the accumulation of IL-8 mRNA as well as increased secretion of IL-8 protein. Upregulation of the IL-8 gene in T84 cells in response to *E. histolytica* components is likely due to the accumulation of IL-8 mRNA through a posttranscriptional mechanism. Thus, increased IL-8 secretion from mucosal epithelial cells in response to *E. histolytica* components may play a novel role in the initiation of the host acute inflammation in intestinal amebiasis prior to cell-cell contact or injury leading to amebic colitis.

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CONNECTING STATEMENT I

Results in Manuscript I clearly demonstrate that *E. histolytica* trophozoites release soluble components which can enhance IL-8 mRNA expression and protein production from human colonic epithelial cells. Thus, amebae can initiate inflammatory responses in the gut prior to making contact with mucosal epithelial cells. This observation coupled with the fact that *E. histolytica* themselves can produce PGE_2 and can stimulate macrophages to produce PGE_2 suggests that PGE_2 may be central in intestinal amebiasis. Accordingly, we determine the molecular mechanism whereby PGE_2 stimulates IL-8 gene expression in human colonic epithelial cells.

MANUSCRIPT II

Prostaglandin E_2 stimulates interleukin-8 gene expression in human colonic epithelial cells by a posttranscriptional mechanism*

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ABSTRACT

Intestinal mucosal epithelial cells produce IL-8, a neutrophil chemoattractant, which contributes to mucosal inflammation in various infectious and inflammatory diseases. However, the mediators involved and the molecular regulation of IL-8 production are poorly understood. As prostaglandin E_2 is central in gut inflammation and modulates a variety of mucosal epithelial cell functions, we determined whether PGE_2 can affect the expression of IL-8. Exogenous PGE_2 induced the accumulation of IL-8 mRNA and protein production in a dose- and time-dependent manner in T84 human colonic epithelial cells. Forskolin and dibutyryl cAMP, which increase intracellular cAMP, stimulated IL-8 in a similar fashion to PGE_2. PGE_2 and PGE_2 receptor agonists coupling through EP_4 receptors elevated intracellular cAMP and upregulated IL-8 mRNA expression by activating protein kinase A. Unlike PMA, PGE_2 and forskolin did not increase IL-8 gene transcription. However, PGE_2, forskolin and PMA enhanced the stability of IL-8 mRNA transcripts, suggesting the involvement of posttranscriptional regulation. Chloramphenicol acetyltransferase reporter gene transfection studies confirmed the presence of PGE_2 responsive cis-element(s) in the IL-8 3'-UTR. Furthermore, dexamethasone inhibited PGE_2-, forskolin- and dibutyryl cAMP-induced, but not PMA-induced IL-8 protein production. These results highlight a novel role for PGE_2 in upregulating IL-8 gene expression by colonic epithelial cells which may contribute to exacerbation of inflammation in the gastrointestinal tract.

INTRODUCTION

Interleukin-8, originally called monocyte-derived neutrophil chemotactic factor, is a potent chemokine causing recruitment and infiltration of neutrophils and T-cells into local inflammatory sites. Infiltration of neutrophils contributes to inflammation and has been implicated in various diseases.

A wide variety of cells produce IL-8 in response to different stimuli and certain pathogens (1). In respiratory inflammation induced with *Pseudomonas areuginosa*, soluble bacterial products were shown to cause marked induction of IL-8 mRNA expression and protein production in human transformed bronchial epithelial cells (2, 3). Epithelial cells are a potential source of various proinflammatory cytokines, such as IL-1, TNF-α, and IL-8 (4, 5). It is becoming clear that intestinal mucosal epithelial cells are key mediators for bidirectional communication between pathogens and host responses. They also play a crucial role in the pathogenesis of inflammatory bowel diseases. Local acute inflammation evoked by the induction of IL-8 contributes to tissue lesions during invasive or noninvasive bacterial infections, such as *Salmonella sp.* or *Helicobacter pylori* (6-8). Recent studies demonstrated that the colonic protozoan parasite, *Entamoeba histolytica*, enhanced IL-8 production in human colonic epithelial cells by secretory components released from the amebae (9) or through a paracrine effect elicited by proinflammatory cytokine (IL-1α) released from damaged host cells (10). In inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, IL-8 production from mucosal epithelial cells has been suggested to play a role in tissue injury (11).

While it is certain that a variety of infectious agents and proinflammatory cytokines can stimulate IL-8, little is known of the role of lipid mediators of inflammation in the regulation of mucosal epithelial cell functions. Low m. w. arachidonic metabolites,

particularly PGE(s), are primarily involved in inflammatory reactions (12). Among the PGs, PGE_2 is considered to be the most potent mediator of inflammation. PGE_2 causes vasodilation and has pyretic effects during host inflammatory responses. Four subtypes of G protein coupled PGE_2 receptors termed EP_1, EP_2, EP_3 and EP_4 mediate PGE_2's function and trigger intracellular signal transduction (13, 14). Recently it was reported that PGE_2 caused osteoblast formation through the induction of IL-1ß by activation of PKA signal transduction pathway (15). A clear role for PGE_2 in up-regulating the production of IL-6 has been demonstrated in mouse peritoneal macrophages following injection of mineral oil (16); activation of cyclo-oxygenase (Cox) 2, but not Cox 1 enzyme activity contributed to the induction of IL-6 (17). There is also evidence that PGE_2 has regulatory functions on IL-8 gene expression in various cell types in response to certain stimuli. For example, it enhances IL-8 production in human synovial fibroblasts stimulated with IL-1ß (18), but has no effect on neutrophil-derived IL-8 evoked by LPS (19). Moreover, in human alveolar macrophages and blood monocytes, PGE_2 down-regulated IL-8 in response to LPS (20). The role of PGE_2 in secretory responses from intestinal epithelial cells has been well addressed (21-23); however, there are no reports to date documenting PGE_2 modulation of IL-8 gene expression in mucosal epithelial cells and this is the focus of the present study.

Herein, we demonstrate that exogenous PGE_2 coupling through EP_4 receptors on human colonic epithelial cells up-regulate IL-8 gene expression and protein production by a posttranscriptional mechanism. These results highlight a potential novel role for PGE_2 in the exacerbation of intestinal inflammation in the gastrointestinal tract.

MATERIALS AND METHODS

Reagents

PGE_2, 13,14-dihydro-15-keto PGE_2, 1-hydroxy-PGE_1, and sulprostone were purchased from Cayman Chemical (Ann Arbor, MI). Forskolin, PMA, dibutyryl cAMP (dcAMP), actinomycin, and dexamethasone (Dex) were purchased from Sigma Chemical Co. (St. Louis, MO). Iloprost was a gift from Dr. F. McDonald (Schering AG, Berlin, Germany). Butaprost was a gift from Dr. P. Gardiner (Bayer, UK). SQ22536 (9-(Tetrahydro-2'-furyl)adenine) and H89 (N-[2-(p-Bromocinnamy(amino)ethyl]-5-isoquinolinesulfonamide, HCl) were purchased from Calbiochem (San Diego, CA). M&B28767 was a gift from Rhône-Poulenc Rorer (Dagenham, UK). Lipofectamine reagent was purchased from Gibco BRL Life Technologies (Burlington, Canada). 2-Nitrophenyl-ß-D-galactopyranoside was purchased from Boehringer Mannheim Canada (Laval, Canada). [^{14}C] Chloramphenicol was purchased from Amersham Canada Limited (Oakville, Canada). Acetyl coenzyme A was purchased from Pharmacia Biotech Inc (Baie Durfé, Canada). pcDNA3 and pRc/CMV plasmids were purchased from Promega Biotch (Madison, WI).

Cell culture

The human colonic adenocarcinoma cells, T84, were obtained from the American Type Culture Collection (Rockville, MD) and grown in 24-well plates in 1 ml of a 1/1 mixture of DMEM with 4.5 g of D-glucose/litre and Ham's F12 nutrient mixture (Life Technologies) containing 5% fetal bovine serum (Hyclone Laboratories, Logan, UT), 100 U/ml of Penicillin, 100 µg/ml of Streptomycin sulphate and 20 mM HEPES (Sigma). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

RNA preparation and semi-quantitative RT-PCR

Total RNAs were isolated from T84 cells with TRIzol reagent (Gibco) and semi-quantitative RT-PCR was performed as previously described (9). A consistent concentration of competitor (644bp) in pGEM plasmid was used to compete the target IL-8 fragment (284 bp). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Measurement of IL-8 protein

An IL-8 enzyme-linked immunosorbent assay (ELISA) was used to measure IL-8 production in the supernatant of T84 cell (about 7 x 10^5) cultures. 96-well ELISA plates (Immunn II, Dynatech Lab San Louis Obispo, CA) were coated with anti-human IL-8 neutralizing Ab (200 ng; R&D Systems, Minneapolis, MN) in 50 µl of carbonate buffer, pH 9.6, overnight at 4°C. The plates were washed with PBS and blocked with 1% BSA overnight at 4°C. Samples (50 µl) were added to each well and incubated for 1 h at 37°C, while different concentrations of recombinant human IL-8 protein (R&D Systems) were used as the standard. After washing, 50 ng of rabbit anti-human IL-8 polyclonal Ab (Endogen, Boston, MA) was added for a further 1 h incubation at 37°C. Subsequently, goat anti-rabbit IgG Ab horseradish peroxidase-conjugate (50 µl; Bio-Rad Laboratories, Richmond, CA) at a 1/1500 dilution was added and incubated for 1 h at 37 °C. The plates were washed and 100 µl of ABTS (Bio-Rad) was added to each well to quantify the results. The OD was measured 30 min later at room temperature with an ELISA reader at 405 nm. IL-8 protein levels are expressed as nanogram per milliliter.

Measurement of intracellular cAMP

Confluent T84 cells were stimulated with PGE_2 (1 µM) or forskolin (10 µM) for 10 min and extracted with absolute ethanol. The supernatant was dried in a speed vacuum and the sample resuspended in phosphate buffer. The cAMP assay was performed using a sensitive protein binding EIA kit (Cayman Chemicals).

Nuclear run-on assay

T84 cells were collected; homogenized in lysis buffer containing 320 mM sucrose, 10 mM Tris-HCl(pH 7.8), 0.1 mM EDTA(pH 8.0), 2 mM $MgCl_2$, 1mM DTT, 0.2% Triton X-100; and kept on ice for 10 min. After centrifugation at 1500 x g for 15 min at 4°C, nuclei were resuspended in 100 µl of storage buffer containing 50% glycerol, 50 mM Tris. HCl(pH 8.0), 5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM DTT. The reaction was performed in 200 µl of reaction buffer containing 25% glycerol; 25 mM Tris-HCl(pH 8.0), 5 mM $MgCl_2$; 0.1 mM EDTA; 1 mM DTT; 120 mM KCl; 500 µM each of ATP, GTP and CTP, 50 µM UTP; and 100 µCi [α-^{32}P] UTP(3000 Ci/mM) for 60 min at 26°C. Subsequently, α-^{32}P labeled nuclear RNA was extracted with Trizol reagent and hybridized onto denatured membrane-bound cDNA probes at 65 °C for 48 h in hybridization buffer containing 5 x Denhart solution, 6 x SSC; 0.05% SDS, 0.1 mM EDTA, and 100 µg/ml of salmon sperm DNA. Three micrograms of IL-8 or GAPDH cDNAs generated by PCR using specific primers as previously described (9) was immobilized onto nitrocellulose membrane using a Minifold I (Schleicher & Schuell, Keene, NH). pGEM vector was used as a control for nonspecific hybridization. After sequential washing with 6 x SSC and 1% SDS at room temperature for 10 min and with 1% SSC at 65 °C, autoradiography was performed. OD was measured using the National Institute of Health Image software, and the ratio of IL-8/GAPDH represents the transcription rate.

Construction of chloramphenicol acetytransferase (CAT) reporter gene plasmids

The IL-8 3'-UTR (bp 1902-3710; Genebank accession no. M28130) was amplified by PCR using the human colonic epithelial cell line T84 genomic DNA as a template with the primers: gatatcTAAAAAAATTCATTCTCTGTGGTATCC (sense) and

ctcgagTGAATTCCGAAGTTCTTTTTGTTC (antisense). This fragment was ligated into the *EcoR*V and *Xho*I sites of the plasmid pcDNA3 which contains the CAT reporter gene (pcDNACAT) in the *Hind*III and *BamH*I sites yielding the reporter gene construct pcDNACAT-UTR. The polyadenylation site was determined at bp 3152, and the sequence from bp 3153-3710 was not transcribed into mRNA (our unpublished observations).

Transfections, CAT and ß-galactosidase assays

T84 cells were plated at a density of 7 x 10^6 in 60-mm dish in complete medium. After 24 h, 3 µg of pcDNACAT-UTR or pcDNACAT plasmids together with 3 µg of pRc/CMV plasmid with ß-galactosidase gene in *Not*I site (pRc/CMV-gal) to correct the difference in the transfection efficiency were transfected into cells using Lipofectamine reagent. To determine the effect of PGE_2 on CAT reporter gene expression, 1 µM PGE_2, 10 µM forskolin, or 1 µM PMA was added into the culture medium 26 h after transfection. The cytoplasmic extract from each sample after 20-h incubation was then assayed for CAT and ß-galactosidase activity as described previously (24). Briefly, transfected T84 cells were washed with PBS and suspended in 300 µl of CAT buffer containing 40 mM Tris-HCl(pH 7.5), 1 mM NaCl, and 1 mM EDTA. After freeze-thawing, the cell extract was used for ß-galactosidase activity in the presence of 67 mM sodium phosphate(pH 7.5), 1 mM $MgCl_2$, 0.045 mM ß-ME, and 150 µg of 2-nitrophenyl-ß-D-galactopyranoside (ONPG) at 37 °C for 24 h. OD was measured at 415 nm. After normalized by ß-galactosidase activity, the cell extract was heated at 65 °C for 10 min and used for measuring CAT activity in reaction buffer containing 100 mM Tris-HCl(pH 8.0), 125 nCi [^{14}C] chloramphenicol, and 25 µg of acetyl coenzyme A at 37°C for 24 h. After incubation, chloramphenicol and its derivative were extracted with ethyl acetate, and the acetylated chloramphenicol and substrate were separated on a thin layer

silica gel plate (VWR Calab, Mississauga, Canada) in the presence of the solvent chloroform/methanol (19/1) and then autoradiographed. The precent conversion of [^{14}C] chloramphenicol to the acetylated form was quantified in comparison to the control groups using National Institute of Health Program.

Statistical analysis

The data were calculated as means $\pm$ SD. The results were analyzed by Student'*t* test. A value of $p<0.05$ was considered statistically significant.

RESULTS

Time and dose dependency for IL-8 mRNA accumulation and protein production in T84 cells exposed to PGE_2, forskolin, and dcAMP. PGE_2 has been shown to elevate intracellular cAMP by interacting with specific EP receptors (13, 14). To determine whether PGE_2 can modulate IL-8 mRNA expression and protein production through a receptor-coupling mechanism, T84 cells were exposed to various concentrations of PGE_2 and IL-8 mRNA and protein levels were quantified. As shown in Figure 1*A*, IL-8 mRNA accumulation occurred in a dose-dependent fashion with increasing concentrations (0.01-10 μM) of PGE_2. Two agents that increase intracellular cAMP, forskolin (0.01-25 μM) and dcAMP (0.1-1000 μM), showed similar dose-dependent effects on IL-8 mRNA expression. A similar dose- and time-dependent accumulation of IL-8 mRNA was also observed in the well-characterized human colonic adenocarnicoma cell line, LS174T in response to PGE_2 (data not shown). Figure. 1*B* and *C* shows the kinetics of IL-8 mRNA expression and protein production. Rapid increases in IL-8 mRNA levels were observed 1 h after treatment with 1 μM PGE_2, peaked after 2 h, and remained

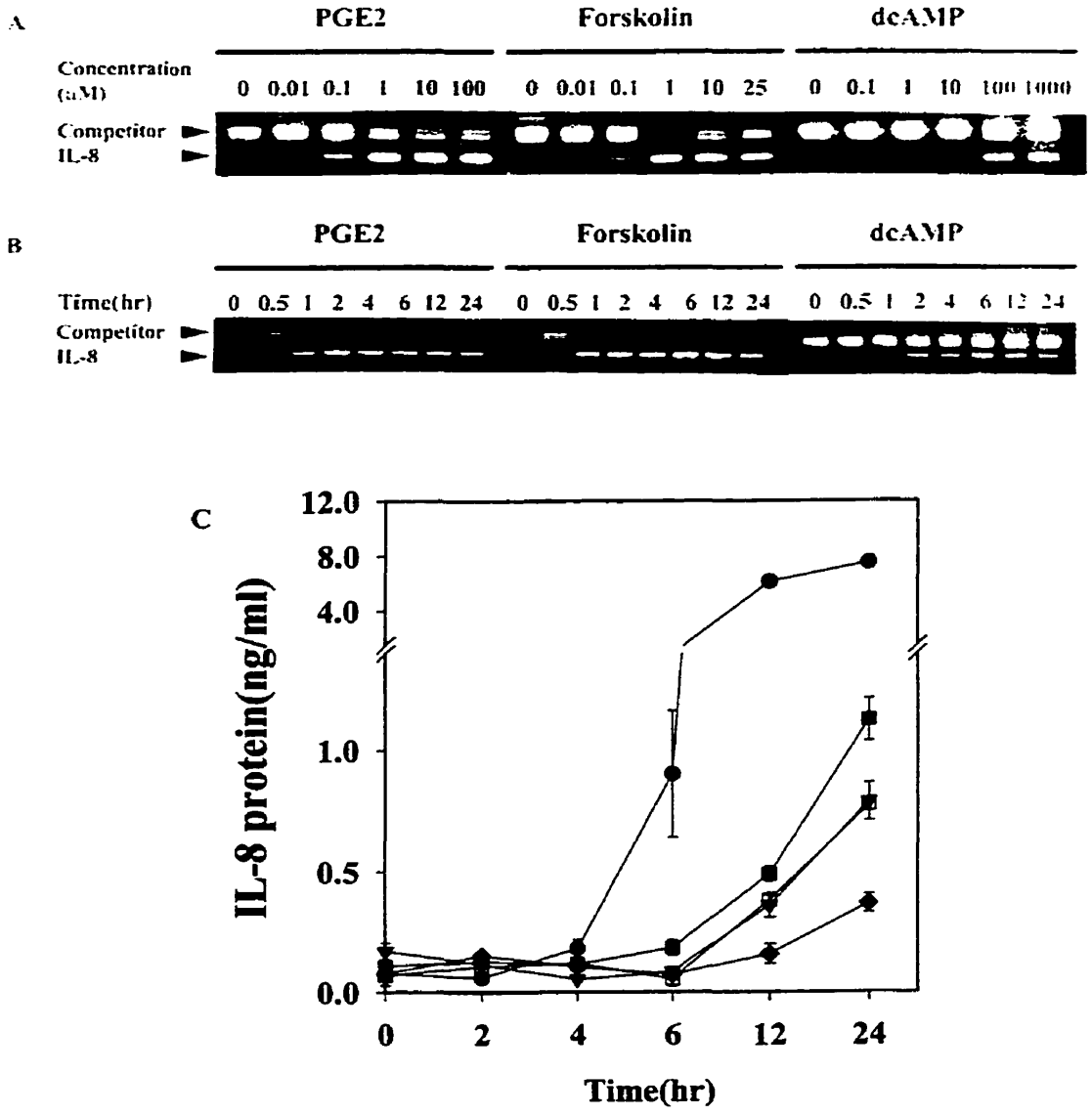

FIGURE 1. Dose- and time-dependent accumulation of IL-8 mRNA and protein production. IL-8 mRNA levels were examined by semi-quantitative RT-PCR in T84 cells after 2 h stimulation with *A*) various concentration of PGE_2 (0.01-10 μM), forskolin (0.01-25 μM), or dcAMP (0.1-1000 μM); and *B*) with 1 μM PGE_2, 10 μM forskolin or 100 μM dcAMP at different time periods. Similar results in *A* and *B* were obtained from two separate experiments. *C*, IL-8 protein production was measured in the supernatants of T84 cells by double sandwich ELISA with specific anti-human IL-8 Abs. Cells were stimulated with 1 μM PGE_2 (□), 1 μM PMA (●), 10 μM forskolin (■), 100 μM dcAMP (▼), or medium (◆) at various time periods. The data present the mean ± SD from four separate experiments.

consistently high up to 24 h. The onset of IL-8 mRNA expression was delayed in response to forskolin (10 μM) and dcAMP (100 μM), with peak expression occurring at 6 h. Interestingly, IL-8 protein was not detected before 12 h of incubation with PGE_2, forskolin, or dcAMP, whereas after 6 h, significant production of IL-8 was observed in response to PMA (1 μM). The effect of PGE_2 on IL-8 protein production was similar to those of forskolin and dcAMP. These results suggest that PGE_2 is able to elevate not only IL-8 mRNA expression but also IL-8 protein production in human colonic cells.

Studies done in the presence of the selective Cox-2 inhibitor: NS-398 (20 μM), and the nonspecific inhibitors for Cox-1 and 2: acetylsalicylic acid (200 μM) and naproxen (130 μM) (25), did not alter basal or PGE_2-stimulated IL-8 mRNA expression in T84 cells. These results clearly indicate that PGE_2 could not induce endogenous PGE_2 production and that T84 Cox-1 and Cox-2 enzyme activity did not contribute to the induction of IL-8 in response to exogenous PGE_2.

Receptor-coupling is involved in IL-8 mRNA induction in response to exogenous PGE_2. It is well known that PGE_2 functions through receptor-coupling events. Among the PGE_2 receptors (EP_1, EP_2, EP_3 and EP_4), EP_2 and EP_4 were shown to elevate intracellular cAMP, which leads to the activation of protein kinase A (13, 14). Accordingly, we determined whether PGE_2 receptor agonists could modulate IL-8 mRNA expression. As shown in Figure 2, PGE_2 caused a significant increase in IL-8 mRNA expression compared with that in unstimulated controls. In comparison, the PGE_2 metabolite, 13, 14 dihydro-15-keto PGE_2 had only a weak effect on IL-8 mRNA expression, demonstrating specificity for PGE_2. Iloprost, an EP_1 agonist; sulprostone,

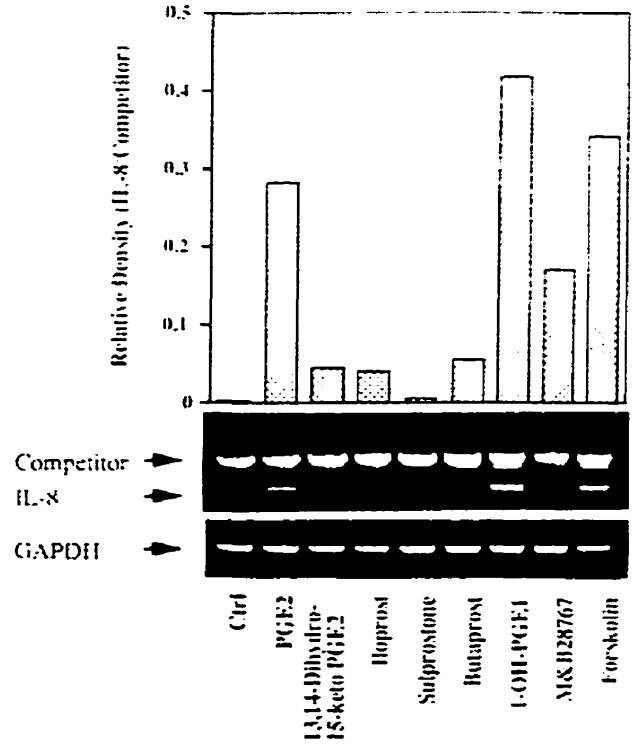

FIGURE 2. Effect of PGE_2 receptor agonists on IL-8 mRNA expression. T84 cells were incubated with medium, 1 μM PGE_2, 1 μM 13, 14-dihydro-15-keto PGE_2, 1 μM iloprost, 1 μM sulprostone, 1 μM butaprost, 1 μM 1-hydroxy-PGE_1, 1 μM M&B28767, and 10 μM forskolin. After 2-h incubation, total RNA was extracted for semi-quantitative RT-PCR to measure IL-8 mRNA levels. GAPDH was used as internal control. Lane designations are identical to the histograms using the National Institute of Health Image software. Similar results were obtained from three separate experiments.

an EP1/EP3 agonist; as well as butaprost, an EP_2 agonist, had no significant effect on IL-8 mRNA expression. In contrast, 1-hydroxy-PGE_1, which has been shown to have high affinity for EP_2 and EP_4 (26,27), and M&B28767, an EP_3/EP_4 agonist, caused a marked or moderate increase in IL-8 mRNA expression. T84 cells express 3 subtypes of PGE_2 receptors, EP_2, EP_3, and EP_4, as revealed by RT-PCR (our unpublished observation). As butaprost, an EP_2 agonist, and sulprostone, an EP_3 agonist, had no

significant effect on IL-8 mRNA expression, these results strongly suggest that PGE_2 coupling through EP_4 resulted in IL-8 gene expression.

PGE_2 elevates intracellular cAMP and triggers cAMP-dependent PKA signal transduction. Based on the results above, we then determined whether PGE_2 coupling through EP_4 receptors could elevate intracellular cAMP in T84 cells (Table I). PGE_2 and forskolin increased intracellular cAMP similar to those observed in previous studies (22, 28). However, in cells pretreated with the adenylate cyclase inhibitor, SQ22536 (100 μM), intracellular cAMP levels in response to PGE_2 or forskolin were markedly inhibited ($p< 0.05$).

Table I. *Intracellular cAMP levels in T84 cells stimulated with PGE_2 or forskolin*[a]

	cAMP (pmol/10^6 cells)	
	- SQ 22536	+ SQ 22536
Control	12.85 ± 3.13	16.28 ± 2.89
PGE_2	151.75 ± 17.53	107.68 ± 12.37 *
Forskolin	297.03 ± 9.78	198.70 ± 21.80 *

[a] Confluent T84 cells were pretreated with or without SQ22536 (100 μM) for 1 h and then stimulated with PGE_2 (1 μM), forskolin (10 μM) or medium. After 10 min incubation, cells were extracted with absolute ethanol and intracellular cAMP measured by EIA. Values are means ± SD of four samples. * $p < 0.05$ compared with homologous untreated controls.

To determine if cAMP-dependent signal tranduction was involved in IL-8 signaling, studies were performed in the presence of H89, a selective protein kinase A inhibitor. As shown in Table II, cells pretreated with H89 produced significantly ($p< 0.05$) less IL-8 protein following stimulation with PGE_2 or forskolin. In contrast, IL-8 protein production in response to PMA, a protein kinase C activator, was not inhibited. Depletion of protein kinase C in cells following treatment with 1 μM PMA for 24 h also did not affect IL-8 mRNA expression stimulated by PGE_2, forskolin or

Table II. *Effect of H89 on IL-8 protein production by T84 cells stimulated with PGE2, forskolin or PMA*[a]

	IL-8 protein (ng/ml)	
	- H89	+ H89
Control	0.144 ± 0.046	0.059 ± 0.031 *
PGE_2	0.394 ± 0.056	0.286 ± 0.003 *
Forskolin	0.821 ± 0.113	0.397 ± 0.078 *
PMA	7.815 ± 0.789	6.545 ± 1.194

[a] Confluent T84 cells were pretreated with or without H89 (10 μM) for 0.5 h and then stimulated with PGE_2 (1 μM), forskolin (10 μM), PMA (1 μM) or medium. After 24 h, the cell supernatant was collected from each sample and examined for IL-8 protein by ELISA. Values are means ± SD of four samples. * $p < 0.05$ compared with homologous untreated controls.

dcAMP, whereas PMA-induced IL-8 mRNA expression was inhibited (our unpublished observation). Taken together, these data clearly show that PGE_2 coupling through EP_4 receptors results in cAMP-dependent PKA signal transduction.

A posttranscriptional event is involved in the regulation of IL-8 gene expression in response to PGE_2. To determine whether IL-8 gene expression is transcriptionally or posttranscriptionally regulated by PGE_2, nuclear run-on assays were performed to examine the rate of IL-8 gene transcription. Unlike PMA which increased the transcriptional rate of IL-8 by two-fold, PGE_2 and forskolin had no effect on IL-8 gene transcription compared with that in the unstimulated controls (Figure 3). Based on these results, we then measured the stability of IL-8 mRNA in response to PGE_2, forskolin or PMA by RT-PCR in the presence of actinomycin D, a transcription inhibitor. As shown in Figure 4, PGE_2 (84% mRNA remaining after 10 h), forskolin (130%) and PMA (111%) delayed the degradation of IL-8 mRNA compared with that in the untreated controls (51%). These results were confirmed by Northern blot analysis. T84 cells were treated with cycloheximide to elevate IL-8 mRNA levels (9) and washed, and then the fate of IL-8 mRNA was measured following treatment with actinomycin D in the presence or absence of the stimuli. Regardless of the stimuli, IL-8 transcripts remained consistently higher than that in the untreated controls (our unpublished observations). Taken together, these data strongly suggest that the stimuli enhanced IL-8 gene expression by stabilizing IL-8 mRNA.

To determine whether IL-8 3' UTR was involved in postranscriptional regulation of the IL-8 gene, we examined the effect of PGE_2, forskolin, and PMA on CAT reporter gene expression in T84 cells transfected with pcDNACAT or pcDNACAT-UTR (Figure 5). CAT gene expression was dramatically reduced after 3' UTR was constructed in the 3' flank of CAT, clearly implicating the down-regulatory effect of IL-8 3'-UTR. Moreover, treatment with PGE_2 or forskolin increased CAT gene expression in cells transfected with pcDNACAT-UTR (157 % and 167%, respectively), while PGE_2 (95%)

had no effect on CAT activity in cells transfected with pcDNACAT, and forskolin (119%) slightly increased CAT activity in comparison with the control groups (100%). In response to PMA, CAT gene expression was markedly increased in cells transfected with either pcDNACAT-UTR (267%) or pcDNACAT (198%). Clearly, the increase in CAT activity in the cells transfected with pcDNACAT-UTR is much higher than that in the cells transfected with pcDNACAT in response to PGE_2, forskolin, or PMA. Taken together, these results suggest that PGE_2 caused the accumulation of IL-8 mRNA through

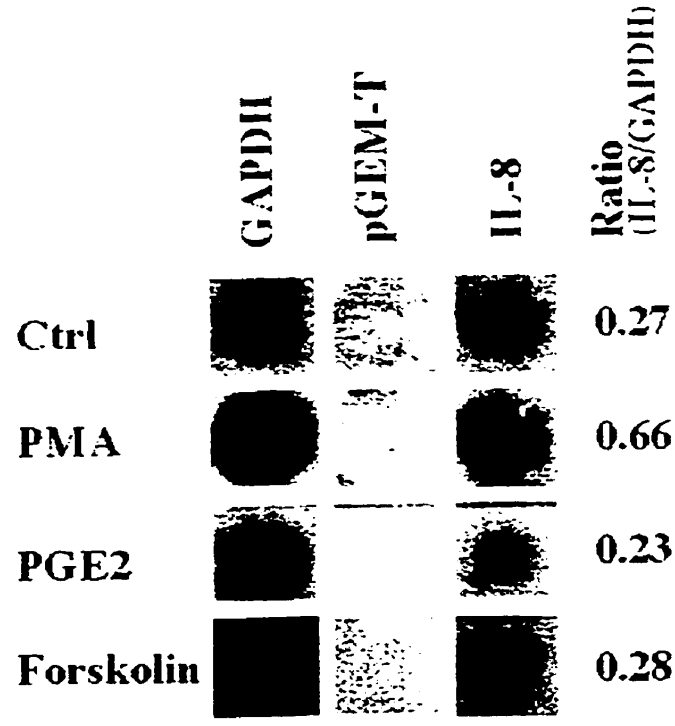

FIGURE 3. Transcriptional regulation is not involved in IL-8 gene expression in T84 cells stimulated with exogenous PGE_2. Confluent T84 cells (70%) cultured in 50-ml culture flask were incubated with 1 μM PMA, 1 μM PGE_2, or 10 μM forskolin for 2 h, and nuclei were purified from each sample. Nuclear run-on assay was performed to examine the transcriptional rate of the IL-8 gene. Similar results were obtained from two separate experiments.

posttranscriptional regulation, and that PGE_2-, forskolin- or PMA-responsive *cis*-element(s) in IL-8 3' UTR contributed to stabilizing the newly synthesized IL-8 mRNA.

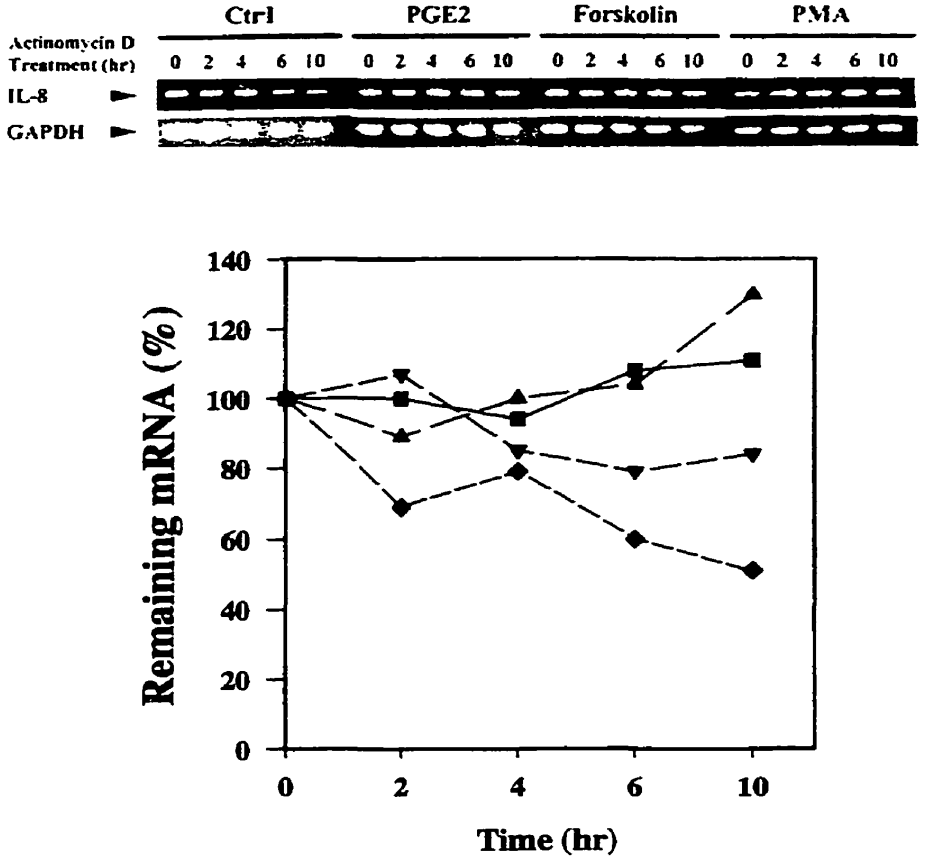

FIGURE 4. Stability of IL-8 mRNA in T84 cells in response to PGE_2, forskolin, or PMA stimulation. Cells were stimulated with 1 μM PGE_2 (▼), 10 μM forskolin (▲), 1 μM PMA (■), or medium (◆) for 2 h. Following the addition of 10 μg/ml actinomycin D total, RNA was extracted at various time periods, and RT-PCR performed. Densitometry of IL-8 and GAPDH was measured using National Institute of Health Image software, and the ratio of IL-8 and GAPDH represents IL-8 mRNA expression. IL-8 mRNA levels are expressed as a percentage of the mRNA levels determined before the addition of actinomycin D. One hundred precent of IL-8 mRNA represents the IL-8 mRNA level 2 h post-stimulation with PGE_2, forskolin, PMA, or medium only. The *top panel* shows IL-8 mRNA in agarose gel with ethedium bromide and the *lower panel* the densitomtry analysis from above. Similar results were obtained from two separate experiments.

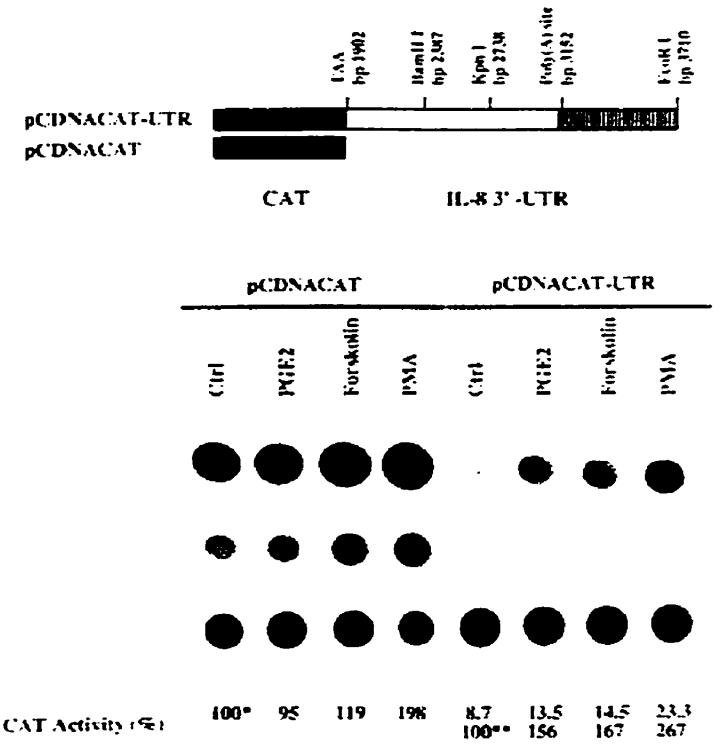

FIGURE 5. Effect of PGE_2 on reporter gene expression in T84 cells. Cells were transiently transfected with 3 μg of pcDNACAT or pcDNACAT-UTR, together with 3 μg of pRc/CMV-gal. PGE_2(1 μM), 10 μM forskolin, or 1 μM PMA was added into the cell culture 26 h after transfection. After 20-h incubation the cytoplasmic extract from each sample was assayed for CAT and ß-galactosidase activity. ß-Galactosidase activity was used to normalize the transfection effeciency. Similar results were obtained from three separate experiments. TAA, stop codon; Poly(A), polyadenylation. CAT activity from control cells (Ctrl) transfected with either pcDNACAT(*) or pcDNACAT-UTR(**) is shown.

Dexamethasone inhibits IL-8 gene expression by PGE_2, forskolin, and dcAMP, but not by PMA. Glucocorticoids suppress IL-8 gene expression in various cell lines in response to certain stimuli either by inhibiting IL-8 gene transcription or by decreasing mRNA stability at the posttranscriptional level (29). We have previously shown (9) that the accumulation of IL-8 mRNA in response to *E. histolytica* secretory components occurs by a posttranscriptional mechanism and is sensitive to Dex. However, Dex could not alter PMA-induced IL-8 mRNA expression, which is regulated both transcriptionally and

posttranscriptionally (9). To determine whether Dex could affect PGE_2-induced IL-8 expression, T84 cells were pretreated with Dex and then stimulated with PGE_2, PMA, forskolin, or dcAMP. After 24 h stimulation, the supernatant was collected and

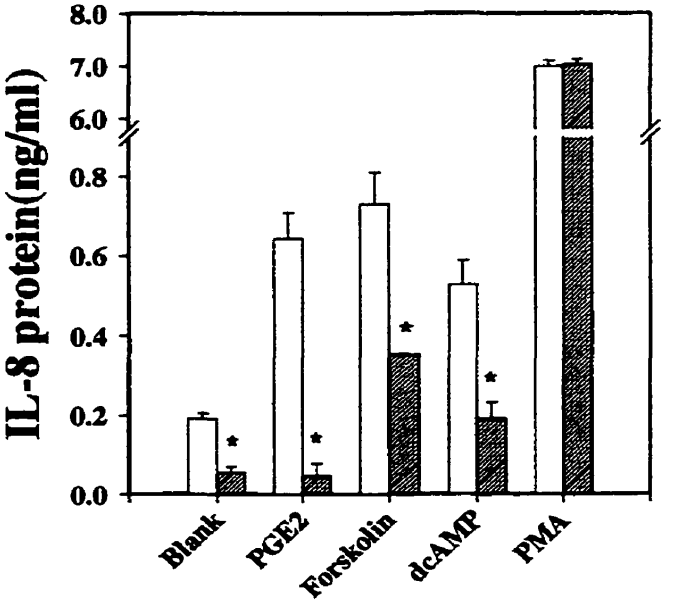

FIGURE 6. Effect of Dex on IL-8 protein production from T84 cells stimulated with PGE_2. Cells were pretreated with 1 μM Dex for 0.5 h (▨) and incubated with 1 μM PGE_2, 10 μM forskolin, 100 μM dcAMP, or 1 μM PMA for 24 h. The cell culture supernatantwas collected and quantified for IL-8 protein by ELISA. The data present the mean ± SD from four separate experiments. *, $p < 0.05$ compared with the untreated control group (□).

quantified for IL-8 protein (Figure 6). Dex pretreatment significantly inhibited IL-8 protein production in cells stimulated with PGE_2, forskolin, and dcAMP as well as in control unstimulated cells. However, Dex pretreatment had no effect on IL-8 production in response to PMA. These data suggest that Dex exerts an inhibitory action on PGE_2-induced IL-8 gene expression in T84 cells by destabilizing newly synthesized mRNA.

DISCUSSION

PGE_2 is a potent modulator of immune and inflammatory responses. While most studies implicate an immunosuppressive effect for PGs, recent studies suggest that under certain conditions PGs may up-regulate the expression of proinflammatory cytokines (15-17).

Our studies demonstrate that exogenous PGE_2 coupling through EP_4 receptors on T84 human colonic epithelial cells markedly enhanced the expression of IL-8 mRNA and stimulated the production of IL-8 protein. This effect was also observed in another human colonic epithelial cell line, LS174T, whereas, in human macrophages, PGE_2 inhibited basal expression of IL-8 mRNA (our unpublished observations). The ability of PGE_2 to stimulate IL-8 from mucosal epithelial cells may play a central role in pathogenesis and immunopathology of inflammatory bowel diseases. The accumulation of IL-8 mRNA in response to exogenous PGE_2 occurred in a dose- and time-dependent manner. Following PGE_2 stimulation IL-8 mRNA expression was observed as early as 1 h, with peak levels occurring after 2 h. Interestingly, IL-8 secretion was first noted after 12-h stimulation with PGE_2, forskolin, or dcAMP, whereas PMA induced IL-8 protein production occurred as early as 6 h. These results implies that different signal transduction pathways and regulatory mechanisms are involved in IL-8 gene expression and protein production. Even after 24 h stimulation with PGE_2, forskolin or dcAMP, the amount of IL-8 produced was seven-fold less than that from PMA stimulated cells. Endogenous PGE_2, derived for T84 cells, played no role in the expression of IL-8 mRNA, as Cox-1 and Cox-2 specific inhibitors had no effect on basal or PGE_2-simulated

IL-8 mRNA levels. These results suggest a role for exogenous PGE_2 in the up-regulation of IL-8 mRNA expression and protein secretion in human colonic epithelial cells.

Inflammation of the intestinal mucosa is characterized by infiltration of neutrophils, macrophages, lymphocytes, and plasma cells (30). Macrophages and neutrophils produce high output of PGE_2 and thus, provide the majority of the PGs in the inflamed gut. In animal models and human cases of intestinal inflammation, elevated levels of arachidonic acid metabolites have been detected. It is well documented that exogenous PGE_2 causes physiological changes in intestinal mucosal epithelial cells, such as secretion of water, electrolytes, and mucins in *in vivo* and *in vitro* models (21-23, 28). Inflammatory bowel disease is associated with the production of high levels of proinflammatory cytokines. IL-8 released from epithelial cells, macrophages, and mesenchymal cells are present in the tissues of the inflamed intestine (11). Proinflammatory cytokines, such as TNF-α and IL-1α/ß have the ability to regulate IL-8 gene expression in intestinal epithelial cells (11). However, it is not known if lipid mediators of inflammation can modulate IL-8 gene expression in mucosal epithelial cells. PGE_2 was shown to have no effect on IL-8 gene expression in neutrophils (18) and to exert a negative regulation on IL-8 production in human alveolar macrophages and blood monocytes (19). In this study, we clearly demonstrate that exogenous PGE_2 is a potent mediator in the regulation of IL-8 gene expression in human colonic epithelial cells. The consequence of overproduction of IL-8 by intestinal epithelial cells may lead to exacerbation of tissue injury or damage through the amplification of mucosal inflammation.

PGE_2 exerts its function on target cells through receptor-coupling events. Four

subtypes of PGE_2 receptors have been identified, namely EP_1, EP_2, EP_3 and EP_4 (13, 14). Among them, EP_2 and EP_4 have the capability of elevating intracellular cAMP (26, 27), which presumably triggers the cAMP-dependent PKA signal transduction cascade. Coupling through EP_1 increases intracellular Ca^{2+}, and coupling through EP_3 decreases intracellular cAMP (13, 14). The EP_1/EP_3 agonist, sulprostone, had no effect on IL-8 mRNA expression and the EP_2 selective agonist, butaprost, had only a week effect. In contrast, 1-hydroxy-PGE_1, an EP_2/EP_4 agonist, and M&B28767, an EP_3/EP_4 agonist, significantly stimulated the expression of IL-8 mRNA. Although the increase in IL-8 mRNA levels evoked by M&B28767 was not as potent as PGE_2, these results clearly demonstrate the involvement of EP_4 receptors. PGE_2 coupling through EP_4 receptors stimulated intracellular cAMP which was specifically inhibited by a selective adenylate cyclase inhibitor. This is consistent with previous studies (22, 28). Furthermore, a selective PKA inhibitor reduced IL-8 production in response to PGE_2, confirming the contribution of cAMP- dependent PKA signal transduction in this event. Taken together, these results unequivocally show that PGE_2 coupling through EP_4 receptors play a major role in the initiation of cAMP-dependent PKA signal transduction resulting in IL-8 gene expression in T84 cells.

Numerous studies have shown that the IL-8 gene can be regulated by various stimuli, such as LPS, cytokines, PMA, and agents that elevate intracellular cAMP or calcium in various cell types (1). Transcriptional regulation of IL-8 gene expression is well defined. A variety of transcription factors, such as NF-κB, NF-IL-6, activator protein-1, octomer-1 or CCAAT/Enhancer-binding protein, are responsible for IL-8 gene transcription regulation (31-36). However, there is limited information with regard to

posttranscriptional regulation of IL-8 gene expression. Previous studies have shown that IFN-γ can up-regulate IL-8 gene expression in human monocytic cells (U937) concomitant with the stabilization of mRNA levels (37). As well, IL-1ß caused a delay in the degradation of IL-8 mRNA in human diploid fibroblasts (38). Villarete et al. (39) reported that transcriptional and posttranscriptional regulation are involved in IL-8 gene expression in human blood cells stimulated with LPS. The IL-8 gene contains AU-rich sequences in its 3'UTR which may cause its mRNA to be more susceptible to degradation (40). We have previously shown that *E. histolytica* secretory components can stimulate IL-8 gene expression in T84 cells by a posttranscriptional mechanism (9). In the present study, nuclear run-on assays revealed that there are no differences in the rate of IL-8 gene transcription following stimulation with PGE_2 and forskolin and in the untreated controls, clearly implicating a posttranscriptional effect. This is in contrast to PMA stimulation which markedly increased the rate of IL-8 gene transcription. Intrestingly, PMA, PGE_2 and forskolin stabilized the degradation of IL-8 mRNA as compared to those in the untreated groups. This suggests that these stimuli can regulate the expression of IL-8 mRNA at the posttranscriptional level. The presence of PGE_2-responsive *cis*-element (s) in IL-8 3' UTR was clearly shown by the ability of PGE_2 to elevate CAT gene expression in T84 cells transfected with CAT reporter gene and IL-8 3' UTR, but not with the CAT reporter gene only. Based on these results it is clear that the accumulation of IL-8 mRNA elicited by exogenous PGE_2 is caused by a delay in the degradation of newly synthesized IL-8 mRNA.

Overproduction of IL-8 results in neutrophil-dependent tissue injury (41). IL-8 is considered one of the primary targets for the treatment of intestinal inflammation (11). Thus, suppression of IL-8 production could be beneficial for the control of inflammation.

Glucocorticoids are the most effective inhibitors of IL-8 gene expression in several types of cells (1, 42). Dex, a synthetic glucocorticoid, has an inhibitory effect on IL-8 gene expression through transcriptional regulation or by destabilizing mRNA levels (29). However, it had no effect on IL-8 gene expression in peripheral blood monocytes in response to PMA, fibroblasts stimulated with leukoregulin, or airway epithelial cells stimulated with elastase (43-45). Our results show that Dex can inhibit IL-8 protein production in cells stimulated with PGE_2 and forskolin, but had no effect on PMA-induced IL-8 production. Therefore, the signal transduction pathways initiated by PGE_2 and forskolin (PKA pathway) and PMA (PKC pathway) are independently regulated by Dex. Dex has an inhibitory effect on IL-8 gene expression in T84 cells stimulated by PGE_2 through a posttranscription mechanism that is cAMP-dependent (PKA pathway); however, no effect was observed on PKC-dependent signaling with PMA which resulted in both transcriptional and posttranscriptional regulation of IL-8.

In conclusion, our findings unravel a novel role for exogenous PGE_2 in up-regulating IL-8 gene expression and protein production by human colonic epithelial cells. PGE_2 coupling through EP_4 receptors triggered cAMP-dependent PKA signal transduction cascade for posttranscriptional regulation of IL-8 gene expression. This study highlights a potential mechanism explaining how mucosal epithelial cells exacerbate intestinal inflammation in the inflamed intestine via production of IL-8 in response to exogenous PGE_2.

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CONNECTING STATEMENT II

Although PGE_2 has regulatory effects on IL-8 gene expression in various cell types evoked by proinflammatory cytokines or LPS, this is the first report (Manuscript II) to demonstrate that PGE_2 can enhance IL-8 gene expression in human colonic epithelial cells. Thus, PGE_2 can initiate inflammation in amebiasis or exacerbate inflammatory responses in IBD by stimulating IL-8. Interestingly, activation of the PKC pathway by PMA enhanced IL-8 gene expression through both transcriptional and posttranscriptional events whereas, activation of the PKA pathway by PGE_2 or forskolin caused posttranscriptional regulation of the IL-8 gene. At present, it is not known what role intracellular Ca^{2+} plays in IL-8 regulation induced by protein kinases or whether it has distinct signaling effects. In the next study, we investigate the mechanisms involved in Ca^{2+}-dependent IL-8 gene expression in human colonic epithelial cells.

MANUSCRIPT III

Calcium-dependent interleukin-8 gene expression in T84 human colonic epithelial cells*

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ABSTRACT

Background/Aims: IL-8 is a chemokine which activates and recruits neutrophils and plays a major role in intestinal inflammation. Signal transduction pathways mediated by protein kinases are central in regulating IL-8 gene expression, however, little is known about the role of Ca^{2+} in this event. Herein, we characterized the effect of intracellular Ca^{2+} on interleukin-8 gene expression in T84 human colonic epithelial cells. *Methods*: Cells were stimulated with Ca^{2+} ionophore, A23187 or thapsigargin, a Ca^{2+}-ATPase inhibitor. Semi-quantitative RT-PCR was used to examine IL-8 mRNA and ELISA for protein quantification. Reporter gene techniques were used to determine transcription rate. *Results*: A23187 and thapsigargin caused a dose- and time-dependent accumulation of IL-8 mRNA and protein production which was dependent on the release of Ca^{2+} from intracellular stores. FK506, a specific inhibitor of calcineurin, inhibited A23187- and thapsigargin-induced IL-8 mRNA expression in a dose-dependent manner. Reporter gene studies and actinomycin D chase experiments showed that A23187 and thapsigargin enhanced IL-8 gene transcription and stabilized IL-8 mRNA transcripts, respectively. Newly synthesized proteins were not necessary for this event and cycloheximide caused superinduction of IL-8 mRNA. *Conclusions*: Intracellular Ca^{2+} plays an important role in regulating IL-8 transcriptionally and posttranscriptionally through calcium/calmodulin-dependent calcineurin.

INTRODUCTION

Interleukin-8 is a chemokine which belongs to the C-X-C superfamily. It is produced by various cell types including monocytes, macrophages, T-cells, epithelial cells, endothelial cells, hepatic cells, gastric and intestinal carcinoma cells, and neutrophils; and is chemotactic for neutrophils, T-lymphocytes, basophils, and eosinophils (1, 2). IL-8 activates neutrophils through its two high affinity receptors, CXCR1 and CXCR2, which leads to chemotaxis and activation of neutrophils characterized by neutrophil shape change, exocytosis, up-regulation of specific receptors and adhesion molecules, formation of bioactive lipids, and respiratory burst to release oxygen intermediates (1). Intestinal epithelial cells produce large amounts of IL-8 and are considered the first line of defense through bidirectional communication between host and infectious pathogens (3, 4). IL-8 mediates physiological responses in normal and disease stages (5-7). Mucosal epithelial cell-derived IL-8 plays a major role in inflammation in the respiratory and gastrointestinal tracts in bacterial and parasitic infections (8-12) and in inflammatory bowel diseases (13, 14).

IL-8 gene is regulated transcriptionally (15-18), posttranscriptionally (19-22), and translationally (23). Different signal transduction pathways, such as protein kinase C (PKC), protein kinase A (PKA), protein tyrosine kinase, phosphatidylcholine-specific phospholipase, or mitogen-activated protein kinase (22-29), can regulate IL-8 gene expression in various cell types. Ca^{2+} ionophores (A23187 or ionomycin) and thapsigargin, a Ca^{2+} ATPase inhibitor, was shown to enhance IL-8 gene expression in monocytes, neutrophils, and T-cells through an increase in intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) (23, 30-32). Calmodulin (CaM) is involved in Ca^{2+}-dependent signal transduction pathway mediating many cellular responses (33, 34). Under the control of

Ca^{2+}/calmodulin, a group of CaM-dependent kinases (e.g., CaM kinases I, II, and IV) and protein phosphatase 2B (calcineurin) can be activated (33-35). CaM-dependent calcineurin regulates IL-8 gene expression in human neutrophils and Jurkat cells by elevating $[Ca^{2+}]_i$(31, 32).

We have previously reported that IL-8 gene expression in T84 human colonic epithelial cells is up-regulated in response to either PKC or PKA activators (12, 22). However, it is not known what role intracellular Ca^{2+} plays in IL-8 gene expression, and this is the focus of the present study. Herein, we demonstrate that A23187 or thapsigargin stimulates IL-8 mRNA expression and protein production in T84 cells following the release of Ca^{2+} from intracellular Ca^{2+} stores. Furthermore, A23187 and thapsigargin regulated IL-8 gene expression transcriptionally and posttranscriptionally which was mediated by CaM-dependent calcineurin.

MATERIALS AND METHODS

Reagents

A23187, thapsigargin, ionomycin, 1,2-*bis*(o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM), Ethyleneglycol-*bis*(ß-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), and FK506 were purchased from Calbiochem (San Diego, CA). Forskolin, phorbol 12-myristate 13-acetate (PMA), cycloheximide, actinomycin D, and DMEM/F12 base medium were purchased from Sigma Chemical Co. (St. Louis, MO). Dialyzed fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT) and Lipofectamine reagent was purchased from GIBCO BRL Life Technologies (Burlington, Canada). 2-Nitrophenyl-ß-D-

galactopyranoside (ONPG) was purchased from Boehringer Mannheim Canada (Laval, Canada). [^{14}C] Chloramphenicol and acetyl coenzyme A were purchased from Amersham Pharmacia Biotech Inc (Baie d'Urfé, Canada). pcDNA3 and pRc/CMV plasmids were purchased from Promega Corporation (Madison, WI). Plasmid pUC18 containing an IL-8 fragment (bp -1841 to +44 in genomic DNA sequence) was a gift from Dr. N. Mukaida (Kanazawa, Japan). pBLCAT-6 was purchased from the American Type Culture Collection (ATCC, Rockville, MD).

Cell culture

The human colonic adenocarcinoma cells, T84, were obtained from the ATCC (Rockville, MD) and grown in 24-well plates in 1 ml of a 1:1 mixture of Dulbecco's modified Eagles medium (DMEM) with 4.5 g of D-glucose/litre and Ham's F12 nutrient mixture (GIBCO) containing 10 % fetal bovine serum (Hyclone Laboratories), 100 U/ml of Penicillin, 100 µg/ml of Streptomycin sulphate and 20 mM HEPES (Sigma). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. To examine the involvement of extracellular Ca^{2+}, cells were incubated with low Ca^{2+} medium (LCM) containing DMEM/F12 base medium supplemented with L-Glutamine, L-Leucine, L-Lysine, L-Methionine, $MgCl_2.6H_2O$, and $MgSO_4$, low Ca^{2+} medium supplemented with 1.05 mM Ca^{2+} (LCMCa), or normal DMEM/F12 medium (NDM) with 1.05 mM Ca^{2+}. Ten percent dialyzed BSA (about 0.0014 mM Ca^{2+}) was used to substitute regular fetal bovine serum.

RNA preparation and semi-quantitative RT-PCR

Total RNA (1µg) was isolated from T84 cells with TRIzol reagent (GIBCO) and semi-quantitative RT-PCR was performed as previously described (22). A consistent concentration of competitor (644 bp, 0.34 ng) in pGEM-T plasmid was used to compete

the target IL-8 fragment (284 bp). The house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Plasmid Construction, transfection, and CAT assay

A IL-8 fragment (bp -1841 to +44) was amplified from plasmid pUC18 containing IL-8 (bp -1841 to +44 in genomic DNA sequence) (15) using a pair of primers: *aagctt*AGGTTGGTTGGAGAAAG(sense) and AAGCTTGGATCCTGC-TCCGGTGGCTTTTTATATC(antisense) and ligated into *Hind* III site in pBLCAT-6 plasmid to generate pBLCAT-5UTR. T84 cells were plated in 60 mm dishes in complete medium. After 24 hours, 3 μg of pBLCAT-5UTR construct, together with 3 μg of pRc/CMV plasmid with the ß-galactosidase gene in *Not* I site (pRc/CMV-gal) to correct the difference in the transfection efficiency, were transfected into cells using Lipofectamine reagent. After 24-hour incubation, various stimuli were added, and the cytoplasmic extract from each sample was then assayed for ß-galactosidase and CAT activity after 16-hour stimulation as described previously (22). The conversion of [^{14}C] chloramphenicol to the acetylated form was quantified using NIH Program 1.6.

Measurement of IL-8 protein

IL-8 protein was measured by an enzyme-linked immunosorbent assay (ELISA) as described previously (22).

Statistical analysis

The data were calculated as mean ± standard error of the mean (SEM). The results were analyzed by Student' *t*-test. A value of $p<0.05$ was considered statistically significant.

RESULTS

Dose- and time-dependence of IL-8 mRNA expression in response to A23187 and thapsigargin.

We have previously shown that IL-8 gene expression in T84 cells is up-regulated in response to the PKC activator, PMA, and the PKA activator, forskolin. Activation of PKC regulated IL-8 gene expression transcriptionally and posttranscriptionally; whereas, activation of PKA stabilized IL-8 mRNA (12, 22). To determine whether an increase in $[Ca^{2+}]_i$ can up-regulate the IL-8 gene, we examine the effect of two Ca^{2+} elevating agents on IL-8 mRNA expression and protein production in T84 cells. Ca^{2+} ionophore A23187 releases Ca^{2+} from intracellular stores (36) and thapsigargin, an irreversible Ca^{2+}-ATPase inhibitor, empties Ca^{2+} from the endoplasmic reticulum (37). Cells were stimulated with various concentrations of A23187 or thapsigargin for 2 hours and IL-8 mRNA levels measured by semi-quantitative RT-PCR. As shown in Figure 1*A*, both A23187 and thapsigargin enhanced IL-8 mRNA expression in a dose-dependent manner. Peak IL-8 mRNA accumulation occurred with 10 μM A23187 (0.01-100 μM) while thapsigargin increased IL-8 mRNA accumulation with increasing concentrations (0.01-100 μM). DMSO, the solvent used for A23187 and thapsigargin, had no effect on IL-8 mRNA expression (data not shown). Figures 1*B* and 2 show the time-dependent kinetic analysis of IL-8 mRNA expression and protein production. Notable accumulation of IL-8 mRNA levels were observed 0.5 hour after treatment with A23187 or thapsigargin, with peak levels after 2 hours, and remained consistently high up to 24 hours (Figure 1*B*). Significant IL-8 protein production was detected after 4 hour stimulation and increased steadily up to 24 hours (2.42±0.68 ng/ml for A23187 and 1.47±0.26 ng/ml for

thapsigargin as compared to 0.157±0.037 ng/ml for the control group after 24 hour incubation) (Figure 2). These data indicate that both A23187 and thapsigargin can up-regulate IL-8 mRNA expression and protein production in T84 cells.

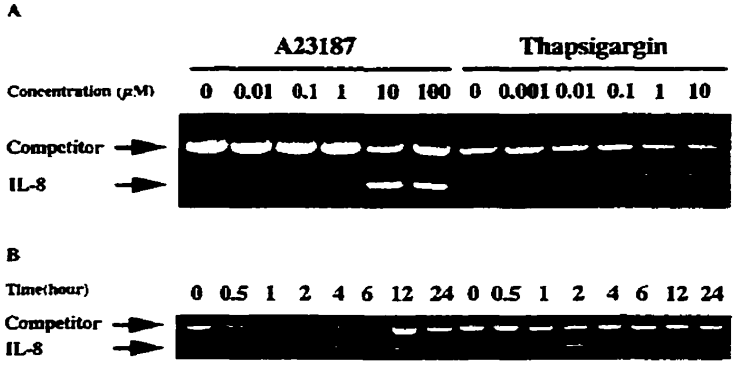

Figure 1. Dose- and time-dependent accumulation of IL-8 mRNA. IL-8 mRNA levels were examined by semi-quantitative RT-PCR in T84 cells after 2 hour stimulation with *A*) various concentration of A23187 (0.01-100 μM) or thapsigargin (0.01-10 μM) and *B*) with 10 μM A23187 or 1 μM thapsigargin at different time periods. Similar results in *A* and *B* were obtained from two separate experiments.

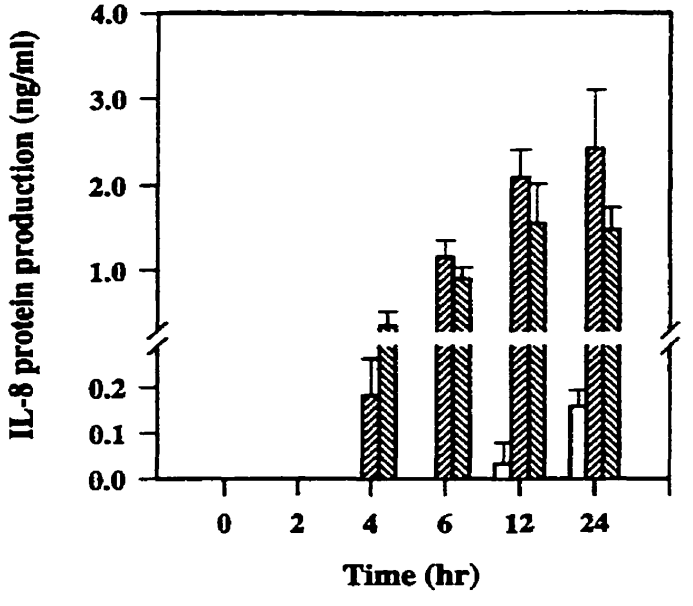

Figure 2. Time-dependent IL-8 protein production. IL-8 protein production was measured in the supernatants of T84 cells by double sandwich ELISA with specific anti-human IL-8 Abs. Cells were stimulated with 10 μM A23187 (▨), 1 μM thapsigargin (▧), or medium (□) at various time periods. The data represent the mean ± SEM from four separate experiments.

A23187- and thapsigargin-induced IL-8 mRNA expression is dependent on elevation of $[Ca^{2+}]_i$ by the release of Ca^{2+} from intracellular stores

A recent study (31) has shown that thapsigargin-induced IL-8 gene expression in human neutrophils is dependent on an influx of extracellular Ca^{2+} since chelation of extracellular Ca^{2+} by EGTA diminished the effect of thapsigargin on IL-8 protein production. To determine whether the induction of IL-8 in T84 cells induced by A23187 or thapsigargin is dependent on an increase in $[Ca^{2+}]_i$ through the entry of extracellular Ca^{2+} or the release of Ca^{2+} from intracellular stores, cells were pretreated with EGTA or BAPTA/AM and then stimulated with A23187 or thapsigargin for 2 hours. Under the same conditions cells were also stimulated with PMA, forskolin, and another Ca^{2+} ionophore, ionomycin for comparison. As shown in Figure 3*A*, addition of EGTA which chelates extracellular Ca^{2+}, surprisingly caused an elevation of basal IL-8 mRNA levels, resulting in an additive increase in IL-8 mRNA expression in response to A23187, thapsigargin, ionomycin, and PMA. However, there was no additive effect on IL-8 mRNA expression induced by forskolin in the presence of EGTA. The addition of cell-permeable BAPTA/AM which chelates intracellular Ca^{2+}, reduced IL-8 mRNA levels in response to all the stimuli (Figure 3*A*). Furthermore, experiments done in the presence of low Ca^{2+} medium (LCM) containing about 0.0014 mM Ca^{2+}, had no significant effect on IL-8 mRNA expression induced by A23187, thapsigargin, ionomycin or forskolin, but slightly inhibited the effect of PMA in comparsion to low Ca^{2+} medium supplemented with 1.05 mM Ca^{2+} (LCMCa), or normal DMEM/F12 medium containing 1.05 mM Ca^{2+} (Figure 3*B*). Cells treated with verapamil, a Ca^{2+}-channel blocker, had no effect on IL-8

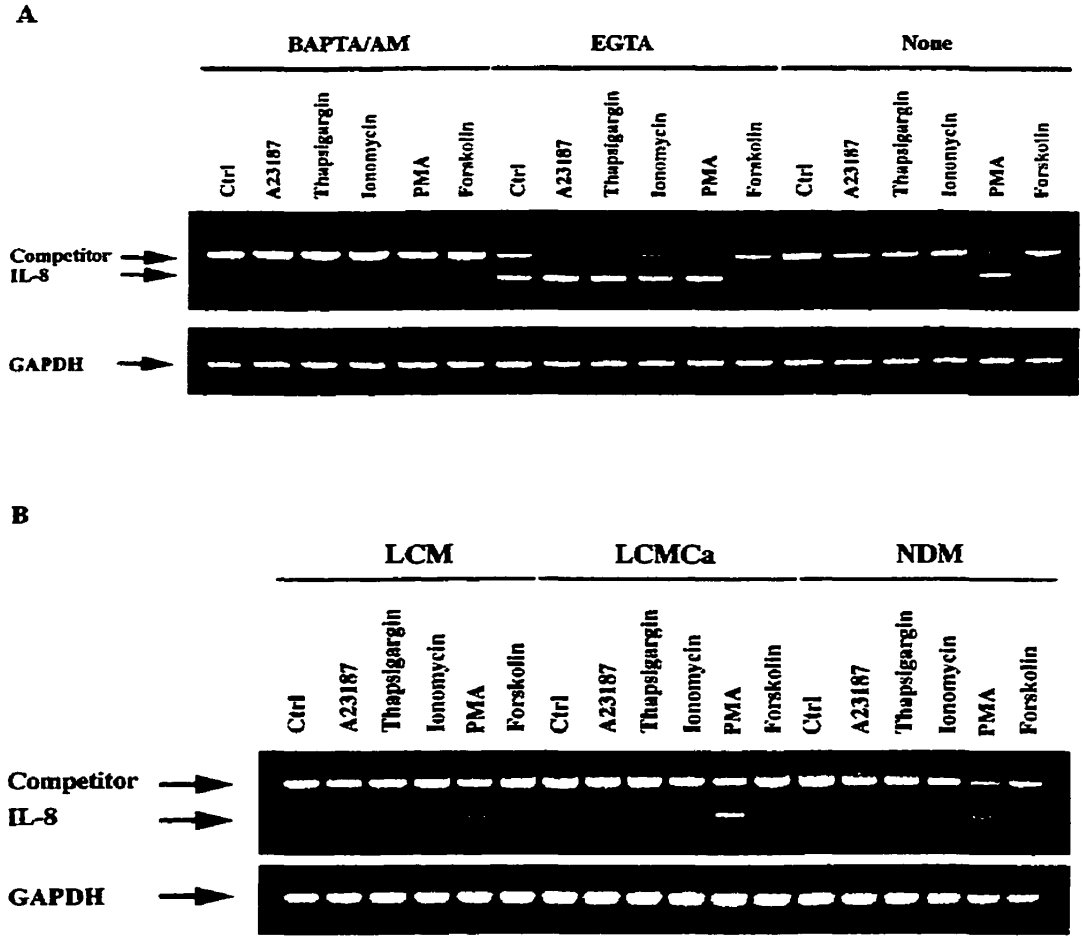

Figure 3. A23187- or thapsigargin-induced IL-8 mRNA expression is dependent on the increase in $[Ca^{2+}]_i$ by the release of Ca^{2+} from intracellular stores. *A)* T84 cells were pretreated with 2.5 mM EGTA or 50 μM BAPTA/AM, or without Ca^{2+} chelators for 15 min and then stimulated with 10 μM A23187, 1 μM thapsigargin, 10 μM ionomycin, 1 μM PMA, or 10 μM forskolin for 2 hours. IL-8 mRNA levels were measured by semi-quantitative RT-PCR. GAPDH is used as an internal control. *B)* T84 cells were washed with DMEM/F12 base medium twice and cultured with either low Ca^{2+} medium (LCM), LCM plus 1.05 mM Ca^{2+} (LCMCa), or normal DMEM/F12 medium with 1.05m mM Ca^{2+} (NDM) in the presence of 10 μM A23187, 1 μM thapsigargin, 10 μM ionomycin, 1μM PMA, or 10 μM forskolin for 2 hours (see **Materials and Methods**). Dialyzed fetal bovine serum (0.0014 mM Ca^{2+}) was used to substitute regular fetal bovine serum. Similar results were obtained from three separate experiments.

mRNA expression evoked by A23187 or thapsigargin (data not shown). Taken together, these results clearly demonstrate that the induction of IL-8 mRNA in response to A23187

and thapsigargin is dependent on elevation of $[Ca^{2+}]_i$ by the release of Ca^{2+} from intracellular stores.

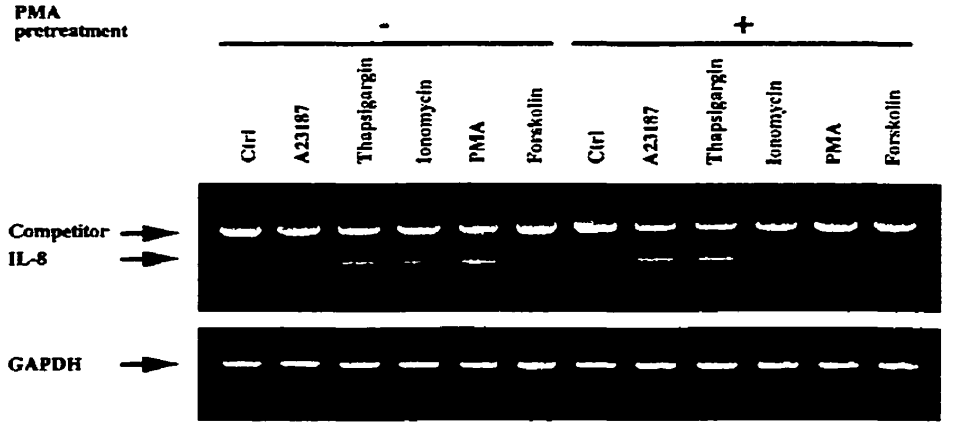

Figure 4. PMA-sensitive PKC is not involved in IL-8 mRNA expression in response to either A23187 or thapsigargin. T84 cells were pretreated with or without 10 μM PMA for 24 hours and then stimulated with 10 μM A23187, 1 μM thapsigargin, 10 μM ionomycin, 1 μM PMA, or 10 μM forskolin for 2 hours. Total RNA was extracted and IL-8 mRNA levels were measured by semi-quantitative RT-PCR. GAPDH was used as an internal control. Similar results were obtained from three separate experiments.

A23187- and thapsigargin-induced IL-8 mRNA expression is independent of PMA-sensitive PKC.

Elevation of $[Ca^{2+}]_i$ can activate PKC (38). We have previously shown that PMA activates PKC signalling which led to an up-regulation of IL-8 gene expression in T84 cells (12, 22). In AGS gastric epithelial cells, elevation of $[Ca^{2+}]_i$ by A23187 increased IL-8 gene expression through the activation of PKC (28). To determine whether the up-regulation of IL-8 mRNA by A23187 or thapsigargin is PKC-dependent, cells were pretreated with or without PMA for 24 hours to deplete PKC (22), and then stimulated for 2 hours with A23187, thapsigargin, ionomycin, PMA, or forskolin. As indicated in Figure 4, depletion of PMA-sensitive PKC impaired PMA-induced IL-8 mRNA expression but had no attenuation at A23187-, thapsigargin-, ionomycin- or forskolin-

induced IL-8 mRNA expression. As an internal control, GAPDH mRNA expression was not altered. These results clearly indicate that elevation of intracellular Ca^{2+} by A23187 or thapsigargin is not dependent on PMA-sensitive PKC signalling for IL-8 gene expression.

Effect of cycloheximide on A23187- and thapsigargin-induced IL-8 mRNA expression

To determine whether IL-8 gene expression in T84 cells required *de novo* protein synthesis, cells were stimulated with A23187, thapsigargin, ionomycin, PMA, or forskolin in the presence or the absence of cycloheximide (CHX), a protein synthesis inhibitor. As depicted in Figure 5, regardless of the stimuli, CHX treatment resulted in superinduction of IL-8 mRNA. Basal IL-8 mRNA levels were also up-regulated in CHX control unstimulated cells. CHX was specific for IL-8 mRNA induction as the expression of the GAPDH gene remained unaltered. These results suggest that *de novo* protein synthesis is not necessary for the effect of A23187 and thapsigargin.

A23187- and thapsigargin-induced IL-8 gene expression is calcineurin-dependent.

Increase levels of $[Ca^{2+}]_i$ can activate CaM-dependent calcineurin (35). Previous studies (32) have shown that in Jurkat cells, FK506, a calcineurin inhibitor, abolished PMA plus thapsigargin-stimulated IL-8 gene expression. Accordingly, we determined whether IL-8 mRNA expression induced by A23187 or thapsigargin in T84 cells was sensitive to FK506. As shown in Figure 6, treatment of FK506 inhibited both A23187- and thapsigargin- induced IL-8 mRNA expression in a dose-dependent manner but had no effect on IL-8 mRNA induced by PMA. Under the same conditions, GAPDH mRNA expression was not altered by FK506. These data indicate that A23187 and thapsigargin-induced IL-8 gene expression in T84 cells is dependent on CaM-dependent calcineurin.

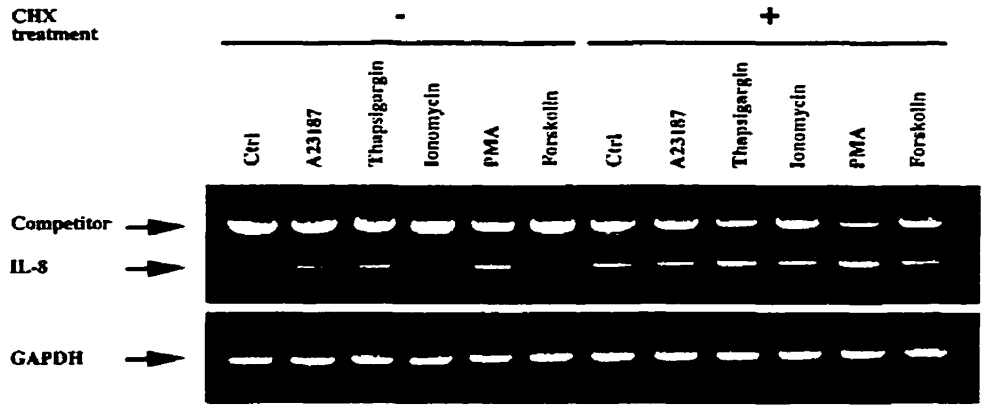

Figure 5. Effect of cycloheximide (CHX) on expression of IL-8 in T84 cells in response to A23187 or thapsigargin. T84 cells were incubated with 10 μM A23187, 1 μM thapsigargin, 10 μM ionomycin, 1 μM PMA, or 10 μM forskolin in the presence or absence of cycloheximide (10 μg/ml) for 2 hours. Total RNA was extracted for semi-quantitative RT-PCR to examine the levels of IL-8 mRNA. GAPDH gene was used as an internal control. Similar results were observed from three separate experiments.

A23187 and thapsigargin induced IL-8 gene transcription and stabilized IL-8 mRNA

To determine whether A23187 and thapsigargin can enhance IL-8 gene transcription, we cloned the IL-8 5' UTR (bp -1841 to +44) into the 5' flanking end of CAT reporter gene in pBLCAT-6 and transiently transfected the construct into T84 cells. After 24-hour incubation, cells were stimulated with the agonists and CAT activity measured after 16-hour incubation. As shown in Table 1, stimulation with A23187 and thapsigargin caused 177% and 165% increased in CAT reporter gene transcription, respectively, and was similar to the positive control, PMA (147%). These results clearly show that elevation of $[Ca^{2+}]_i$ by A23187 and thapsigargin can enhance the transcription rate of the IL-8 gene.

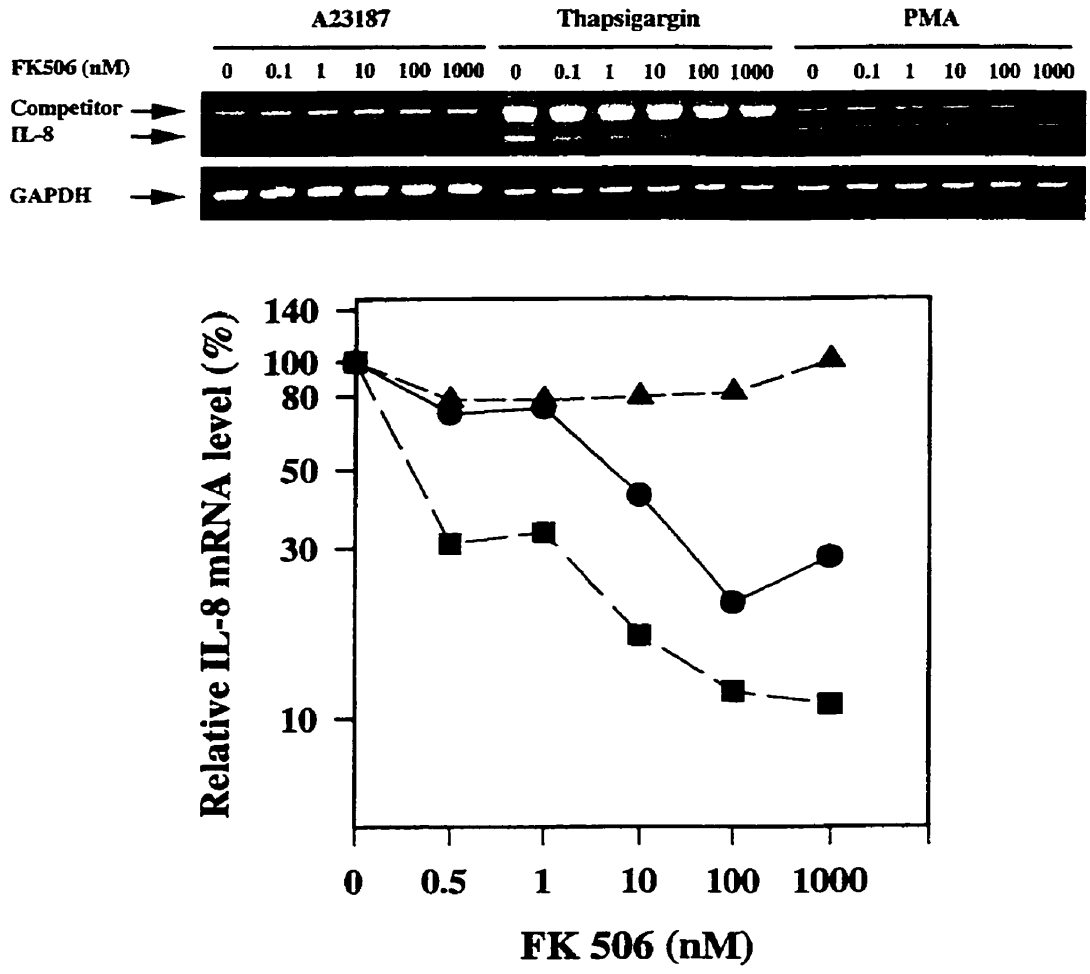

Figure 6. Calcineurin is involved in IL-8 gene expression in T84 cells in response to either A23187 or thapsigargin. T84 cells were stimulated with 10 μM A23187 (●), 1 μM thapsigargin (■), or 1 μM PMA (▲) in the presence various concentrations of FK506 (0-1 μM) for 2 hours. Total RNA was extracted and IL-8 mRNA was measured by semi-quantitative RT-PCR. Densitometric units of IL-8 and GAPDH was measured using the NIH Image Program 1.6, and the ratio of IL-8 and GAPDH represents IL-8 mRNA expression. One hundred precent of IL-8 mRNA represents the IL-8 mRNA level 2 hour post-stimulation with A23187, thapsigargin, or PMA in the absence of FK506. The *top panel* shows IL-8 mRNA in agarose gel with ethedium bromide and the *lower panel* the densitomtric analysis from above. Simlar results were observed from three separate experiments.

We have previously shown that PMA and forskolin can stabilize IL-8 mRNA (12, 22), and it was of interest, therefore, to determine whether A23187 or thapsigargin can

regulate IL-8 at the posttranscriptional level. Cells were stimulated with the agonists for 2 hours and then treated with actinomycin D (ACT D), a transcription inhibitor. As shown in Figure 7, in the control group IL-8 mRNA degraded rapidly following treatment with ACT D and was reduced by about 67% within 2 hours. In contrast, both A23187 and thapsigargin impaired IL-8 mRNA degradation by reduction of only 20% and 10%, respectively, at the same time period. Taken together, these results indicate that both transcriptional and porttranscriptional events are involved in IL-8 gene expression in T84 cells in response to A23187 and thapsigargin.

Table 1. Effect of A23187 and thapsigargin on IL-8 5'-UTR-driven CAT reporter gene expression

Stimuli	Relative CAT Activity (%)
Control	100
A23187	177±30 *
Thapsigargin	165±16 *
PMA	147±10 *

T84 cells were transiently transfected with 3 μg of pBLCAT-5UTR, together with 3 μg of pRc/CMV plasmid with ß-galactosidase gene to correct the difference in the transfection efficiency. After 24 hours, tranfected cells were stimulated with 10 μM A23187, 1 μM thapsigargin, or 1 μM PMA for another 16 hours. The cytoplasmic extract from each sample was assayed for ß-galactosidase and CAT activity after another 16 hour incubation. The conversion of [^{14}C] chloramphenicol to the acetylated form was quantified using NIH Image Program 1.6. Results are shown as relative CAT activity of pBLCAT-5UTR without stimulation (control) defined as 100%. Each value represent the mean ± SD. of three separate experiments. * A value of $p<0.05$ was considered statistically significant.

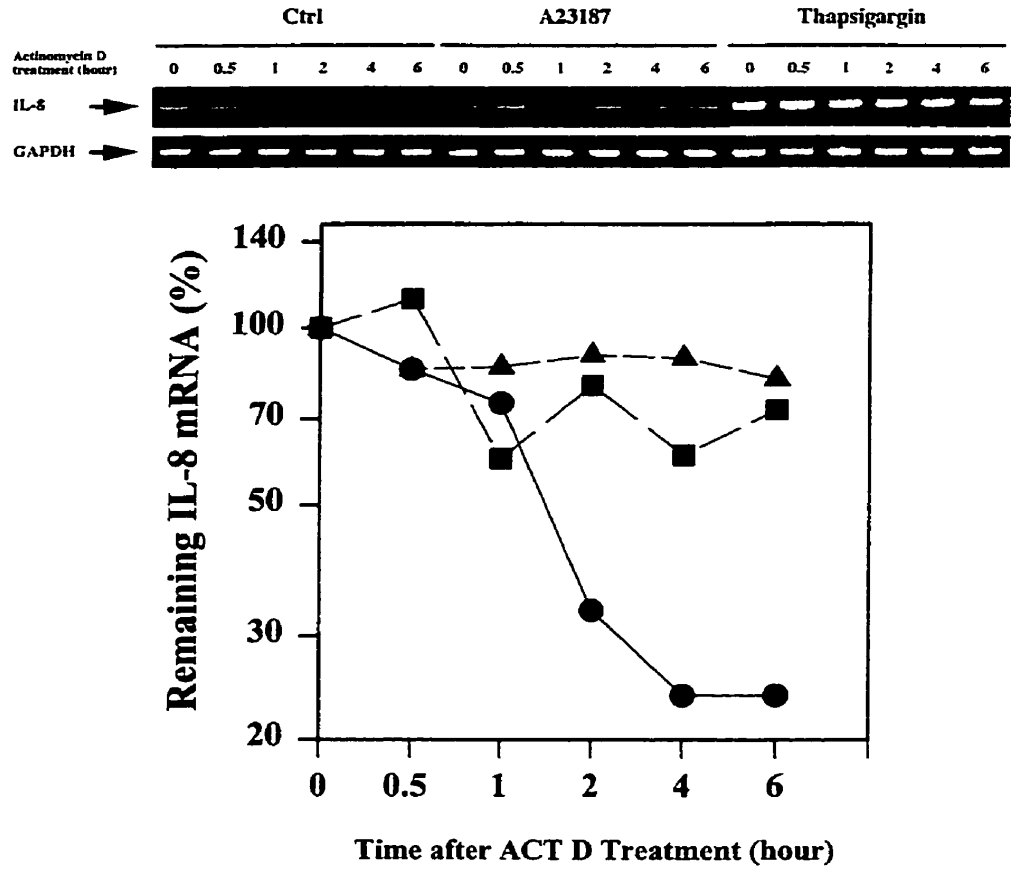

Figure 7. Stability of IL-8 mRNA in T84 cells in response to A23187 or thapsigargin stimulation. Cells were stimulated with 10 μM A23187 (■), 1 μM thapsigargin (▲), or medium (●) for 2 hours. Following the addition of 10 μg/ml actinomycin D, total RNA was extracted at various time periods and semi-quantitative RT-PCR performed. Densitometric units of IL-8 and GAPDH were measured using NIH Image Program 1.6, and the ratio of IL-8 and GAPDH represents IL-8 mRNA expression. IL-8 mRNA levels are expressed as a percentage of the mRNA levels determined before the addition of actinomycin D. One hundred percent of IL-8 mRNA represents the IL-8 mRNA level 2 hour post-stimulation with A23187, thapsigargin, ionomycin or medium only. The *top panel* shows IL-8 mRNA in agarose gel with ethidium bromide and the *lower panel* the densitometric analysis from above. Similar results were obtained from three separate experiments.

DISCUSSION

IL-8 is a potent chemokine which activates and recruits neutrophils into local sites

resulting in inflammation (1, 2). However, the signal transduction pathways that regulate IL-8 gene expression are not well understood. We have previously shown that activation of either PKC or PKA signal transduction pathways resulted in IL-8 gene expression in T84 cells (22). In the present study, we provide evidence that IL-8 expression in T84 cells is also regulated by Ca^{2+} ionophore A23187 or thapsigargin. In particular, we demonstrated that A23187 and thapsigargin elevated $[Ca^{2+}]_i$ which stimulated IL-8 mRNA accumulation and protein production. The accumulation of IL-8 mRNA occurred in a dose- and time-dependent manner and protein secretion was significantly increased as early as 4 hours. Other studies have shown that elevation of $[Ca^{2+}]_i$ can evoke mucin (5, 39) and electrolyte secretion (such as Cl^-) (6, 40) in human colonic epithelial cell lines (TH29, T84). Thus, Ca^{2+}-dependent signal transduction in intestinal epithelial cells is not only involved in the regulation of normal physiological functions but also in the development of mucosal inflammation by stimulating IL-8 (this study).

A23187 and thapsigargin elevated $[Ca^{2+}]_i$ through the release of Ca^{2+} from intracellular stores (36, 37). In other studies (31, 41, 42) it was shown that thapsigargin regulated plasma membrane Ca^{2+} permeability which resulted in an influx of extracellular Ca^{2+} in human neutrophils and rat lymphocytes. In our study, treatment with EGTA which chelates extracellular Ca^{2+}, drastically increased the basal levels of IL-8 mRNA and additively increased IL-8 mRNA expression in response to A23187, thapsigargin, ionomycin and PMA but not to forskolin. At present, it is not known why EGTA increased basal levels of IL-8 mRNA. Furthermore, we found that forskolin combined with EGTA failed to have an additive effect on IL-8 mRNA expression suggesting that EGTA may affect the action of forskolin; however, it is clear that Ca^{2+} was not involved

in the action of EGTA in IL-8 mRNA expression since forskolin plus A23187 or thapsigargin showed an additive effect (data not shown). As expected, chelation of intracellular Ca^{2+} by cell-permeable BAPTA/AM almost completely abolished IL-8 mRNA expression evoked by the agonists. Attenuation of the effect of PMA or forskolin after chelation of intracellular Ca^{2+} by BAPTA/AM suggests that a physiological concentration of intracellular Ca^{2+} is necessary for the action of PKC and PKA (38, 43) or that BAPTA/AM itself alters PKC and PKA activity or interferes with their actions (44). Furthermore, low Ca^{2+} medium had no significant effect on IL-8 mRNA expression induced by A23187 or thapsigargin in comparison to low Ca^{2+} medium supplemented with 1.05 mM Ca^{2+} or normal medium with 1.05 mM Ca^{2+} clearly demonstrating a dependence for elevated $[Ca^{2+}]_i$ from intracellular Ca^{2+} stores. Moreover, treatment with verapamil, a Ca^{2+}-channel blocker, had no inhibitory effect on IL-8 mRNA expression evoked by A23187 or thapsigargin. Other studies have shown that in human neutrophils, the release of Ca^{2+} from thapsigargin-sensitive stores contributed to IL-8 mRNA expression and protein production to a minimal degree, whereas, capacitative entry of extracellular Ca^{2+} resulted in maximal IL-8 gene expression (31). Interestingly, PKC appeared not to be downstream of the Ca^{2+}-dependent signal for IL-8 gene expression evoked by A23187 (or ionomycin) and thapsigargin as depletion of PKC activity had no inhibitory effect on IL-8 mRNA expression. This is in marked contrast to other studies (28) which reported that depletion of PKC activity abolished A23187-induced IL-8 gene expression in AGS gastric epithelial cells. Therefore, Ca^{2+}, PKC, and PKA have distinct signal transduction pathways that stimulate IL-8 gene

expression with no counteracting effects in T84 cells.

Activation of CaM-dependent calcineurin can up-regulate the expression of a variety of cytokines such as IL-2, IL-4, or IL-8 (45). FK506 and cyclosporin A are potent immunosuppressive agents which specifically bind to FK506 binding protein 12 and cyclophilin A, respectively, which then interact and inactivate calcineurin (35, 45, 46). Cyclosporin A was shown to inhibit IL-8 mRNA expression in human T cells in response to PMA plus phytohemagglutinin or phytohemagglutinin alone (47). More recently (31), it was also reported that cyclosporin A could inhibit thapsigargin-induced IL-8 gene transcription in human peripheral blood neutrophils. In Jurkat cells stimulated with PMA plus ionomycin, elevation of $[Ca^{2+}]_i$ increased IL-8 gene transcription which was dependent on both AP-1- and NF-κB (32). However, FK506 inhibited the induction of IL-8 by changing the NF-κB complex formation but had no effect on the AP-1 complex. In our studies, A23187 and thapsigargin stimulated IL-8 gene expression by activating CaM-dependent calcineurin which was inhibited dose-dependently by FK506. Furthermore, activation of calcineurin through elevation of $[Ca^{2+}]_i$ by A23187 and thapsigargin enhanced IL-8 gene transcription and stabilized IL-8 mRNA. Other studies (31) have shown that thapsigargin can enhance IL-8 gene transcription whereas, A23187 can increase IL-8 protein production exclusively by a translational mechanism without altering its transcription in human peripheral blood neutrophils (23). In contrast to other studies (31), our findings showed that *de novo* protein synthesis was not involved in IL-8 gene expression evoked by the elevation of $[Ca^{2+}]_i$. CHX caused superinduction of IL-8 mRNA in unstimulated and agonists stimulated cells which was probably due to the inhibition of the biosynthesis of negative factors (proteins) which are involved in the degradation of newly synthesized mRNA.

In conclusion, our studies clearly demonstrate that A23187 and thapsigargin can increase IL-8 gene expression in T84 cells both transcriptionally and posttranscriptionally, which is dependent on the elevation of $[Ca^{2+}]_i$ from intracellular Ca^{2+} stores resulting in the activation of CaM-dependent calcineurin. Thus, pathogens that interact at mucosal surfaces or proinflammatory mediators that increase $[Ca^{2+}]_i$ can stimulate IL-8 gene expression and protein production which can recruit and activate neutrophils in the initiation or exacerbation of intestinal inflammation. Unravelling the multiple mechanisms involved in IL-8 gene expression in intestinal epithelial cells may lead to novel treatments to control IL-8 production to eleviate mucosal inflammation in the gastrointestinal tract.

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CONNECTING STATEMENT III

In our previous studies (Manuscript I, II, and III), it is clear that IL-8 is regulated transcriptionally and/or posttranscriptionally in human colonic epithelial cells. Moreover, posttranscriptional regulation appears to be a common phenomenon in IL-8 gene expression in T84 cells. Transcriptional regulation of the IL-8 gene has been well documented but there is limited information with regards to posttranscriptional regulation. Although IL-8 3'-UTR containing AU rich elements has been suggested to contribute to the degradation of IL-8 mRNA, more evidence is required to demonstrate the molecular mechanism involved in IL-8 mRNA turnover. In the following study, we examine the role of IL-8 3'-UTR in reporter gene expression in COS 7 cells which may reflect its role in IL-8 gene expression.

MANUSCRIPT IV

Identification of a 3'-untranslated region of human interleukin (IL)-8 mRNA that suppresses IL-8 gene expression: secondary structure is more important than AU rich elements*

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ABSTRACT

Although AU rich sequences in the 3'-untranslated region (UTR) of the interleukin (IL)-8 gene have been suggested to contribute to its posttranscriptional regulation, the molecular basis by which this occurs is still not known. To investigate the role of the 3'-UTR on human IL-8 gene regulation, chimeric reporter genes were generated by adding full length or differentially deleted 3'-UTR to chloramphenicol acetyltransferase (CAT). The addition of the entire IL-8 3'-UTR resulted in a reduction of CAT mRNA and protein expression in transfected COS 7 cells. A 357-base sequence (nt 2387-2743 in genomic DNA) within the 3'-UTR designated **e**, suppressed CAT gene expression by accelerating CAT mRNA turnover. A 26-base AU rich sequence (nt 2552-2577) within **e**, containing four AUUUA pentamers that form two UAUUUAUU and one UUAUUUAU octamers, did not suppress CAT gene expression. However, deletion of the AU rich sequences attenuated the inhibitory effect of **e** on CAT gene expression implicating its functional involvement. Elimination of the first 100 bases (nt 2386-2486) containing one hairpin structure attenuated the potency of fragment **e**, but much weaker than elimination of the first 146 bases (nt 2387-2533) containing two hairpin structures. RNA gel shift assays demonstrated the presence of proteins in COS 7 cells that bind to **e**. Thus, secondary structure but not AU rich sequences of **e** play a major role in IL-8 mRNA turnover.

INTRODUCTION

Interleukin-8 (IL-8), also called monocyte-derived neutrophil chemotactic factor (1) or neutrophil activating peptide-1(2), is a potent chemokine that belongs to the CXC family. Various cells produce IL-8, including monocytes, macrophages, T-cells, epithelial cells, endothelial cells, heptatic cells, gastric carcinoma cells, and neutrophils. IL-8 is the most potent chemokine that attract neutrophils and T-lymphocytes. It activates neutrophils through its two high affinity receptors, CXCR1 and CXCR2. The interaction of IL-8 with its receptors leads to neutrophil shape change, exocytosis, up-regulation of receptors and adhesion molecules, formation of bioactive lipids, and respiratory burst to release oxygen intermediates (3, 4). IL-8 has been demonstrated to be involved in various inflammatory responses and infectious diseases (5-8).

Transcriptional regulation of the IL-8 gene in various cell types has been extensively studied (9, 10). A variety of transcription factors, such as NF-κB, NF-IL-6, AP1, AP2, and Oct-1, are regulated by various stimuli including cytokines and viral or bacterial products (9-12). Studies have shown that the IL-8 gene could be regulated by a posttranscriptional mechanism in the presence of various stimuli by prolonging the stability of the IL-8 transcripts (13-18). Glucocorticoids suppress IL-8 gene expression by destabilizing its mRNA but not by decreasing its transcription rate (17). AU rich sequences such as AUUUA pentamers were identified in the IL-8 3'-UTR and were suggested to be involved in the degradation of IL-8 mRNA (9, 19); however, there is not enough evidence to demonstrate the involvement of the AU rich elements in IL-8 mRNA turnover. There is a wealth of information to suggest a crucial role for the 3'-UTR of many genes containing stabilizing elements (20-22), destablizing elements (23-27), and iron regulating elements (28, 29) in controlling the fate of mRNA. AU rich sequences in the 3'-UTR of mRNA of various genes, especially cytokines and growth factors, are the

major cause in mediating rapid degradation of RNA in depolyadenylation-dependent or independent pathway (30-32).

We have previously shown with CAT reporter gene linked to the IL-8 3'-UTR, an elevation of CAT gene expression in response to PGE_2 stimulation in human colonic epithelial cells without any alteration of the transcription rate (18). The objective of this study was to determine the role of the IL-8 3'-UTR on IL-8 gene regulation. Using chimeric reporter genes, we demonstrate that the IL-8 3'-UTR [nucleotides (nt) 1902-3152 in genomic DNA] down-regulates CAT reporter gene expression through both posttranscriptional and translational mechanisms. We have identified a 357-base fragment (nt 2387-2743) within the IL-8 3'-UTR, designated **e**, that contributes to the degradation of CAT mRNA leading to the suppression of CAT gene expression. RNA gel shift assays demonstrated the interaction of protein(s) from COS 7 cells with this fragment. AU rich sequences within this fragment did not contribute to the degradation of CAT mRNA but rather, hairpin structures in **e** may play a key role in its inhibitory function.

MATERIALS AND METHODS

Cell culture

The monkey kidney cell line COS 7 was cultured in DMEM medium (GIBCO) containing 10% fetal bovine serum (Hyclone), 100 U/ml of penicillin, 100 μg/ml of streptomycin sulfate, and 20 mM HEPES (Sigma Chemical Co.). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Plasmid Construction

pcDNACAT was generated by inserting the CAT gene, which was amplified from pBLCAT 6 (ATCC) using a pair of primers: *aagctt*GGCGAGATTTTCAG (sense) and

*cctagg*AATGCGGGCGGGACGGTG (antisense), into the *Hind* III and *Bam*H I sites of the pcDNA3 vector (Invitrogen Corp.). A fragment (**a**) (nt 1902-3710 in genomic DNA; Genebank, M28130) was amplified by PCR using the human genomic DNA as a template and the primers *gatatc*TAAAAAAATTCATTCTCTGTGGTATCC (sense) and *ctcgag*-TGAATTCCGAAGTTCTTTTTGTTC (antisense) and cloned into the *Eco*R V and *Xho* I sites of the pcDNACAT to generate pcDNACAT-**a**. pcDNACAT-**a** was digested with *Kpn* I/*Eco*R I, *Bam*H I, or *Bam*H I/*Kpn* I, respectively, and then self-ligated to generate pcDNACAT-**b**, **c**, or **f**. pcDNACAT-**d** and **e** were generated by inserting either fragments from nt 1902 to 2387 or from nt 2387 to 2743 into the *Bam*H I and *Xho* I sites of pcDNACAT. Several fragments were obtained by PCR using specific primers: CAGGATCCACAAGTCCTTG (sense) and *ctcgag*GGTTAATAAGTTACACTTG (antisense) for **g** (nt 2387-2551); *ggatcc*GCATCAAATATTTGTGCAAG (sense) and *ctcgag*CTGGGTACCCAATTGTT (antisense) for **i** (nt 2578-2743); *ggatcc*AT-GTGGAAGCACTTTAAG (sense) and *ctcgag*CTGGGTACCCAATTCTTTG (antisense) for **j** (nt 2488-2743); *ggatcc*AAGTGTAACTTATTAACC (sense) and *ctcgag*CTG-GGTACCCAATTGTTTG (antisense) for **k** (nt 2534-2743), and CAGGATCCAC-AAGTCCTTG (sense) and *ctcgag*TGCACAAATATTTGATGC for **l** (nt 2387-2595), respectively. These fragments were ligated into the *Bam*H I and *Xho* I sites of pcDNACAT to generate pcDNACAT-**g**, pcDNACAT-**i**, pcDNACAT-**j**, pcDNACAT-**k**, and pcDNACAT-**l**. pcDNACAT-**h** (nt 2552-2577) was generated by annealing the two oligonucleotides: *ccggatcc*TATTTATTATTTATGTATTTATTTAA*ctcgagcc* and *ggctcgag*TTAAATAAATACATAAATAATAAATA*ggatccgg*, digested with the *BamH* I and *Xho* I, and then ligated into pcDNACAT. pcDNACAT-**m** (-nt 2552-2577) was generated by deleting a fragment (nt 2552 to 2577) from pcDNACAT-**e**.

Transfection, ß-galactosidase, and CAT assays

COS 7 cells were plated at a density of 3-4 x 10^5 in 60-mm culture dishes in

complete medium. After 24 hr, 1 μg of CAT construct together with 0.5 μg of pRc/CMV (Invitrogen) plasmid containing ß-galactosidase gene in the *Not* I site (pRc/CMV-gal) to correct the difference in the transfection efficiency was transfected into cells using Lipofectamine reagent (GIBCO). After 48 hr incubation, the cytoplasmic extract from each sample was assayed for ß-galactosidase and CAT activity as described previously (33). Briefly, transfected COS 7 cells were washed twice with PBS and resuspended in 500 μl of CAT buffer containing 40 mM Tris-HCl (pH 7.5), 1 mM NaCl, and 1 mM EDTA. After repetitive cycles of freeze-thawing, 10 μl of cell extract was used for ß-galactosidase activity in the presence of 67 mM sodium phosphate (pH 7.5), 1 mM $MgCl_2$, 0.045 mM ß-mercaptoethanol, and 150 μg of 2-nitro-phenyl-ß-D-galactopyranoside at 37 °C for 30 min. Optical density was measured at 415 nm. After normalized by ß-galactosidase activity, the cell extract was heated at 65 °C for 10 min and used to measure CAT activity in a reaction buffer containing 100 mM Tris-HCl (pH 8.0), 125 nCi [^{14}C] chloramphenicol, and 25 μg of acetyl coenzyme A at 37°C for 40 min. After incubation, chloramphenicol and its derivative were extracted with ethyl acetate. The acetylated chloramphenicol and the substrate were separated on a thin layer silica gel plate (VWR Canlab) in the presence of the solvent chloroform:methanol (19:1) and autoradiographed using Kodak X-Omat AR film (Eastman Kodak Co.). The percent conversion of [^{14}C] chloramphenicol to the acetylated form was quantified in comparison to the control groups using NIH Program 1.6.

Reverse transcription PCR

Total RNA was extracted with TRIzol reagent (GIBCO) 48 hr after transfection. Total RNA was digested with RNase-free DNase (Promega), extracted with phenol/chloroform and precipitated with ethanol. 100-200 ng of RNA was used for reverse transcription (RT) to make cDNA in a 25 μl reaction mixture containing 4 U

RNasin ribonuclease inhibitor (Promega), 1 mM dNTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.1 μg Oligo $(dT)_{12-18}$, 4 mM DTT, and 50 U Moloney MuLV reverse transcriptase (GIBCO) at 37 °C for 1 hr. Reactions were stopped by heat inactivation at 95 °C for 5 minutes. Subsequently, 2.5 μl of RT reaction product was used for PCR in 100 μl of reaction mixture containing 25 pmoles of both sense and anti-sense primers, 50 μM dNTP, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, and 5 U Taq polymerase. PCR was carried out in a PROGENE thermal cycler (Mandel Scientific Co.) and consisted of denaturation at 95 °C for 30 sec, annealing at 56 °C for 1 min, and extension at 72 °C for 2 min for a total of 25 cycles followed by a final cycle with extension at 72 °C for 8 min. A pair of primers ATGACTTCCAAGCTGG-CCGTGGC (sense) and AATGCGGGGCGGGACGGTG (antisense) were used for the amplification of the CAT gene to generate a 659 bp fragment. The Neomycin (NEO) gene was used as an internal control where a 401 bp fragment was generated using a pair of primers CAGGACGGCAGCGCGG (sense) and CGGCAAGCAGGCATCGCC (antisense). The PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide to visualize amplification and photographed with Polaroid film. The results were analyzed using the NIH Image Program 1.6. The 3'-end of the IL-8 mRNA was mapped by 3' rapid amplification of cDNA Ends (3'-RACE) (34). cDNAs were obtained by reverse transcription in the presence of oligonucleotide GGCCACGCG-TCGACTAGTACTTTTTTTTTTTTTTT using total RNA from COS7 cells transfected with pcDNACAT-a. PCR products were obtained using a pair of primers *gatatc*TAA-AAAAATTCATTTCTCTGTGGTATCC (sense, nt 1902-1930) specific to the IL-8 gene and an anchor primer GGCCACGCGTCGACTAGTAC (antisense). Subsequently, the PCR products were cloned into pGEM-T (Promega) and sequenced by the Sheldon

Biotechnology Center (McGill University).

Preparation of cytosolic extract

COS 7 cells were washed twice with cold PBS twice and resuspended in a buffer containing 25 mM Tris-HCl (pH7.5), 0.5 mM EDTA, 10 μg/ml Leupeptin, and 0.15 mM PMSF, and lysed by repetitive cycles of freeze-thawing. Nuclei were removed by centrifugation at 15,000 g for 5 min and the cytosolic extract was further centrifuged at 100,000 g for 1 hr. The supernatant was collected and designated S100 and immediately frozen in liquid nitrogen and stored at -70 °C. Protein concentration was determined (35) and adjusted to 120 ng/ml.

In vitro transcription

Fragment e or the full length IL-8 3'-UTR (nt 1902-3152) was cloned into pGEM-T, and the plasmids linearized by digestion with the *Xho* I. RNA transcripts were synthesized with either 50 U T7 or 30 U SP6 RNA polymerase (GIBCO) from 500 ng of DNA constructs according to the manufacturer's recommendation; 400 μM each of adenosine triphosphate, guanosine triphosphate, and cytidine triphosphate, and 10 μM of uridine triphoaphate (UTP), either 10 μCi [α–^{32}P]-UTP (3000 Ci/mmol, Amershan, Corp) for the ^{32}P-labeled probe or 400 μM UTP for the cold probe (competitor) for 60 min at 37 °C. Two units of RNA-free DNase was added and incubated for 15 min. After passing through a Biol 30 column (Bio-Rad), the RNA transcripts were extracted using phenol/chloroform and precipitated with ethanol.

RNA mobility shift assay

S100 was incubated with 1-2 x 10^5 cpm ^{32}P- labeled RNA in the presence of 20 mM HEPES (pH7.6), 5 mM $MgCl_2$, 110 mM sucrose, 2.5 mM DTT, 1.5 mM KCl, and 10 mM NaCl for 30 min at room temperature. For the competition assay, various

amounts of cold probe were preincubated with S100 for 15 min at room temperature before the addition of ^{32}P-labeled RNAs. Samples were electrophoresed in a 4% native polyacrylamide gel (29:1) with a Tris-acetate running buffer containing 40 mM Tris-acetate and 1 mM EDTA at 15 mA for 2-3 hr. The gels were dried and autoradiographed using Kodak X-Omat AR film (Kodak Co.).

RESULTS

IL-8 3'-UTR suppresses CAT protein and mRNA expression in COS 7 cells. Posttranscriptional regulation of the IL-8 gene in various cell types has been demonstrated and the 3'-UTR was suggested to play a crucial role in the degradation of mRNA (13-18). To determine the effect of the IL-8 3'-UTR on IL-8 gene expression, we examined the function of the 3'-UTR on CAT reporter gene expression in COS 7 cells. A fragment (**a**) (nt 1902-3710) was amplified by PCR from genomic DNA and cloned into the 3'-flanking end of the CAT reporter gene construct (pcDNACAT) to generate pcDNACAT-**a**. The polyadenylation site of the IL-8 gene was determined by 3'-RACE using the primer specific to IL-8 3'-UTR after pcDNACAT-**a** was transfected into COS 7 cells. As shown in Fig 1, PCR product was generated from 3'-RACE and sequenced. The polyadenylation site is located at nt 3152 of the 3'- UTR. Thus, the length of IL-8 3'-UTR transcribed in COS 7 cells is 1250 bases (nt 1902-3152). A series of deletions of the IL-8 3'-UTR was generated and cloned into the 3' flanking end of the CAT gene in pcDNA3 to generate various CAT reporter gene constructs (Fig 2A). CAT activity was measured 48 hr after the constructs were transiently transfected into COS 7 cells. Either the IL-8 poly(A) site or bovine growth hormone poly(A) site in pcDNA3 vector was used for the polyadenylation of transcribed CAT mRNA. As shown in Fig 2B, fragment **a**

which includes the full length 3'-UTR, reduced CAT activity up to 80% as compared with CAT alone while fragment **e** and **f** inhibited CAT activity up to 97% and 89%, respectively. Fragments **b** and **c**, which include **e**, reduced CAT activity approximately 86% and 89%, respectively. Thus, **e** had a very strong effect on the function of both **b** and **c**. In contrast, fragment **d** had only weak effect and reduced CAT activity by 24%. Therefore, the IL-8 3'-UTR is capable of suppressing reporter gene expression, implicating a similar effect on IL-8 gene expression. The elements which down-regulated CAT gene expression to varying degrees may be scattered along the IL-8 3'-UTR.

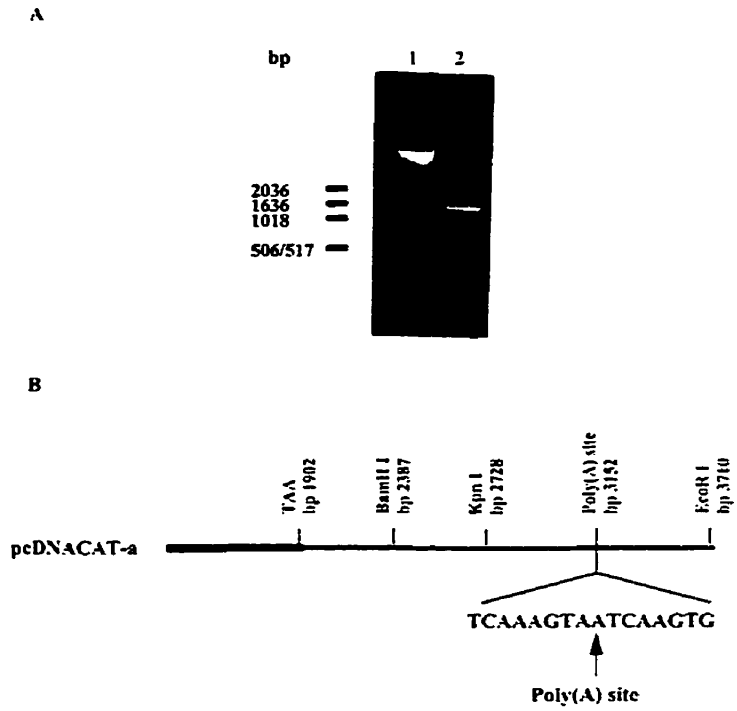
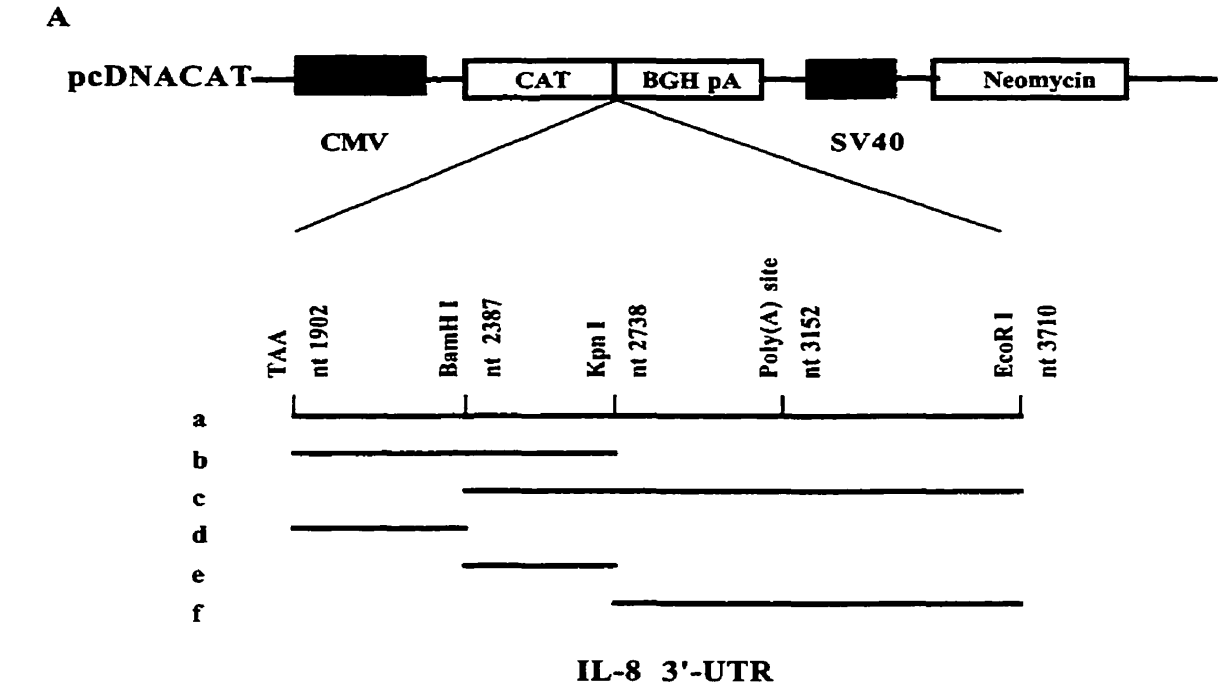


FIGURE 1. Mapping the polyadenylation site of the IL-8 gene. Panel A. Product derived from RT-PCR to map the 3' end of transcript derived from pCDNACAT-**a**. RNA was isolated from transfected cells and RT-PCR performed using anchor primer and the primer specific to the IL-8 gene *gatatc*TAAAAAAATTCATTTCTCTGTGGTATCC (nt 1902-1930). PCR product (lane 2) was cloned into pGEM-T and sequenced. DNA marker is shown in lane 1. Panel B. Diagram showing the location of the poly(A) addition site for the transcript derived from the pCDNACAT-**a** construct. The poly(A) addition site occurred approximately 1250 bases downtream from the stop code of IL-8 gene.



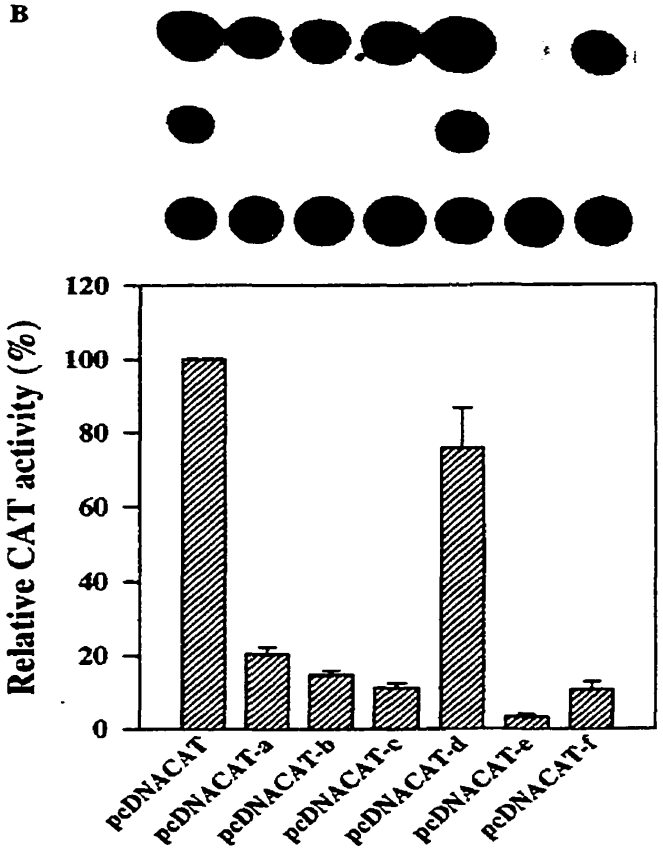


FIGURE 2. Effect of the IL-8 3'-UTR on CAT reporter gene expression. A schematic diagram of the pcDNA3 vector cloning site and the CAT reporter gene constructs with or without IL-8 3'-UTR is shown in panel *A*. Differentially deleted fragments of the IL-8 3'-UTR were cloned into pcDNACAT using PCR primers or by using the indicated restriction sites. Panel *B* shows the CAT activity in COS7 cells transfected with CAT alone or its mutants. 3-4 x10^5 cells in 60 mm dish were transiently transfected and CAT activity measured 48 hrs after transfection. β-Galactosidase gene was cotransfected to normalize the transfection efficiency. Results are shown as relative CAT activity with pcDNACAT defined as 100%. Each value is the mean ± SD. of the triplicate measurements of one representive experiment. Similar results were obtained from three separate experiment. CMV, human cytomegalovirus promoter; BGH pA, bovine growth hormone polyadenylation signal for polyadenylation of transcribed mRNA; SV40, SV40 promoter.

To determine whether the decrease in CAT protein expression is due to a reduction of CAT mRNA expression, we examined CAT mRNA levels by RT-PCR in COS 7 cells transiently transfected with pcDNACAT, pcDNACAT-**a**, pcDNACAT-**d**, pcDNACAT-**e**, and pcDNACAT-**f** during the same time period used to examine CAT activity. The neomycin (NEO) gene in the pcDNA3 vector was used as an internal control. NEO is constantly driven by the SV40 promoter and is not affected by the

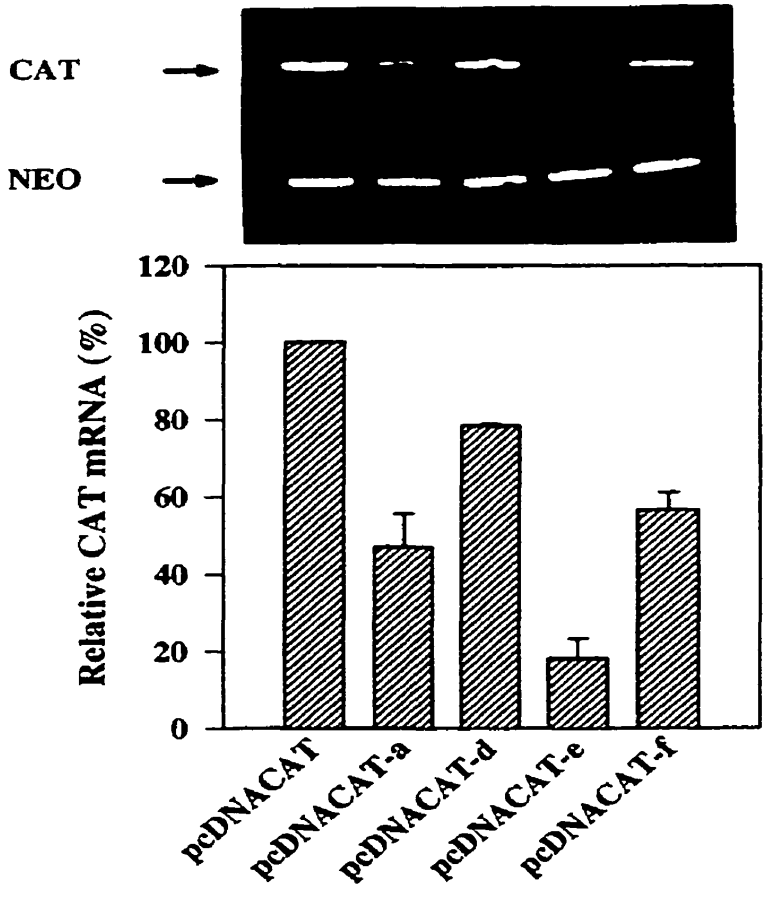


FIGURE 3. Effect of the IL-8 3'-UTR on CAT mRNA expression. COS 7 cell were transiently transfected with pcDNACAT, pcDNACAT-**a**, pcDNACAT-**d**, pcDNACAT-**e**, and pcDNACAT-**f**. Total RNA was extracted after 48 hr and CAT mRNA levels measured by RT-PCR as described in Materials and Methods. The neomycin (NEO) gene was used as an internal control. The ratio of CAT mRNA and NEO represents the CAT mRNA level. Results are shown as relative CAT mRNA level with pcDNACAT defined as 100%. Each value is the mean ± SD of three separate experiments.

addition of IL-8 3'-UTR in the 3'-flanking end of the CAT gene. The ratio of CAT to NEO represents the relative CAT mRNA level. As depicted in Fig 3, fragments **a**, **d**, **e**, and **f** caused 53, 22, 82, and 44% reduction in CAT mRNA levels, respectively. Therefore, addition of the fragments into CAT reporter gene constructs reduced CAT mRNA expression presumably by destabilizing its transcripts, as the CMV promoter drives the same transcription rate in all reporter gene constructs in COS 7 cells (36). It is very likely that translational regulation may also be involved in regulating CAT reporter gene expression because **a** resulted in up to 80% reduction in CAT activity but only 53% reduction in CAT mRNA. It is apparent that **e** reduced CAT gene expression by accelerating mRNA turnover as the reduction of mRNA (82%) contributed to a reduction of CAT protein expression (97%). Both posttranscriptional and translational regulation were also involved in the effect of **f** on CAT gene expression since it reduced CAT mRNA by 44% (Fig 3) and protein by 89% (Fig 2B), respectively. The involvement of the 3'-UTR in translational regulation has been demonstrated in various studies (37, 38). Furthermore, reduction of CAT protein (24%) by **d** was concomitant with a decrease in CAT mRNA levels (22%). Taken together, these results suggest that IL-8 3'-UTR can regulate reporter gene expression posttranscriptionally and translationally, and that fragment **e** (nt 2387-2743) is engaged in the suppression of reporter gene expression mainly by destabilizing mRNA.

Mutational analysis of a distinct 357-base fragment (e). Fragment **e** contains a unique AU rich sequence, UAUUUAUUAUUUAU-GUAUUUAUUUAA (nt 2552 to 2577) which contains four AUUUA pentamers (Fig 4A). This AU rich sequence can also be grouped into two UAUUUAUU and one UUAUUUAU octamers, which have been shown to have destablizing activity (39, 40). It is well known that AU rich elements are involved in the destabilization of mRNA (25-28). Thus, it was important to define the role of the AU rich sequences in IL-8 gene expression. CAT reporter gene constructs with various

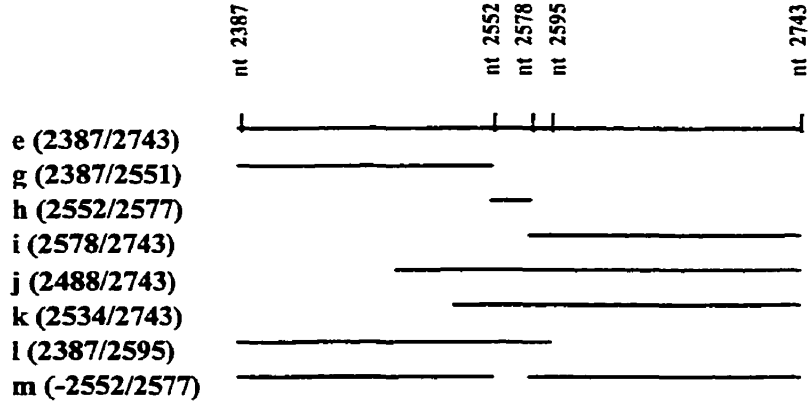
A

2387

ggauccacaaguccuuguuccacugugccuugguuucuccuuuauuucuaaguggaaaaaguauuagc
caccaucuuaccucacagugauguugugaggacauguggaagcacuuuaaguuuuuucaucauaacaua
aauuauuuucaaguguaacuuauuaaccuauuuauuauuuauguauuuauuuaagcaucaaauauuugu
gcaagaauuuggaaaaauagaagaugaaucauugauugaauaguuauaaagauguuauaguaaauuuau
uuuauuuuagauauuaaaugauguuuuauuagauaaauuucaaucaggguuuuuagauuaaacaaacaa
acaauuggguacc

2743

B



C

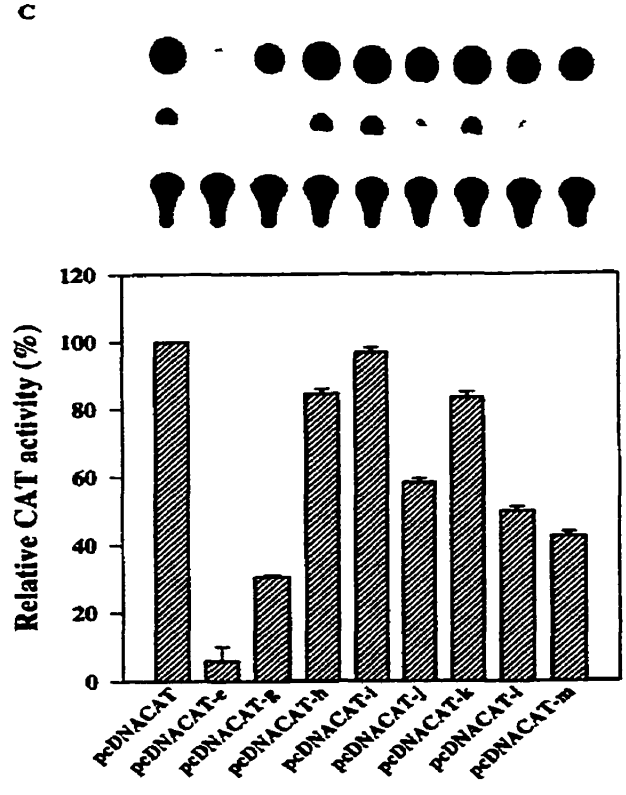


FIGURE 4. Mutational analysis of e (nt 2378-2743) on CAT reporter gene expression. (A). Ribonucleotide sequence of e. AUUUA pentamers are underlined. (B). The schematic diagram of CAT reporter gene constructs with or without e and various mutants. Differentially deleted fragments of e were cloned into pcDNACAT using PCR primers. Panel C shows CAT activity in COS 7 cells transfected with CAT alone or mutants of e. 3-4 x10^5 cells in 60 mm dish were transiently transfected and CAT activity measured 48 hr after transfection. ß-galactosidase gene was cotransfected to normalize the transfection efficiency. Results are shown as relative CAT activity with pcDNACAT defined as 100%. Each value is the mean ± SD of triplicate measurements of one representative experiment. Similar results were obtained from three separate experiments.

deletions in fragment **e** (Fig 4B) were generated and transiently transfected into COS 7 cells. The bovine growth hormone poly(A) site in pcDNA3 vector was used for the polyadenylation of transcribed CAT mRNA for all the reporter gene constructs. As shown in Fig 4C, full length fragment **e** had the most potent effect on the suppression of reporter gene expression (up to 94%) since any deletion attenuated its inhibitory effect. Fragment **h** containing the AU rich sequence (nt 2552 to 2577) had a weak inhibitory effect on CAT activity (16% reduction), while **m**, generated by deleting **h** from **e**, had a stronger effect (58%) but less than **e**. This clearly indicates the involvement of this AU rich sequence in suppression of CAT reporter gene expression. Moreover, **g** (nt 2387-2551) strongly reduced CAT activity up to 70%, whereas **i**, containing one AUUUA pentamer, had no significant effect. Surprisingly, **h** attenuated the effect of **g** (70%) since **l** containing **g** and **h**, had less potency (50%). These data indicate that AU rich elements are not the major destabilizing factor. Computational prediction of the secondary structure by RNA-folding algorithm (41, 42) clearly show that fragment **e** contain many hairpin structures (Fig 5) with a folding energy of -64.6 kcal/mol. Deletion of the first 100 (**j**) bases which eleminated hairpin HP 1, or 146 (**k**) bases which eliminated hairpins HP 1 and HP 2, caused 42 and 17% reduction of CAT gene expression, respectively. This suggests that the first 146 bases are very important for the inhibitory function of **e**. Taken together, these data demonstrate that the AU rich sequences (nt 2552 to 2577) in **e** are not the major cause for suppression of reporter gene expression. Thus, it is very likely these hairpin structures are crucial for the degradation of mRNA.

Interaction of e with RNA binding proteins in COS 7 cells. To determine the presence of trans-acting binding protein(s), fragment **e** was *in vitro* transcribed with α-^{32}P-UTP, and RNA electrophoretic mobility shift assay performed using the COS 7 cytosolic extract S100. As shown in Fig 6A, a single RNA-protein complex was formed and the degree

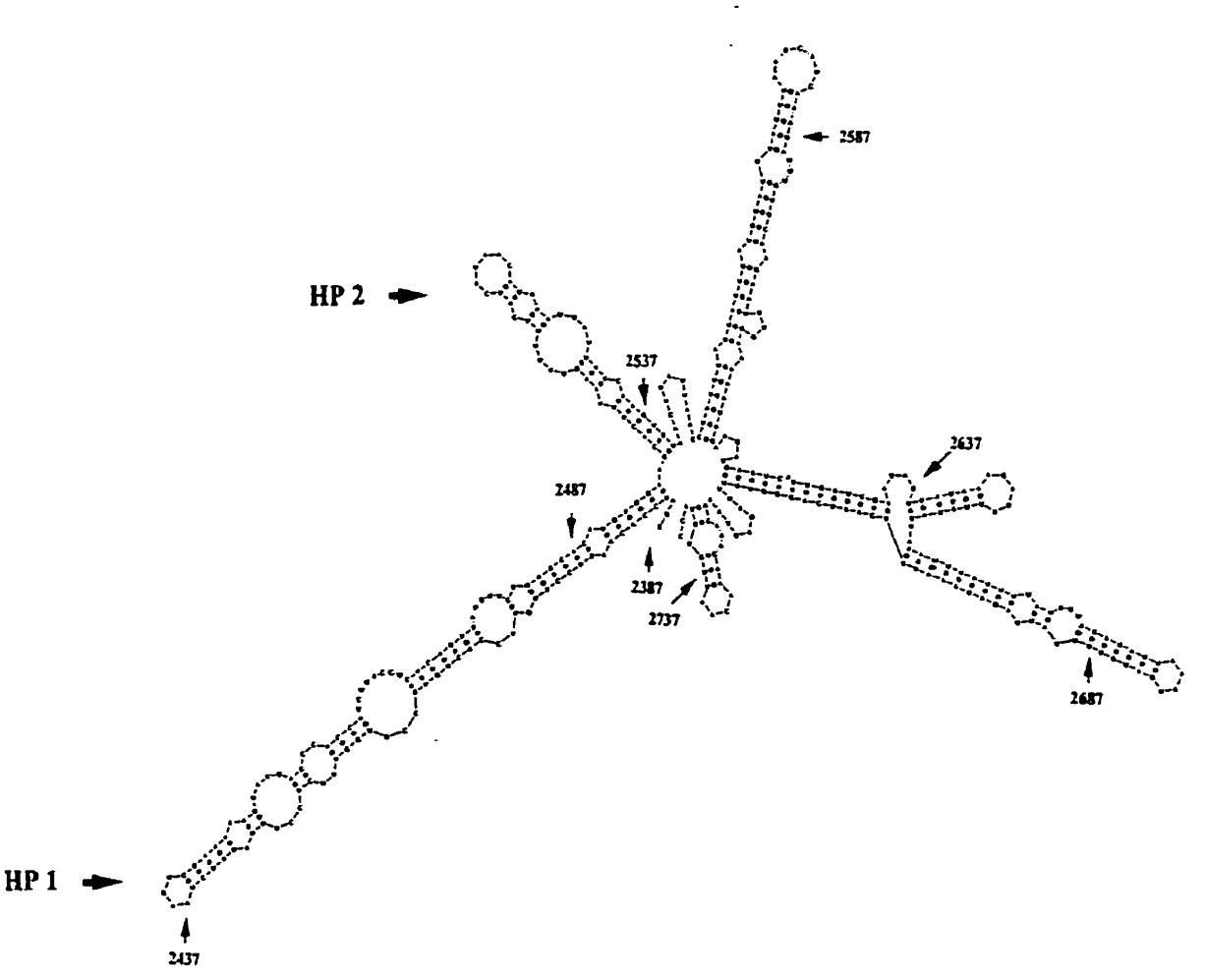


FIGURE 5. Possible folding of fragment e (nt 2387- 2743). The secondary structure of e within IL-8 3'-UTR was determined using computer algorithm. The minimum free energy is -64.6 kcal/mol. HP 1 and HP 2 represent the first and second hairpin structures.

of the supershift was dependent on the ratio between RNA and protein. Specificity for the formation of RNA-protein complex was demonstrated by competition assays using various concentration of cold probes, either e or full length IL-8 3'-UTR (Fig 6B). The RNA-protein complex formed by e and S100 was inhibited by cold e and full length 3'-UTR in a dose-dependent manner. These data indicate that COS 7 cells contain protein(s) which bind to e, suggesting that the interaction of RNA and protein(s) leads to the destabilization of mRNA.

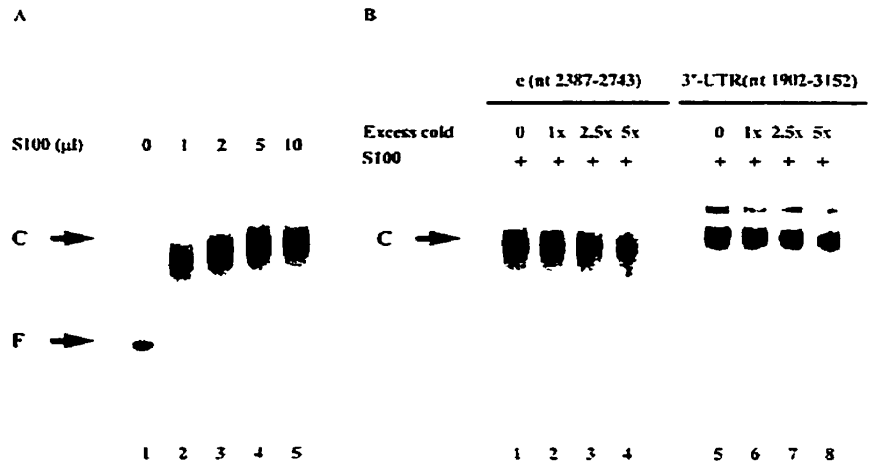


FIGURE 6. Formation of RNA-protein complex. [α-^{32}P]-labeled e and full length 3'-UTR RNAs were transcribed *in vitro* as described in Materials and Methods. RNA gel shift assay was performed in the presence of 100,000 to 200,000 cpm [α-^{32}P]-labeled RNA and various concentrations of S100 extract of COS 7 for 30 min (Panel A). Samples were electrophoresed in a 4% native polyacrylamide gel (29:1). The gel was dried and autoradiographed on Kodak X-Omat AR film. For the competition assay (Panel B), various amount of cold probe (competitor) was preincubated with S100 for 15 min at room temperature before the addition of [α-^{32}P]-labeled RNA probe. The ratio of cold probe and ^{32}P-labeled RNA is on weight/weight basis. Cold e: 0x (lane 1), 1x (lane 2), 2.5x (lane 3), and 5x (lane 4) and cold full lenght 3'-UTR: 0x (lane 5), 1x (lane 6), 2.5x (lane 7), and 5x (lane 8). Similar results were obtained from three separate experiments. C (arrow) shows the RNA-protein complex and F (arrow) shows the free probe.

DISCUSSION

There is accumulated evidence that IL-8 gene expression can be up-regulated by stabilizing its transcripts in various cell types in response to different stimuli (13-16, 18). The effect of the IL-8 3'- UTR on posttranscriptional regulation is not fully understood. AUUUA pentamers have been suggested to contribute to IL-8 mRNA turnover (9, 19). Recently, Chaudhary and Avioli (17) reported that dexamethasone inhibited IL-1ß-induced IL-8 gene expression in human bone marrow stromal cells in a posttranscriptional fashion and that the mRNA detabilizing sequence AUUUA pentamers in the IL-8 3'-UTR may contribute to the inhibitory effect of dexamethasone. However,

the molecular basis for the involvement of these AU rich elements in the destabilization of IL-8 mRNA is still unclear. To determine the effect of the IL-8 3'-UTR on IL-8 gene expression, we investigated CAT reporter gene expression in COS 7 cells transfected with chimeric reporter genes linked to full length IL-8 3'-UTR or deletion mutants. Addition of the full length 3'-UTR reduced CAT mRNA levels by 53% and CAT protein production by 80% as measured by CAT activity, suggesting that the inhibitory effect of the IL-8 3'-UTR may be through both posttranscriptional and translational events. More detailed deletions of the 3'-UTR showed that a distinct 357-base fragment, designated **e** (nt 2387-2743), caused a 97% reduction of CAT protein production which was concomitant with a 82% reduction in CAT mRNA levels. In contrast, fragment **d** (nt 1902-2386) reduced CAT protein production by only 24% and CAT mRNA by 22%, suggesting that this region is not potent in the suppression of CAT reporter gene expression. Many studies have shown a critical role for 3'-UTR binding proteins in modulating the fate of mRNAs, especially those containing AU rich elements (42-45). By RNA gel shift assays we clearly show that fragment **e** had RNA-binding activity for COS 7 cytosolic extracts forming a single RNA-protein complex. This RNA-protein interaction is presumably required for the suppressive effect of **e**. Therefore, it was of interest to further investigate the function of **e**.

The IL-8 3'-UTR has nine AUUUA pentamers which has been suggested to destabilize IL-8 mRNA (11). There are five AUUUA elements in fragment **e**, four of them between nt 2552 to 2577 within a unique AU rich sequence, UAUUUAUUA-UUUAUGUAUUUAUUUAA, and one between nt 2653 to 2657. This AU rich sequence also contains two UAUUUAUU and one UUAUUUAU octamers. AU rich elements, especially AUUUA, have been well documented to accelerate the degradation of mRNA of many genes, which include cytokines, growth factors, and proto-oncogenes (26, 27, 30, 46). However, in the mouse fibroblast cell line NIH 3T3, a

UUAUUUA(U/A)(U/A) nonamer was suggested to be the minimum functional sequence necessary for the destabilization of the mRNA, whereas AUUUA alone is not sufficient to cause degradation of mRNA. Several derivatives from the UUAUUUA(U/A)(U/A) nonamer, UUAUUUAU and UAUUUAUU octamers, were capable of destabilizing mRNA but had reduced potency (38, 39). In our study, we demonstrated that the AU rich sequence in IL-8 3'-UTR was not a major contributor to the degradation of IL-8 mRNA since addition of the AU rich sequence UAUUUAUUAUUUAUGUAU-UUAUUUAA (nt 2552 to 2577) into the CAT reporter gene only caused a weak reduction in CAT activity, whereas deletion of this fragment from **e** attenuated the potency of **e**. Therefore, this unique AU rich sequence in the IL-8 3'-UTR is not the major cause but essential for maximal IL-8 mRNA turnover. The presence of distinct mRNA-destabilizing elements in the 3'-UTR of GM-CSF, IL-2, and IL-6 mRNA have been demonstrated, but are structurally and functional different from AU rich elements (47). There is a possibility that the effect of these AU rich elements in the IL-8 3'-UTR may need additional cis-elements or secondary structure elements.

Using computational prediction of RNA folding, we found that **e** contains many hairpin structures. Deletion of the hairpin structures drastically reversed the inhibitory effert of **e** on CAT reporter gene expression. Furthermore, addition of the first 165 base (**g**, nt 2387-2551) markedly reduced CAT gene expression (up to 70%) but was not able to reach the potency of **e** (94%). The last 172 bases (**i**, nt 2578-2743), containing one AUUUA pentamer, had no effect. These data suggest that the native conformation of **e** exerts a maximum inhibitory effect and the first two hairpins play a very important role in IL-8 mRNA turnover. They appear to be required for the proper interaction between RNA and protein(s).

In summary, our studies clearly demonstrate the down-regulatory function of the IL-8 3'-UTR. A distinct 357-base fragment (**e**) between nt 2387-3743 was shown to

play a crucial role in IL-8 mRNA turnover and required the presence of RNA-protein interaction. The detailed mutational analysis of this fragment revealed that AU rich sequences do not play a important role but is essential for maximum degradation of IL-8 mRNA. Hairpin sturctures may play a major role in the inhibitory function of e.

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SECTION III: GENERAL DISCUSSION

IL-8 produced by intestinal epithelial cells has been demonstrated to be involved in intestinal inflammation associated with bacterial or parasitic infection and in IBD (1-8). Therefore, it is of great interest to elucidate the molecular mechanisms involved in the regulation of IL-8 in human intestinal epithelial cells, which can help us understand the role of IL-8 in initiation and/or exacerbation of intestinal inflammation.

Previous studies have shown that intestinal amebiasis is associated with massive infiltration of neutrophils and macrophages into the intestinal mucosa by an unknown mechanism (9-11). Although *E. histolytica* killing of epithelial cells can up-regulate IL-8 gene expression from intact epithelial cells (8, 12, 13), it is unable to explain why neutrophil infiltration in the lamina propria occurs in animal models of the disease with an intact interglandular epithelium (10, 11). It is apparent that *E. histolytica* may induce IL-8 gene expression in epithelial cells by releasing soluble components prior to amebic invasion since a variety of substances released from amebic trophozoites can modulate intestinal functions through a cascade of signals (14-16). Data in Manuscript I clearly demonstrate that soluble amebic components caused IL-8 mRNA accumulation and protein production, which could contribute to neutrophil infiltration. A major finding was that live amebae released soluble components which stimulated IL-8 gene expression in human colonic epithelial cells without direct parasite-enterocyte contact. Therefore, in addition to the notion that amebic killing of epithelial cells can up-regulate IL-8, our studies provide novel insights into the pathogenesis of amebiasis whereby amebae can initiate intestinal inflammation prior to amebic adherence to the epithelia. The dogma in intestinal amebiasis is that amebae bind to and kill mucosal epithelial cells allowing the parasite to invade the lamina propria. Based on these studies a model for intestinal inflammation and pathogenesis of intestinal amebiasis is proposed (Figure. 1 A, B, C, and D). *E. histolytica* colonizes the intestine by adhering to colonic mucins via the Gal-lectin. During colonization, trophozoites release soluble components that interact with

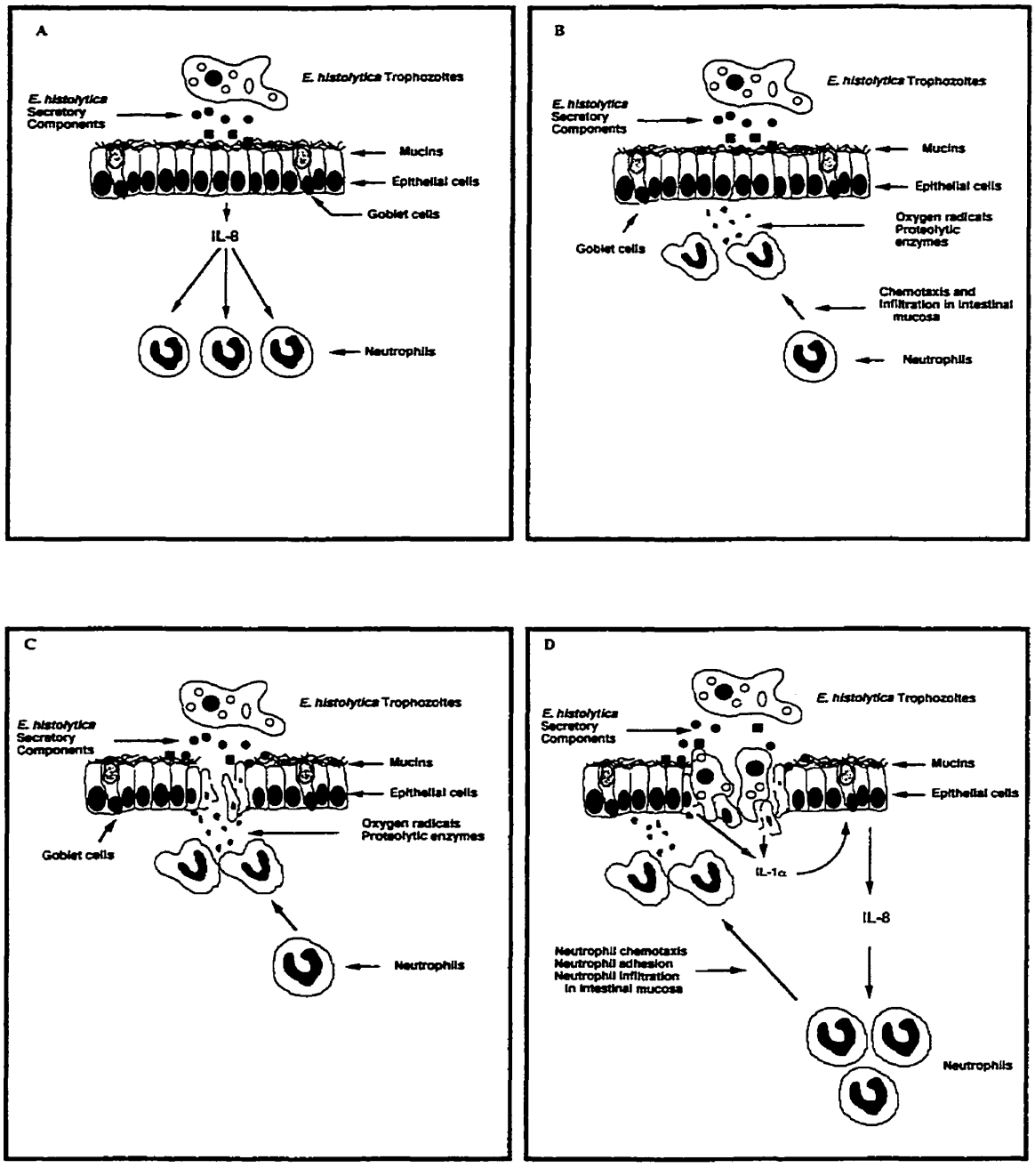

Figure 1. Possible model for the initiation and exacerbation of intestinal inflammation in amebiasis. *E. histolytica* trophozoites colonize the intestine and release soluble components , which induce IL-8 gene expression from colonic epithelial cells (**A**). Activated neutrophils infiltrate into local sites releasing various oxygen radicals and proteolytic enzymes (**B**), resulting in non-specific tissue damage in epithelia (**C**). Damaged epithelia may facilitate amebic invasion; lysed epithelial cells killed by amebae release preformed IL-1α to cause more IL-8 production from intact epithelial cells leading to greater infiltration of neutrophils(**D**).

epithelial cells to trigger a signal transduction cascade to up-regulate IL-8 mRNA expression and protein production. The amount of soluble components released by amebae in the colon may be central in the initiation of this event. IL-8 released in the colon will recruit and activate circulating neutrophils into local sites and thus, initiate intestinal inflammation. Subsequently, amebic invasion can be facilitated through damaged epithelia caused by activated neutrophils (enzyme degradation) and neutrophils killed by amebae. Lysed epithelial cells also release preformed IL-1 α that has a paracrine action on IL-8 gene expression in intact epithelial cells leading to exacerbation of intestinal inflammation. Other studies (4, 17) have showed that soluble bacterial substances can cause the induction of IL-8 in mucosal epithelial cells without contact and cellular entry.

At present, the identity of the amebic substances that caused up-regulation of IL-8 in human colonic epithelial cells are not known. Interestingly, we previously demonstrated that *E. histolytica* can stimulate IFN-γ activated macrophages to produce large amounts of PGE_2 (18, 19). PGE_2 is considered one of the most potent mediators of inflammation (20). In IBD, high output of PGE_2 released from infiltrated neutrophils and macrophages correlated with the development of disease. PGE_2 has been considered one of the best targets for the development of drugs for treatment of IBD (21). To determine the mechanisms whereby PGE_2 plays a role in intestinal inflammation, we examined the effect of exogenous PGE_2 on IL-8 mRNA expression and protein production in human colonic epithelial cells. Results in Manuscript II clearly demonstrate that exogenous PGE_2 can stimulate IL-8 mRNA expression and protein production in T84 cells by coupling to EP_4 receptors to elevate intracellular cAMP which activated cAMP-dependent PKA signal transduction. Unlike the PKC activator PMA, activation of the PKA pathway caused up-regulation of IL-8 gene expression posttranscriptionally or

stabilized IL-8 mRNA without changing its transcription rate. Other studies have shown that PGE_2 inhibited LPS-induced IL-8 production in human alveolar macrophages, blood monocytes, and neutrophils (22, 23) but enhanced IL-8 gene expression in human synovial fibroblasts in response to LPS (24). PGE_2 is well known to modulate physiological functions of intestinal epithelia by causing the secretion of electrolytes and mucin (25-27). Our studies revealed a unique role for PGE_2 in the up-regulation of IL-8 in human colonic epithelial cells, further supporting the importance of PGE_2 in intestinal inflammation. This is the first report documenting that exogenous PGE_2 can enhance IL-8 gene expression in human colonic epithelial cells. Therefore, as a multifunctional mediator, PGE_2 not only regulates normal physiological functions in intestinal epithelia but also initiate and/or exacerbate intestinal inflammation by up-regulating IL-8. A working model for PGE_2 in intestinal inflammation is shown in Figure 2. In the inflamed gut in IBD or caused by bacterial or parasitic infections, infiltrated macrophages and neutrophils in the lamina propria release large amounts of PGE_2 which can up-regulate IL-8 gene expression in epithelial cells. The release of IL-8 in tissues could exacerbate intestinal inflammation by recruiting and activating more neutrophils into local sites to sustain the inflammatory responses.

Moreover, as shown in Manuscript I and II, neither *E. histolytica* components nor PGE_2 could change the transcription rate of IL-8 but stabilized the mRNA. Glucocorticoids inhibited both *E. histolytica* and PGE_2-induced IL-8 mRNA expression and protein production suggesting that amebae and PGE_2 apparently share the same PKA signal transduction pathway; IL-8 induced through PKC was not altered. To determine the possible involvement of second signals involved in the up-regulation of IL-8 in

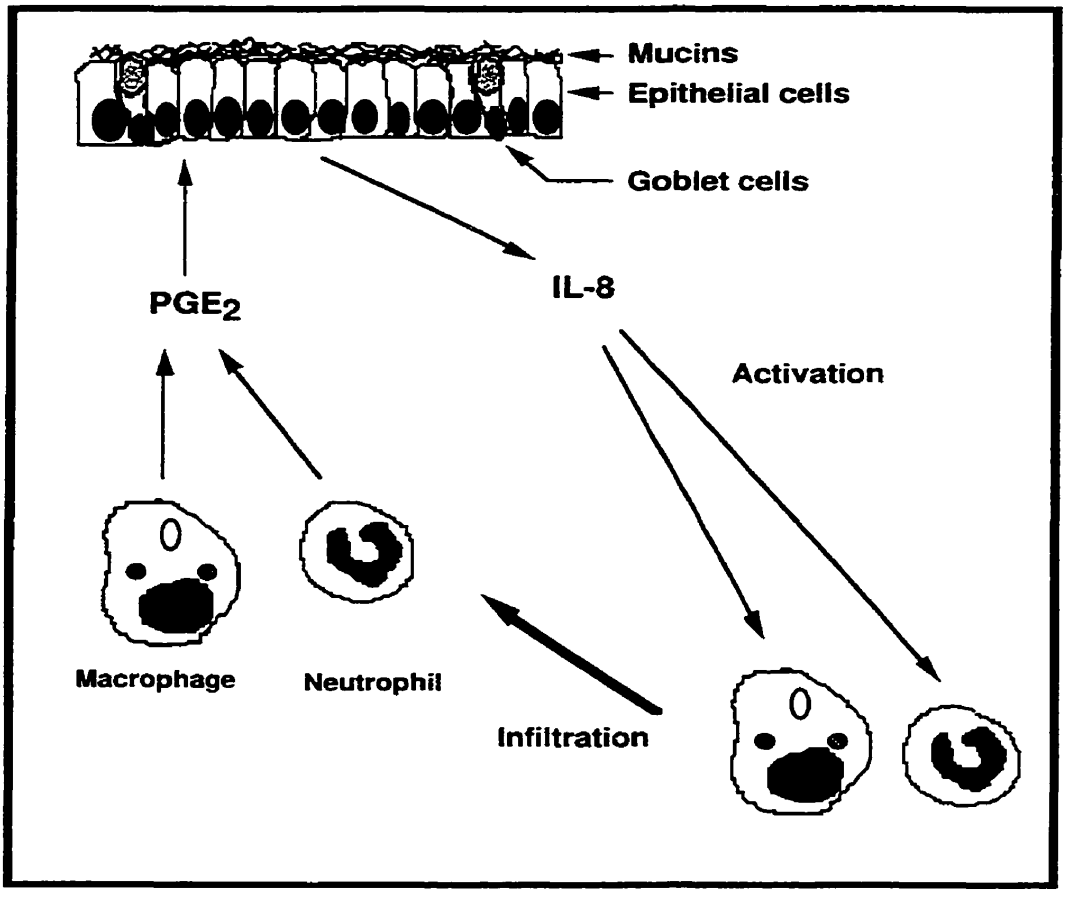

Figure 2. A working model for the action of PGE_2 in intestinal inflammation. Infiltrated macrophages and neutrophils in the inflamed gut caused by pathogens or in IBD release large amount of PGE_2, which in turn stimulates IL-8 production from epithelial cells. The release of IL-8 in tissues recruit more neutrophils to exacerbate inflammatory responses.

human colonic epithelial cells, we examined the role of PKC, PKA, and Ca^{2+} in IL-8 gene expression (Manuscript I, II, and III). Activation of PMA-sensitive PKC, cAMP-dependent PKA, and CaM-dependent calcineurin caused up-regulation of IL-8 gene

Table 1. Signal transduction pathways involved in the up-regulation of IL-8 in T84 cells

Stimuli	Receptor	Signaling	Transcription	Posttranscription
E.histolytica components	?	?	-	+
PGE_2	EP4	PKA	-	+
Forskolin	-	PKA	-	+
A23187 /thapsigargin	-	Calcineurin	+	+
PMA	-	PKC	+	+

expression. Each one of the pathway seems to have a distinct signal in IL-8 gene expression which does not counteract with each other (Table 1). These data suggest that a variety of insults are able to stimulate IL-8 gene expression in intestinal epithelial cells leading to intestinal inflammation through various mechanisms (PKC, PKA, or Ca^{2+}). Interestingly, activation of either PKC or CaM-dependent calcineurin induced both transcriptional and posttranscriptional regulation of IL-8 whereas PKA only caused posttranscriptional regulation. Thus, posttranscriptional regulation of the IL-8 gene clearly is the common phenomenon in human colonic epithelial cells. However, at present, there are no reports showing the molecular mechanism involved in the role of the IL-8 3'-UTR, which contains many AU rich elements (28), in posttranscriptional regulation of IL-8 gene expression (29-32). AU rich elements have been demonstrated

to play a very important role in mRNA turnover (33-35). Data in Manuscript IV clearly show that AU rich elements in the IL-8 3'-UTR were not the major cause for IL-8 mRNA degradation even though there are PKC and PKA responsive elements in the IL-8 3'-UTR. These results provide profound insights into the molecular mechanism(s) involved in the regulatory effect of IL-8 3'-UTR in IL-8 gene expression, which may open new avenues to explore the possibility that drugs could be designed to cause rapid degradation of IL-8 leading to relief of the development of intestinal inflammation by reducing IL-8 protein production.

Another special contribution of our study is the therapeutic strategy in treatment of intestinal inflammation when IL-8 is used as a target. Our studies clearly revealed a different effect for glucocorticoids on IL-8 gene expression in colonic epithelial cells. Dex, inhibited IL-8 production induced by activation of PKA which undergoes posttranscriptional regulation but had no effect by activation of PKC which stimulates both transcriptional and posttranscriptional regulation of IL-8. Therefore, caution should be taken when glucocorticoids are administrated to treat intestinal inflammation in IBD or those associated with infectious diseases.

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