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Inducible nitric oxide synthase activity and expression in a human colonic epithelial cell line, HT-29

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1 We have determined which cytokines regulate the expression of human inducible nitric oxide synthase (iNOS) mRNA and nitrite generation in the human colonic epithelial cell line HT-29.

2 Growth arrested cell cultures were stimulated with the human recombinant cytokines interleukin-1α (IL-1α), tumour necrosisfactor-α (TNF-α), interferon γ (IFN-γ) or vehicle added alone or in combination. Human iNOS mRNA was determined by Northern blot analysis and nitrite generation by the use of a fluorometric assay.

3 Unstimulated cells produced a small time-dependent increase in nitrite generation of 50 ± 4, 75 ± 8, and 103 ± 8 nM per 10^6 cells at 24 h, 48 h, and 72 h respectively. This nitrite generation was unaffected by cycloheximide (5 μg ml^{-1}) pretreatment and iNOS mRNA was not detected.

4 None of cytokines alone induced either iNOS mRNA expression or an increase in nitrite generation. The combination of IL-1α/IFN-γ produced a highly significant ($P < 0.001$) 4 fold increase in nitrite production at 48 h, compared to basal values, while no other pair of cytokines was effective.

5 Time course studies with IL-1α/IFN-γ combination revealed significant ($P < 0.001$) increases in nitrite at 24 h (153 ± 7), 48 h (306 ± 24), and 72 h (384 ± 15) compared to basal values of 50 ± 4, 75 ± 8, and 103 ± 8 nM per 10^6 cells respectively.

6 Studies with IL-1α/IFN-γ combination demonstrated a time dependent expression of iNOS mRNA, first observed at 6 h, peaked at 24 h and was undetectable by 72 h. IL-1α (0.3-10 ng ml^{-1}) and IFN-γ (10-300 u ml^{-1}) in combination induced a concentration-dependent expression of iNOS mRNA at 24 h.

7 Pretreatment with cycloheximide before IL-1α/IFN-γ stimulation reduced nitrite levels to basal values. These data suggest that the IL-1α/IFN-γ-induced nitrite production by HT-29 cells is dependent on *de novo* protein synthesis, probably the iNOS enzyme.

8 The addition of TNF-α produced a significant ($P < 0.001$) 3 fold increase of IL-1α/IFN-γ-induced nitrite generation. In marked contrast the presence of TNF-α had no effect on IL-1α/IFN-γ-induced iNOS mRNA expression by HT-29 cells. These findings suggest that the up-regulation by TNF-α of IL-1α/IFN-γ-induced nitrite generation is at the post-transcriptional level.

9 These data suggest that pro-inflammatory cytokines induce NO production in colonic epithelial cells probably due to the induction of iNOS and these cells may be a major source of NO generation in inflammatory bowel disease.

Keywords: Nitric oxide; inducible nitric oxide synthase; HT-29 cells; colonic epithelial cells; ulcerative colitis; inflammatory bowel disease

Introduction

Nitric oxide (NO) is a free radical gas with important immune, cardiovascular and neurological second messenger functions that is implicated in sepsis, ischaemia and inflammation. This molecule is synthesized from the amino acid L-arginine by a family of enzymes, the nitric oxide synthases (NOS) (Moncada & Higgs, 1993). Two of the NOS are continuously present and are termed constitutive NOS (cNOS) (Nathan, 1992; Michel *et al.*, 1993; Nakane *et al.*, 1993). The two isoforms of constitutive NOS require calcium and calmodulin, produce small amounts of NO and are involved in homeostatic processes (Moncada *et al.*, 1991). A third isoform, which is calcium and calmodulin independent, is expressed after induction by certain cytokines, microbes or microbial products, thus it is called inducible NOS (iNOS) (Nussler & Billiar, 1993). Constitutive and inducible isoforms of NOS differ in structure and regulation, while the inducible NOS is responsible for the main NO production in tissues and it is highly regulated by the action of cytokines (Moncada & Higgs, 1993). The human iNOS isoform has been cloned and its expression and activity have been induced in human hepatocytes (Geller *et al.*, 1993), mesangial cells (Nicolson *et al.*, 1993), lung epithelial cells (Robbins *et al.*, 1994; Asano *et al.*, 1994), monocytes/macrophages (Reiling *et al.*, 1994), astrocytes (Lee *et al.*, 1993), chondrocytes (Palmer *et al.*, 1993; Charles *et al.*, 1993) and smooth muscle cells (Junquero *et al.*, 1992; Nakayama *et al.*, 1994) stimulated with a 'cocktail' of cytokines.

Increasing evidence indicates that NO may be involved in acute and chronic inflammation. In the gastrointestinal tract, nitric oxide synthesis has been shown to be increased in colonic mucosa from patients with ulcerative colitis (Middleton *et al.*, 1993b; Boughton-Smith *et al.*, 1993) and leukocyte derived nitric oxide has been found to induce colonic circular smooth muscle relaxation (Middleton *et al.*, 1993a). Active ulcerative colitis is associated with increased vascular permeability and mucosal vasodilatation. In fulminant ulcerative colitis impaired colonic motility is associated with toxic megacolon, which may lead to perforation (Boughton-Smith, 1994). The generation of high levels of NO by the inflammatory stimuli-induced iNOS in the intestinal mucosa and the subsequent formation of reactive products could underlie all these features (Moncada & Higgs, 1993). The intestinal epithelium represents an important interface between host and external environment serving both as a surface for absorption and a defence against ingested pathogens (McKay & Perdue, 1993a,b). The role of human colonic epithelial cells in inflammatory and immune reactions is becoming increasingly recognized. They can present antigen through class II molecules (Mayer *et al.*, 1991),

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and produce soluble mediators such as complement components (Andoh *et al.*, 1993), cytokines and chemokines (Eckmann *et al.*, 1993; Kolios *et al.*, 1994; Schuerer-Maly *et al.*, 1994), known to be important in the communication between inflammatory cells and cells of the immune system. These observations point to an important role for these cells as an essential outpost of host mucosal defence (Sartor, 1994). Several proinflammatory mediators such as interleukin-1 (IL-1), IL-6, platelet-activating factor (PAF), leukotrienes, tumour necrosis factor-α (TNF-α) prostaglandins, and complement are increased in actively inflamed tissues from patients with inflammatory bowel disease (Lichtman & Sator, 1993). Tissue concentrations of IL-2 and interferon-γ (IFN-γ) remain controversial. Secretion of IL-2 and IFN-γ by stimulated lymphocytes in long term culture is lower in inflammatory bowel disease than control tissues. However, *in vivo* levels of mRNA and proteins for these lymphokines are increased in Crohn's disease but not in ulcerative colitis (Sartor, 1994). Intestinal epithelial cells in rats stimulated with cytokines were found to be a source of NO production (Grisham, 1993). Thus, human colonic epithelial cells might be a major source of NO in intestinal inflammation. The present study explores this hypothesis by examining the cytokine requirements for the regulation of iNOS expression and NO generation by human colonic epithelial cells.

Methods

Cell cultures

HT-29 cell line was purchased from European Collection of Animal Cell Cultures (ECACC). HT-29 cells are a well characterized epithelial cell line derived from a primary colon tumour, which have characteristics of normal intestinal epithelium such as epithelial polarity, presence of the actin-binding protein villin, and the occurrence of an enterocytic differentiation (Chantret *et al.*, 1988). Cell cultures were maintained in McCoy's 5A medium (Gibco) supplemented by 10% foetal calf serum, penicillin-streptomycin (10 u ml^{-1} and 10 μg ml^{-1}) and fungizone (0.5 μg ml^{-1}) (complete medium), incubated at 37°C in an atmosphere of 5% CO_2, and passaged weekly. For experiments HT-29 cells were seeded at $2-3 \times 10^4/cm^2$ in 6 well plates (Nunc, U.K.) in complete medium and incubated at 37°C in 5% CO_2 until confluent. Twenty-four hours before stimulation the confluent cell cultures were washed and cultured in fresh medium without foetal calf serum. Growth arrested cultures were treated with the appropriate concentrations of stimuli in medium without foetal calf serum and incubated as above. In all experiments the final volume of culture fluid was 2 ml in each well, which was comprised of medium with the appropriate concentrations of stimuli. Cell counting and viability were checked by Trypan blue at the beginning and the end of each experiment, using representative wells. Viability was always greater than 95%.

Fluorometric assay for the measurement of nitrite

NO production by HT-29 cells was determined by measuring in culture supernatants the stable-end product nitrite. Nitrite was measured using a fluorometric assay as has been described by Misko *et al.* (1993). This assay is based upon the reaction of nitrite with 2,3-diaminonaphthalene (DAN), a light sensitive substance, to form the fluorescent product 1-(H)-naphthotriazole; 200 μl of freshly prepared DAN (0.05 mg ml^{-1} in 0.62 M HCl) were added in 2 ml of sample and mixed immediately. After 10 min incubation at room temperature in the dark, the reaction was terminated with 100 μl of 2.8 N NaOH. A standard curve was produced with known concentrations of sodium nitrite. The samples were measured by a PTI dual wavelength spectrophotofluorimeter, excitation at 365 nm and emission at 405 nm. The sensitivity of the assay is 10 nM.

Northern blot analysis

Total cellular RNA from HT-29 cells was isolated as has been previously described (Strieter *et al.*, 1989). Briefly, HT-29 monolayers were solubilized in a solution containing Tris 25 mM (pH 8.0), guanidine isothiocyanate 4 M, Sarcosyl 0.5%, and 2-mercaptoethanol 0.1 M. After homogenization, the above suspension was added to an equal volume of Tris 100 mM (pH 8.0), EDTA 10 mM and SDS 1%. The RNA was then extracted with chloroform-phenol (1:1, v:v) and chloroform-isoamyl alcohol (24:1, v:v). The total RNA content was alcohol precipitated and the pellet dissolved in diethyl-pyrocarbonate-treated water. The concentration of RNA was measured by obtaining the absorbance at A_{260} and A_{280} nm, and 10 μg of RNA was loaded into each well of the agarose gel. Total RNA was separated by electrophoresis by use of formaldehyde, 1% agarose gels and transferred overnight to nylon membrane (Boehringer Mannheim) by capillary blotting. The blots were baked at 120°C for 20 min and then hybridized as described by Boehringer Mannheim in their DIG luminescent-detection kit. Briefly, membranes were prehybridized for 1 h at 60°C and then hybridized overnight at the same temperature with DIG-labelled oligonucleotide probes (10 ng ml^{-1}) for human iNOS. Bound probes were detected by anti-DIG Fab fragments conjugated to alkaline phosphatase with lumigen PPD (Boehringer Mannheim) as the chemiluminescent substrate. Blots were exposed after hybridization to x-ray film. Quantitation of specific chemokine mRNA was performed by laser densitometry. Equivalent amounts of total RNA load per gel lane were assessed by monitoring 18 s and 28 s RNA.

Materials

Human recombinant IL-1α (specific activity 5×10^7 u mg^{-1}), and TNF-α (specific activity 6×10^7 u mg^{-1}) were generous gifts from Glaxo (Greenford, U.K.) and Bayer (Slough, U.K.) respectively. Human recombinant IFN-γ (specificity $\geqslant 2.0 \times 10^7$ u mg^{-1}) was purchased from Boehringer Mannheim, U.K. 2,3-Diaminonaphthalene was purchased from Lancaster Synthesis Ltd. Cycloheximide and sodium nitrite were purchased from Sigma Chemical. All cell culture reagents and plastics were from Gibco BRL and Nunc Maxisorp, respectively. The digoxigenin (DIG) chemiluminescent detection kit for Northern blotting was from Boehringer Mannheim (Lewes, U.K.). 5'-Digoxigenin labelled probe for iNOS was a cocktail containing 3 antisense 30-mer oligonucleotides purchased from R & D Systems (Abingdon, U.K.).

Statistical analysis

Statistical significance was assessed by Student's *t* test. A probability value of $\leqslant 0.05$ was taken as the criterion for a significant difference. Data are expressed as means ± s.e. of three experiments. Triplicate determinations were performed in each experiment.

Results

Nitrite generation

Growth arrested monolayers of HT-29 cells when stimulated with vehicle produced a small constitutive amount of nitrite (Figure 1). Time course studies revealed a time-dependent increase in constitutive nitrite generation of 50 ± 4, 75 ± 8, and 103 ± 8 nM per 10^6 cells ($n=3$) at 24 h, 48 h, and 72 h, respectively (Figure 2). The pro-inflammatory cytokines IL-1α (10 ng ml^{-1}), TNF-α (100 ng ml^{-1}), and IFN-γ (300 u ml^{-1}), added alone to HT-29 cells did not induce a significant increase in nitrite generation compared to the constitutive nitrite production in vehicle-treated cells (Figure 1). The combination of IL-1α/IFN-γ was the minimal requirement for enhanced

nitrite production, while other pairs of cytokines were ineffective. Stimulation with IL-1α (10 ng ml^{-1})/IFN-γ (300 u ml^{-1}) produced a highly significant ($P < 0.001$) increase in nitrite production of 306±24 nM per 10^6 cells ($n=3$) at 48 h, compared to the basal production of 75±8 nM per 10^6 cells ($n=3$) (Figure 1). The addition of TNF-α to the combination of IL-1α/IFN-γ produced approximately a three fold enhancement of IL-1α/IFN-γ-induced nitrite generation eg 889±35 nM per 10^6 cells ($n=3$) compared to 306±24 nM per 10^6 cells ($n=3$) at 48 h (Figure 1).

Stimulation of HT-29 cells with IL-1α (10 ng ml^{-1})/IFN-γ (300 u ml^{-1}) induced a time-dependent generation of nitrite at 24 (153±7), 48 (306±24) and 72 (384±15) h compared to basal values of 50±4, 75±8, and 103±8 nM per 10^6 cells respectively ($n=3$) (Figure 2). Stimulation with increasing concentrations of IL-1α (0–10 ng ml^{-1}) in the presence of IFN-γ (300 u ml^{-1}) was found to produce a concentration-dependent generation of nitrite production at 48 h by HT-29 cells (Figure 3a). Similarly, increasing concentrations of IFN-γ (0–300 u ml^{-1}) in the presence of IL-1α (10 ng ml^{-1}) were found to produce a concentration-related production of nitrite at 48 h (Figure 3b). Interestingly, different concentrations of TNF-α (0–100 ng ml^{-1}) in the presence of the combination IL-1α (10 ng ml^{-1})/IFN-γ (300 u ml^{-1}) induced a concentration-dependent enhancement of nitrite production (Figure 4). Pretreatment of HT-29 colonic epithelial cells with the protein synthesis inhibitor cycloheximide (5 μg ml^{-1}) for 1 h before IL-1α (10 ng ml^{-1})/IFN-γ (300 u ml^{-1}) stimulation reduced the nitrite levels from 306±24 to 80±6 nM per 10^6 cells ($n=3$) at 48 h, which was no different to basal nitrite production of 75±8 nM per 10^6 cells. Finally, treatment of unstimulated cells with cycloheximide (5 μg ml^{-1}) did not affect the basal nitrite production (74±4 vs 75±8 nM per 10^6 cells).

Inducible NOS mRNA expression by HT-29 cells

To determine whether the inducible generation of nitric oxide by HT-29 epithelial cells was due to the induction of iNOS, the iNOS mRNA expression was determined by Northern blot analysis. The time course of iNOS mRNA expression in epithelial cells after stimulation with IL-1α (10 ng ml^{-1})/IFN-γ (300 u ml^{-1}) was examined. In unstimulated cells iNOS transcripts were not detected at any of the time points. The combination IL-1α (10 ng ml^{-1})/IFN-γ (300 u ml^{-1}) induced iNOS mRNA expression, which was first observed at 6 h, peaked at 24 h and was undetectable by 72 h (Figure 5). Increasing concentrations of IL-1α (0–10 ng ml^{-1}) in the presence of IFN-γ (300 u ml^{-1}) were found to produce concentration-related iNOS mRNA expression after 24 h treatment (Figure 6a). Similarly, using different concentrations of IFN-γ (0–300 u ml^{-1}) in the presence of IL-1α (10 ng ml^{-1}) we found concentration-dependent iNOS mRNA expression at 24 h in HT-29 cells (Figure 6b). These results demonstrate that both IL-1α and IFN-γ produce concentration-dependent iNOS and mRNA expression in HT-29 cells which induces concentration-dependent nitrite formation by

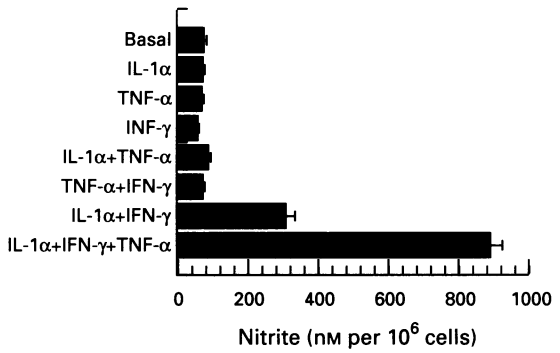

Figure 1 Nitrite production by HT-29 cells following stimulation for 48 h with vehicle, IL-1α (10 ng ml^{-1}), TNF-α (100 ng ml^{-1}), and IFN-γ (300 u ml^{-1}) added alone or in combination. Each column is the mean and horizontal lines s.e. of three experiments.

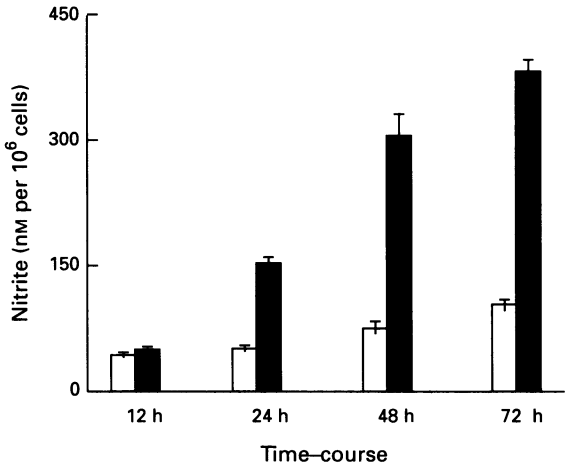

Figure 2 Time course of nitrite production by HT-29 cells following stimulation with vehicle (open columns) or IL-1α (10 ng ml^{-1})/IFN-γ (300 u ml^{-1}) (solid columns). Each column is the mean and vertical lines s.e. of three experiments.

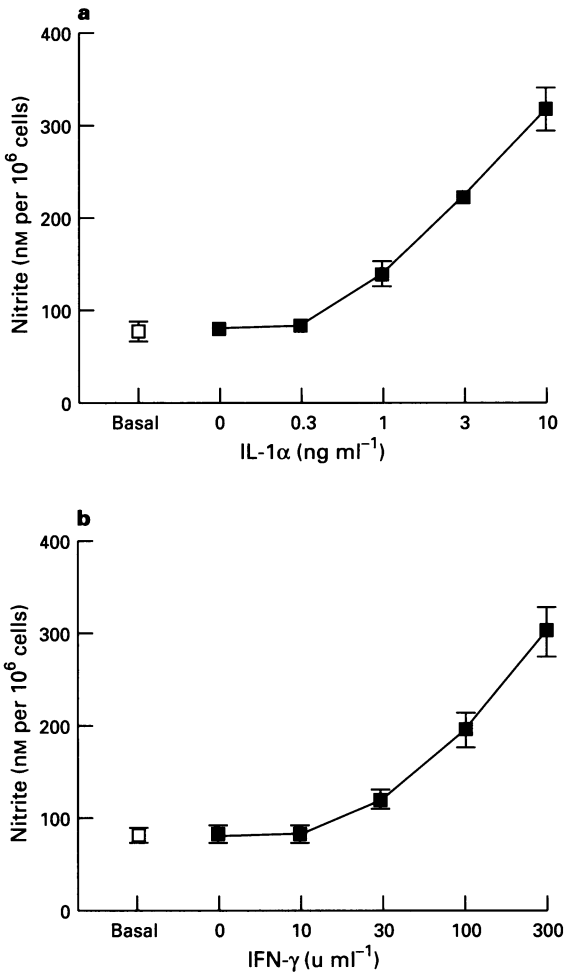

Figure 3 (a) Nitrite production by HT-29 cells after 48 h treatment with different concentrations of IL-1α (0–10 ng ml^{-1}) in the presence of IFN-γ (300 u ml^{-1}) (■). (b) Nitrite production by HT-29 cells after 48 h treatment with different concentrations of IFN-γ (0–300 u ml^{-1}) in the presence of IL-1α (10 ng ml^{-1}) (■). Each point is the mean±s.e. of three experiments.

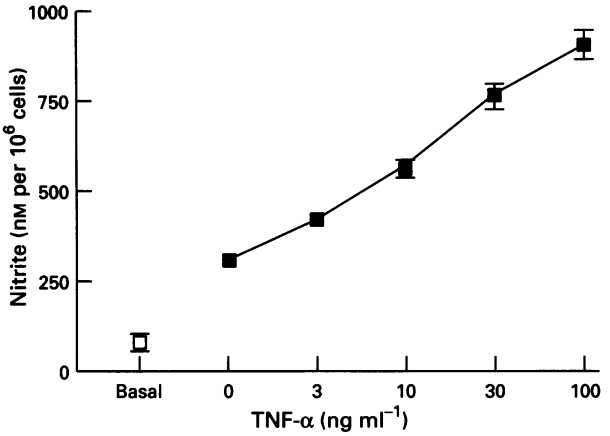

Figure 4 Effect of TNF-α (0–100 ng ml^{-1}) on (■) IL-1α (10 ng ml^{-1})/IFN-γ (300 u ml^{-1})-induced nitrite generation after 48 h treatment by HT-29 cells. Each point is the mean ± s.e. of three experiments.

these cells (Figure 3a and b). In marked contrast, experiments using different concentrations of TNF-α (3–100 ng ml^{-1}) in the presence of IL-1α (10 ng ml^{-1})/IFN-γ (300 u ml^{-1}) demonstrate that the addition of TNF-α was without effect on the IL-1α/IFN-γ-induced iNOS expression in HT-29 colonic epithelial cells (Figure 7). These results suggest that the up-regulation by TNFα of the IL-1α/IFN-γ-induced nitrite generation by HT-29 cells is at the post-transcriptional level.

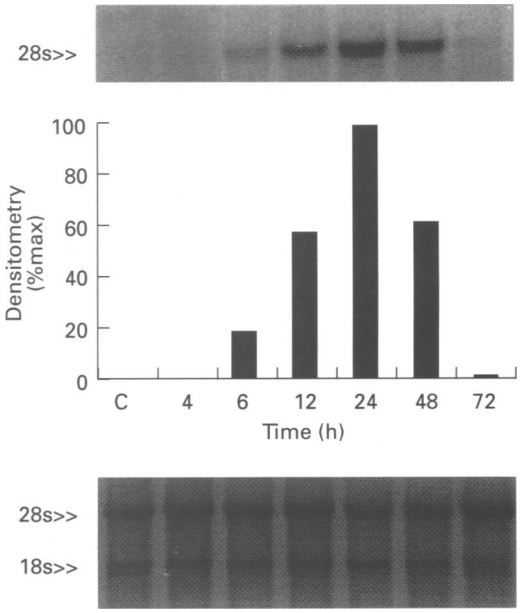

Figure 5 Northern blot analysis of time course of iNOS mRNA expression in HT-29 cells stimulated with IL-1α (10 ng ml^{-1})/IFN-γ (300 u ml^{-1}). The top panel is the Northern blot, the middle panel is the densitometry analysis of blot and the lower panel is the ethidium bromide stained 18 s and 28 s bands indicating equal loading of the lanes. Representative of three experiments. C: control.

Discussion

In this study, we have demonstrated that human HT-29 colonic epithelial cells in response to specific combinations of cytokines express iNOS mRNA and produce large quantities of nitrite. Tissue-derived cytokines are known to be potent inducers of NO synthase in macrophages, neutrophils and endothelial cells but the demonstration of such activity in human colonic epithelial cells is potentially of great importance in colonic inflammation. Although our results were obtained with a colonic epithelial cell line, which has many characteristics in common with 'normal' colonic epithelial cells (Chantret *et al.*, 1988) the possibility exists that 'normal' cells may behave differently from tumour cells. In support of our findings , studies in patients with ulcerative colitis have shown a remarkable increase in NO synthase activity in the inflamed mucosa compared to the uninflamed controls but the types of cells responsible for nitrite generation is not known (Middleton *et al.*, 1993b; Boughton-Smith *et al.*, 1993). More recent studies, using *in situ* hybridization and immunohistochemistry to detect iNOS, demonstrated high expression of iNOS localised to the surface epithelium and crypts in the mucosa from patients with ulcerative colitis (Reynolds *et al.*, 1995). Previous studies, using histochemical analysis, have demonstrated extensive neural and vascular localisation of iNOS expression in rat intestine (Nichols *et al.*, 1993), in addition rat intestinal

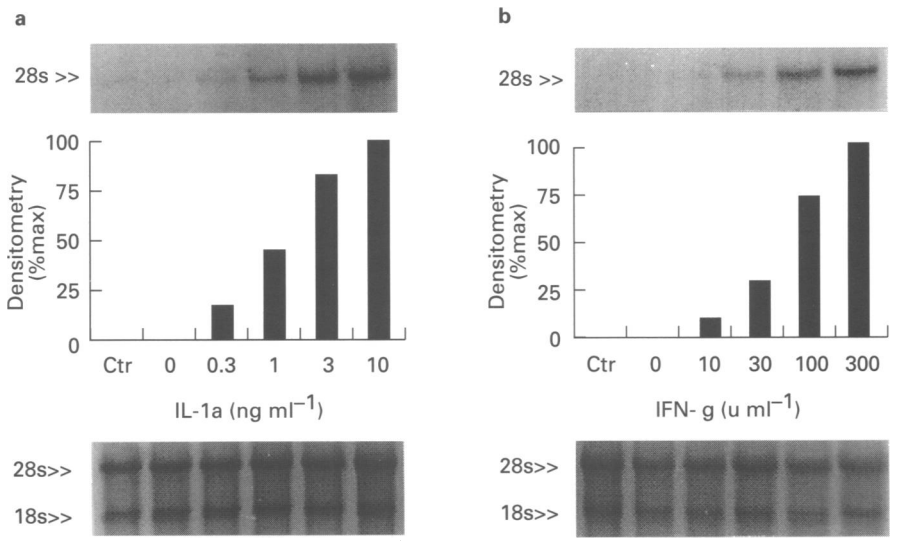

Figure 6 (a) iNOS mRNA expression in HT-29 cells after 24 h treatment with different concentrations of IL-1α (0–10 ng ml^{-1}) in the presence of IFN-γ (300 u ml^{-1}). (b) iNOS mRNA expression in HT-29 cells after 24 h treatment with different concentrations of IFN-γ (0–300 u ml^{-1}) in the presence of IL-1α (10 ng ml^{-1}). The top panel of each figure is the Northern blot, the middle panel is the densitometry analysis of blot and the lower panel is the ethidium bromide stained 18 s and 28 s bands indicating equal loading of the lanes. Representative of two experiments.

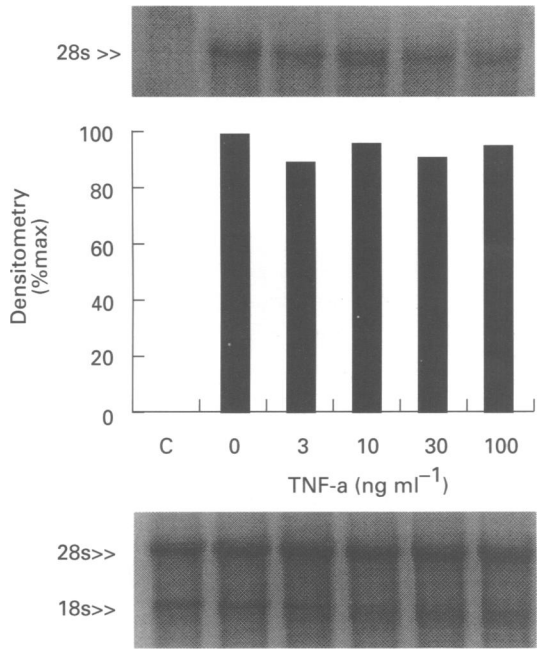

Figure 7 Effect of TNF-α (0–100 ng ml^{-1}) on IL-1α (10 ng ml^{-1})/IFN-γ (300 u ml^{-1})-induced iNOS mRNA expression in HT-29 cells after 24 h treatment. The top panel is the Northern blot, the middle panel is the densitometry analysis of blot and the lower panel is the ethidium bromide stained 18 s and 28 s bands indicating equal loading of the lanes. Representative of three experiments.

epithelial cells in culture when stimulated with cytokines and lipopolysaccharide were found to produce NO (Grisham, 1993).

The expression of iNOS activity in cells and tissues is thought to be controlled by a combination of cytokines and the profile of cytokine responsiveness varies with the cell type. In our study, unstimulated HT-29 cells produced a small amount of nitrite in a time-dependent manner, which appeared to be due to constitutive NOS as no iNOS mRNA expression was detected and the production of nitrite was unaffected by the protein synthesis inhibitor cycloheximide. None of cytokines added alone increased the nitrite generation. These results are similar to those found in cultured human hepatocytes (Nussler *et al.*, 1992; Geller *et al.*, 1993), lung epithelial cells (Asano *et al.*, 1994; Robbins *et al.*, 1994), and mesangial cells (Nicolson *et al.*, 1993). In contrast, treatment of either human vascular smooth muscle cells (Junquero *et al.*, 1992) with IL-1β, or human chondrocytes (Charles *et al.*, 1993; Maier *et al.*, 1994) with single proinflammatory cytokines IL-1β, IFN-γ, or TNF-α caused a marked increase in iNOS mRNA, iNOS protein, and nitrite generation. The combination of IL-1α and IFN-γ was the minimal stimulation required, while no other pair of cytokines was effective, for iNOS mRNA expression or significant increase of nitrite generation in HT-29 cells. Similar results were also obtained with human mesangial cells (Nicolson *et al.*, 1993). Combinations of IL-1α and IFN-γ induced a concentration-dependent nitrite generation and iNOS mRNA expression. Treatment of HT-29 cells with IL-1α/IFN-γ induced up to a 4 fold increase in nitrite generation, which is in contrast to human hepatocytes as the same combination of cytokines induced only a 5% increase in nitrite generation (Nussler *et al.*, 1992). The combination TNF-α/IL-1α/IFN-γ was most effective with nitrite levels increasing 12 fold compared to constitutive values. The presence of these three signals produced the same high increase of nitrite production in human hepatocytes, lung epithelial cells, and mesangial cells. Studies of iNOS mRNA expression revealed that the TNF-α up-regulation of IL-1α/IFN-γ-induced nitrite generation by HT-29 cells was at the post-transcriptional level. Cycloheximide, a protein synthesis inhibitor, has been shown to inhibit the nitrite generation in human cells treated with cytokines (Junquero *et al.*, 1992; Nicolson *et al.*, 1993; Palmer *et al.*, 1993). Similarly in our study pretreatment with cycloheximide reduced the nitrite production to constitutive levels, regardless of maximal stimulation with IL-1α/IFN-γ, suggesting that the nitrite evoked by these cytokines is dependent on *de novo* protein synthesis, probably the iNOS enzyme and/or essential peptide co-factors.

We believe that the increased nitrite generation is due to iNOS enzyme activity because this production was induced by a combination of cytokines and blocked by cycloheximide, a protein synthesis inhibitor. However, more importantly, we have only detected increased nitrite generation with those treatments which induce the expression of iNOS mRNA. Time course studies with the combination of IL-1α and IFN-γ demonstrated that the nitrite generation peaked at 72 h, while peak rate of nitrite production occurs between 24–48 h and slows between 48–72 h. The different rate of nitrite generation could represent peak protein translation or co-factors generation at 24–48 h, which is necessary for optimum enzyme activity. Furthermore Northern analysis demonstrated that iNOS mRNA peaks at 24 h and is undetectable by 72 h, indicating a loss of iNOS mRNA from 24 h onwards available for protein synthesis. These results are consistent with the findings from human hepatocytes (Nussler *et al.*, 1992; Geller *et al.*, 1993) in which the iNOS and mRNA peaked at 8 h after stimulation and the nitrite generation peaked at 48 h. The fact that the maximum nitrite generation occurs more than 24 h after the peak of mRNA indicates that iNOS enzyme is stable and active for long after its synthesis. The gap between maximum enzyme message expression and maximum nitrite generation might suggest the requirement of *de novo* synthesis of a co-factor, e.g. tetrahydrobiopterin (Stuehr & Griffith, 1992; Nussler *et al.*, 1992) for maximal activity. These data suggest that human colonic epithelial cells must now be considered together with human hepatocytes, smooth muscle cells, chondrocytes, lung epithelial cells, and mesangial cells as an important source of nitric oxide production.

Increased expression and production of NO may explain some of the pathophysiological features of active ulcerative colitis. NO is a potent vasodilator and it is known that during active episodes of colonic inflammation blood flow to the mucosa and submucosa of the colon is increased between 2 and 6 fold (Hulten *et al.*, 1977). NO may be an important pathway whereby inflammation induces hyperaemia, mucosal vasodilatation and increased vascular permeability. NO is capable of affecting alterations in intestinal motility in inflammatory bowel disease by mediating the effects of gastrointestinal hormones and non-adrenergic and non-cholinergic mediated relaxation of colonic smooth muscle (Hata *et al.*, 1990). In active ulcerative colitis this can contribute to diarrhoea by depressing segmentation in the colon and in fulminant colitis impaired colonic motility is associated with toxic megacolon, which may lead to perforation. NO is important in the control of absorption from intestine. At physiological concentrations NO promotes absorption but at higher levels may produce secretory effects in the colon (MacNaughton, 1993).

Enhanced NO release via the induction and activation of iNOS in the colon may contribute directly to the mucosal damage due to cytotoxic activity by a variety of mechanisms including inhibition of DNA synthesis, inhibition of mitochondrial function and intracellular iron release (Drapier & Hibbs, 1986; Kwon *et al.*, 1991). NO can freely interact with oxygen metabolites to yield nitrosating species and the formation of nitrosamines may be important in the development of colorectal cancer in ulcerative colitis. High levels of nitrosamines have been demonstrated in rectal dialysates of patients with active inflammatory bowel disease (MacNaughton, 1993).

In conclusion we have shown that the expression of human iNOS mRNA and nitrite generation in HT-29 colonic epithe-

lial cells is differently regulated by combinations of cytokines. If these results can be extrapolated to primary colonic epithelial cells then it is likely that NO and its metabolites derived from epithelial cells may be of central pathogenic significance in intestinal inflammation. These results have stimulated us to search for naturally occurring agents, such as asymmetrical dimethylarginine which is found in renal failure (Vallance *et al.*, 1992), and pharmacological agents that might inhibit the production of active nitrogen species and prove of value in the treatment of inflammatory bowel disease.

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