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The bile acid deoxycholic acid (DCA) at neutral pH activates NF-κB and induces IL-8 expression in oesophageal cells *in vitro*

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Barrett's oesophagus patients accumulate chromosomal defects during the histological progression to cancer, one of the most prominent of which is the amplification of the whole of chromosome 4. We aimed to study the role that the transcription factor NF-κB, a candidate cancer-promoting gene, present on chromosome 4, plays in Barrett's oesophagus, using OE33 cells as a model. Specifically, we wanted to determine if NF-κB was activated by exposure to bile acid (deoxycholic acid) in oesophageal cells. We employed pathway specific cDNA microarrays and real-time PCR, to first identify bile acid induced genes and specifically to investigate the role of NF-κB. An NF-κB reporter system was used, as well as an inhibitor of NF-κB (pyrrolidine dithiocarbamate) to confirm the activation of NF-κB by bile. We show that physiological levels of DCA (100–300 μM) were capable of activating NF-κB in OE33 cells and inducing NF-κB target gene expression (particularly IκB and IL-8). Other gene expression abnormalities were also shown to be induced by DCA. Importantly, preliminary experiments showed that NF-κB activation by bile occurred at neutral pH, but not at acid pH. Acidic bile did however cause over-expression of the c-myc oncogene, as reported previously. Hence, we present data showing that NF-κB may be a key mediator of carcinogenesis in bile exposed Barrett's tissues. In addition, neutral bile acids appear to play a significant part in reflux induced gene expression changes. We postulate that the activation of the survival factor NF-κB by bile may be linked to the previous cytogenetic data from our laboratory showing the amplification of NF-κB's chromosome (chromosome 4), during Barrett's cancer progression. Hence chromosome 4 amplification may provide a survival mechanism for bile exposed oesophageal tissues via NF-κB.

Introduction

The incidence of adenocarcinoma of the oesophagus is increasing dramatically amongst Western populations. A major risk factor for oesophageal adenocarcinoma is the presence of intestinal metaplasia in the lower oesophagus (so-called Barrett's oesophagus). Barrett's oesophagus is caused by prolonged reflux of stomach and duodenal contents and has been reported in up to12% of patients undergoing endoscopy for reflux symptoms (1). In order to provide adequate surveillance for the increasing Barrett's population it is evident that molecular markers of cancer progression are needed (2). A number of specific molecular alterations have already been shown to be correlated with cancer development in Barrett's tissues (2–4). As well as serving as predictive markers of cancer development, these molecular alterations, may help us to understand the mechanism of cancer development and provide the basis for intervention strategies in the future.

In terms of cancer initiation and promotion in Barrett's tissues, it is becoming accepted that refluxed bile acids play a significant role (5–7). Barrett's metaplasia patients have increased bile exposure and increased proportions of secondary bile acids in particular in their refluxate (8). Furthermore, Barrett's patients with early adenocarcinoma have higher exposure to bile acids than uncomplicated Barrett's patients (9). Unconjugated bile acids in particular have been suggested as an important factor in the progression of the neoplastic sequence in Barrett's cancers (10,11). Opinion is still divided as to the relative roles that bile acids and stomach acid play (12). However, due to the fact that patients undergoing acid suppression therapy still progress to adenocarcinoma, it suggests that factors other than acid are important in Barrett's carcinogenesis. Bile acids are known to be capable of inducing DNA damage (13), chromosome aberrations (14), as well as causing gene expression abnormalities (15,16); hence bile acids are promising candidates as oesophageal carcinogens. When coupled to the compensatory proliferation, which has been postulated to occur *in vivo* as a result of bile induced apoptosis (17), we can see a picture of bile as both an initiator and promoter of cancer development.

Our previous cytogenetic studies of Barrett's progression have identified two particular chromosomal abnormalities, which appear to be intrinsically linked to cancer progression. We have shown that chromosome 4 and chromosome 8 are specifically amplified during cancer development (18,19). In the case of chromosome 8 amplification, it can be readily explained by the presence of the c-myc oncogene on its q arm at locus 8q24, which has been linked previously to Barrett's progression (15). This amplification has also been noted by other researchers (20,21). However, the finding of chromosome 4 amplification in Barrett's patients is, as far as we are aware, unique to our laboratory and has no obvious mechanistic basis. Hence, we have been looking to identify candidate cancer-promoting genes on chromosome 4.

NF-κB is an anti-apoptotic, pro-inflammatory transcription factor present on chromosome 4, whose increased activity has been linked to many types of cancer (22). Figure 1 illustrates one of the possible activation pathways of NF-κB. NF-κB when activated enters the nucleus and switches on its target genes, hence NF-κB is controlled post-translationally and does not require *de novo* gene expression. There is already some evidence that NF-κB may play a role in Barrett's progression

Abbreviation: DCA, deoxycholic acid.

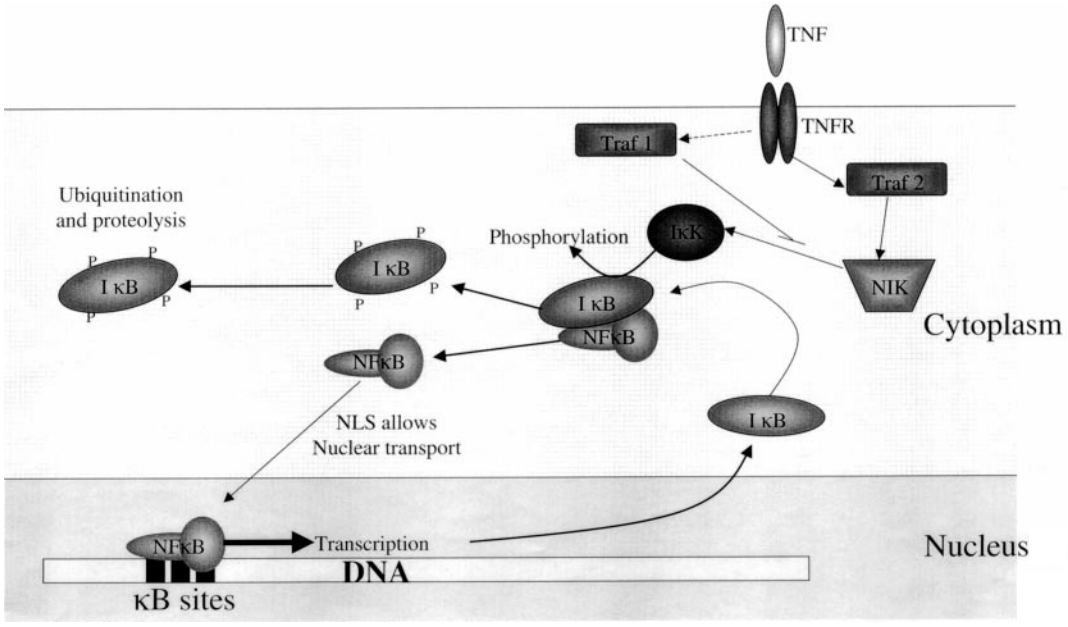

Fig. 1. The TNF/NF-κB pathway. TNF activates NF-κB via a TNFR/Traf2/NIK/IKK pathway, antagonized by TRAF1. This results in IκK phosphorylating IκB leading to its degradation. NF-κB is then free to cross the nuclear membrane (hence post-translational control). NF-κB then switches on its target genes including IκB, resulting in a negative feedback loop.

(23,24). There are also reports of the up-regulation of several members of the NF-κB pathway during cancer development in Barrett's patients. For example, TNF, an upstream activator of NF-κB, has been shown to be progressively up-regulated in Barrett's tissues (25) and IL-8, a downstream transcriptional target of NF-κB has also been shown to be up-regulated in Barrett's related adenocarcinomas (23). Finally, there are reports that bile acids are capable of activating NF-κB in colon cancer cell (26,27) and hepatocytes (28). Therefore, it is possible that refluxed bile acids may be involved in NF-κB activation in Barrett's tissues.

In this study we have taken a gene expression approach in order to answer two inter-related questions. First, using cultured oesophageal cells, are bile acids responsible for gene expression abnormalities known to be present in Barrett's tissues? Secondly, is NF-κB specifically activated in bile exposed cells?

Materials and methods

Cell culture

OE33 cells derived from a Barrett's related adenocarcinoma were purchased from the European Collection of Cell Cultures (Salisbury, UK). These cells, which are basically tetraploid, were chosen due to their widespread use as models of oesophageal cancer and due to our experience of them in previous studies (14). Cells were grown in 25 ml of RPMI 1640 (Life Technologies, Paisley, UK), supplemented with 1% glutamine and 10% bovine calf serum (Life Technologies). The cells were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were detached from the flasks prior to subculturing by the removal of the medium and the addition of 10 ml of trypsin (0.25% trypsin in Hanks solution) for 5–10 min.

Treatment of the OE33 cells

Sub-confluent OE33 cells were treated with varying concentrations (100–300 μM) of the bile acid deoxycholic acid (DCA) (Sigma, Poole, UK) in serum-free medium for the allotted period of time (0–24 h) at 37°C. These doses of DCA were chosen from previous studies of DCA induced chromosomal damage (14). In order to study the effect of acidified bile on OE33 cells, the pH of the serum-free medium was adjusted to the desired level with concentrated HCl prior to the addition of DCA. Furthermore, in the case of treatment with the inhibitor of NF-κB, i.e. pyrrolidine dithiocarbamate (PDTC) (Sigma), it was added at a concentration of 1 or 10 μM, shown previously to be effective at inhibiting NF-κB (26), along with the specified dose of DCA.

RNA extraction and reverse transcription

The OE33 cells were subject to RNA extraction through the use of a Midi RNeasy RNA extraction kit (Qiagen, Crawley, Surrey, UK). The extracted RNA was subsequently DNase treated using a DNA-free kit (Ambion, Huntingdon, UK). The RNA quantity was assessed by spectrophotometry at 260 nm and RNA quality was assessed by RT–PCR using primers for β-actin DNA (forward: gatggccacggctgcttc, reverse: tgcctcagggcagcggaa). Reverse transcription of the RNA samples (100–500 ng) was carried out for 60 min at 42°C using a Retroscript RT kit (Ambion). PCR was subsequently carried out using both cDNA and RNA to confirm the success of the DNase treatment.

Membrane array analysis

Gene expression analysis was carried out using a number of pathway-specific Q series GEA membrane arrays (Superarray, Frederick, MD) containing 96 genes from each particular pathway. Total RNA (2 μg) was labelled with Biotin UTP (Roche, Mannheim, Germany) during reverse transcription with Superscript RNA H^- (Invitrogen, Paisley, UK). The labelled cDNA was then hybridized onto the membranes overnight following the manufacturer's instructions. Post-hybridization washes and a blocking step were carried out, followed by chemiluminescent detection of hybridized signals. Arrays were then exposed to hyperfilm ECL (Amersham Biosciences, Bucks, UK) and the film was developed using a Curix 60 automatic developer (Agfa, Mortsel, Belgium). The arrays all contained 10 housekeeping genes in addition to the 96 pathway specific genes; this allowed the RNA quality and the success of the labelling to be monitored in each experiment. Array experiments were performed in duplicate to ensure that only consistent gene expression changes were identified. The arrays used in this study included a Cancer Pathway array, a Signal Tranduction array, a NF-κB array, a Cell Cycle array and a Chemokine/Chemokine Receptor array.

Real-time PCR

The RNA (2 μg) was first reverse transcribed into cDNA using a Retroscript RT kit (Ambion) as detailed above. Real-time PCR was then carried out using an iCycler PCR machine (Bio-Rad, Hemel Hempstead, UK). The PCR primers used in real-time analysis were as follows: β-actin (see above); IκB (forward: acactagaaaacttcagatgc, reverse: acacagtcatcatagggcag); il8 (forward: caatcctagtttgatactccc, reverse: aattactaatattgactgtggag); NF-κB (forward: ccacaagcaagaagctgaag, reverse: agatactatctgtaagtgaacc). C-myc primers have been published previously (15). cDNA was amplified in three separate reactions and each cDNA batch was aliquoted in triplicate into 96 well plates using the appropriate PCR primers and the IQ Sybr-green supermix (Bio-Rad). Relative

gene expression between samples was calculated through the inclusion of a cDNA dilution series of a reference sample, amplified with each primer set separately. In order to account for RNA loading differences between samples, the measured gene expression was normalized against β-actin expression. The *t*-test was used to assign statistical significance.

NF-κB reporter system

The ability of DCA to activate NF-κB was investigated using a luciferase reporter system in which the luciferase gene was under the control of a NF-κB dependent promoter (Stratagene, Cambridge, UK). In short, the OE33 cells were seeded in triplicate into 6 well tissue culture flasks and the NF-κB luciferase plasmid was transfected into them using the genejammer transfection reagent (Stratagene). Initial experiments validated the transfection using a supplied positive control for NF-κB activity. The cells were then either exposed to DCA for varying periods of time (0–4 h) or remained untreated. After exposure, the cells were processed following the manufacturer's recommendations to release the luciferase enzyme and the subsequent luciferase expression was quantified with the Bright-Glo luciferase assay system (Promega, Southampton, UK). Resulting luminescence was measured using a luminometer (Anthos Labtech Instruments, Salzburg, Austria). Fold increases were calculated based on the background luminescence of the untreated cells (untreated cells were set up for each time point analysed). The *t*-test was used to evaluate differences between the triplicate readings from treated and untreated cells.

Results

DCA specifically switches on IκB and IL-8

RNA extracted from untreated OE33 cells and OE33 cells treated for 24 h with 300 μM DCA at pH 7, was analysed using five different pathway specific membrane arrays. By far the most prominent of the DCA induced gene expression changes were the switching on of IκB and IL-8. As IκB and IL-8 are transcriptional targets of NF-κB, this suggests activation of NF-κB by DCA (as is demonstrated below). As shown in Figure 2, using a cancer pathway array and a signal transduction array, both IκB and IL-8 are substantially up-regulated 24 h after DCA treatment. Figure 2 also shows a number of other changes in gene expression 24 h after DCA treatment (mostly up-regulation), these (and other changes noted from other arrays) are documented in Table I.

The gene expression alterations listed in Table I from the five different arrays used, have been grouped into four separate groups based on the function of the genes involved. As IL-8 (29) and IκB (30) have previously been suggested to be transcriptional

Table I. Other DCA induced gene expression alterations

Gene	↑↓	Time point
NFκB related genes		
Tumour necrosis factor alpha induced protein (TNFaip)	↑↑	4–24 h
Flice inhibitory protein (FLIP, Cflar, Casper)	↑	24 h
Cellular proteases/invasion genes		
Plasminogen activator, Urokinase (Plau)	↑↑↑	1–24 h
Plasminogen activator receptor (PlauR)	↑↑	24 h
Plasminogen activator inhibitor 1 (Serpine 1)	↓↓	24 h
Serine protease inhibitor B5 (maspin, Serpine B5)	↑↑	24 h
Plasminogen activator inhibitor B2 (Serpine B2, PAI-2)	↑↑	24 h
Growth factors/receptors		
Melanoma growth stimulating activity (MGSA, Gro 1)	↑	24 h
Vascular endothelial growth factor (VEGF)	↑	4–24 h
Epidermal growth factor receptor (EGFR)	↑	1–24 h
Adhesion molecules		
β-Catenin	↑	1–24 h
Integrin B1, integrin A6	↑	4–24 h
E-Cadherin	↑	1 h
Intercellular adhesion molecule (Icam)	↑	4–24 h
Neural cell adhesion molecule (Ncam)	↑↑	1–4 h

This table contains a list of genes whose expression was consistently altered after exposure to DCA. By consistent, we mean that the expression change was evident on both duplicate array experiments (although no real-time PCR data available). In the right hand column is the time point at which the expression change was evident. The arrows in the central column reflect the relative strength of the expression changes.

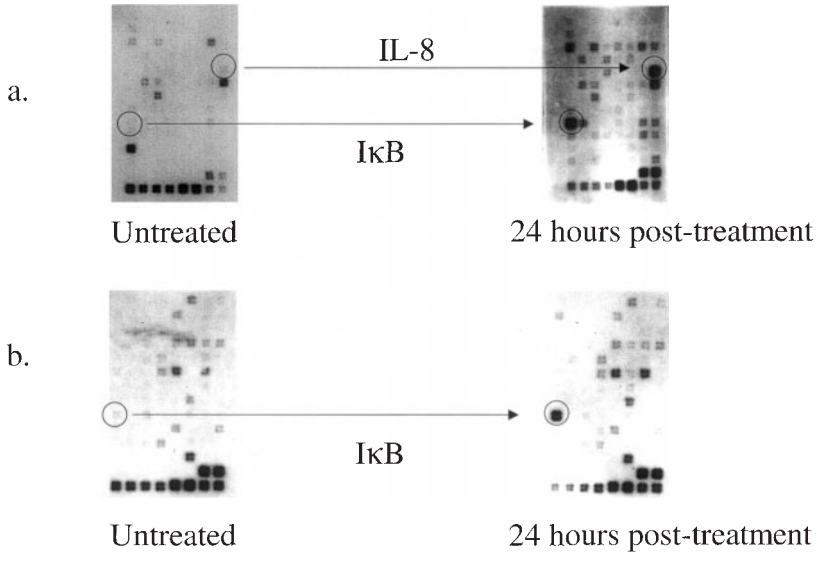

Fig. 2. Membrane array analysis of DCA treated OE33 cells, 24 h post-exposure. (**a**) Cancer pathway arrays were used to study gene expression abnormalities induced by 300 μM DCA relative to untreated OE33 cells. These example arrays show the up-regulation of the IκB and IL-8 genes by DCA. (**b**) Two example signal transduction arrays are shown; those hybridized with cDNA from untreated OE33 cells and cDNA from OE33 cells 24 h after DCA exposure.
A number of other genes are up-regulated by DCA, particularly evident in the cancer pathway array in (a). These genes (and others) are identified in Table I.

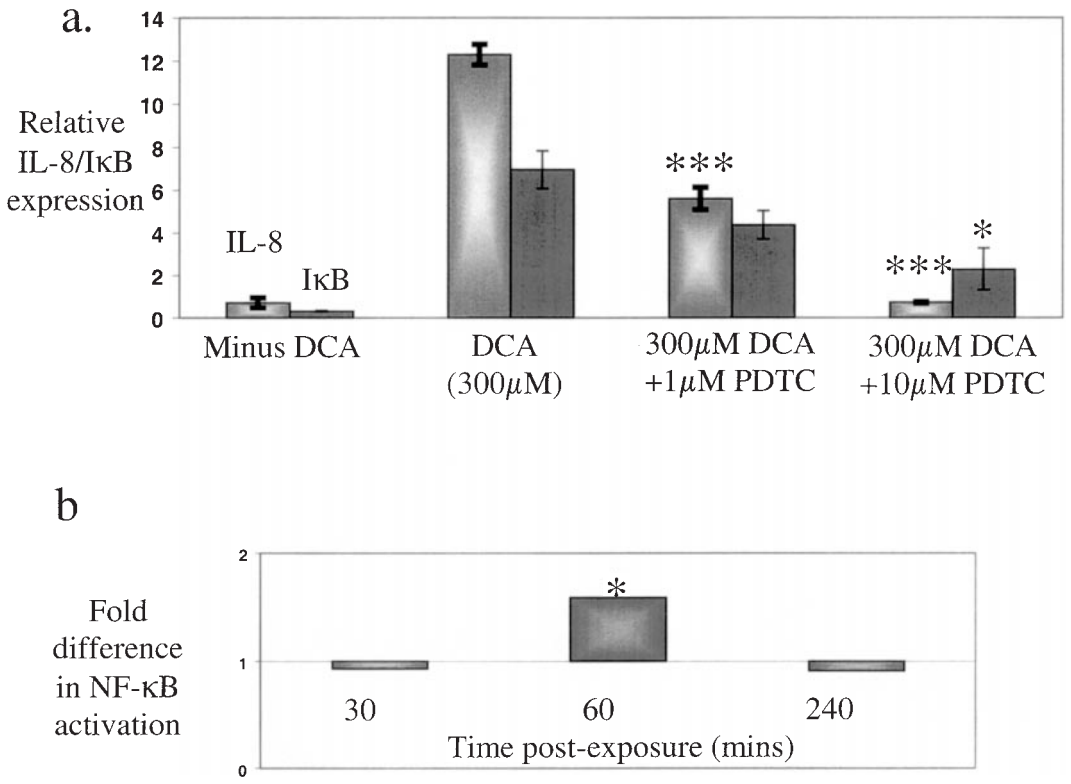

Fig. 3. Demonstration that NF-κB is specifically activated by DCA. (**a**) The effect that an inhibitor of NF-κB (pyrrolidine dithiocarbamate) has on NF-κB activation, as measured by IκB and IL-8 expression. Both levels of IL-8 gene expression inhibition are significant ($P < 0.001$), whereas the inhibition of IκB is only significant ($P < 0.05$) at the 10 μM PDTC concentration. (**b**) Results of the NF-κB reporter system after exposure to DCA. An exposure of 1 h causes a significant increase in NF-κB activity as measured with this reporter system ($P < 0.05$).

targets of NF-kB, we have subsequently used IL-8 and IκB expression as surrogates for NF-κB activation. In order to support the use of IL-8 and IκB as surrogates of NF-κB activation we exposed cells to bile in the presence of an inhibitor of NF-κB, pyrrolidine dithiocarbamate (PDTC). Figure 3a shows the result of this experiment and demonstrates that inhibition of NF-κB results in suppression of DCA induced IL-8 and IκB expression, thus confirming the link between NF-κB activation and IL-8 and IκB up-regulation. Figure 3b further demonstrates the activation of NF-κB by bile acids, using a luciferase reporter system containing NF-κB promoter elements. DCA (300 μM at neutral pH) caused significant luciferase activity 1 h after incubation with the cells bearing the reporter.

Time course study on DCA induced gene expression changes

In order to study the effect of DCA exposure duration on the gene expression changes noted above (particularly IκB and IL-8), a time course study was undertaken whereby OE33 cells were exposed to 300 μM DCA for 1–24 h. For this study only the cancer pathway finder array was employed, as this array contained both the IκB and IL-8 genes. The results of this study showed that the DCA induced expression of IκB and IL-8 was first evident 4 h post-exposure. Real-time PCR was used to confirm these findings and to quantify the increases in gene expression (Figure 4a). NF-κB expression was also measured by real-time PCR, but did not change during the time series, as expected. Figure 4a also shows examples of the individual array signals for the IκB and IL-8 genes across the time series. The fold increases in IκB and IL-8 gene expression as measured by real-time PCR, at 4 h were 46- and 16-fold, respectively. The similar figures at 24 h were 64- and 17-fold, respectively.

In order to assess the role of DCA dose level on the gene expression patterns noted above, DCA exposures of 100 and 200 μM were also performed at the 4 h time point. IL-8 up-regulation was employed here as a measure of NF-κB activation. Real-time PCR showed that the up-regulation of the IL-8 gene at 100 and 200 μM was 3.3- and 5.4-fold, respectively (Figure 4b). Both these levels of up-regulation were statistically significant ($P < 0.05$). The DCA induced activation of NF-κB and up-regulation of IL-8 and IκB has also been demonstrated by us in another cell line (OE21, derived from a squamous cell carcinoma) (Harries *et al.*, manuscript in preparation). Therefore, these data coupled with reports of NF-κB activation in colon cancer cells and hepatocytes (26,27) demonstrate that the effect is not cell line specific.

The effect of pH on DCA induced gene expression

As reflux episodes usually occur in patients at acid pH, we investigated whether lowering the pH of the bile to 4 (the normal pH of the stomach) affected the changes in gene expression noted above. Treating OE33 cells for 4 h with 300 or 200 μM DCA at pH 4 resulted in extensive cell death, hence little RNA was available for expression analysis. Measuring gene expression changes against a background of cytotoxicity could lead to unreliable data. Therefore, DCA doses of 100 μM, shown previously to bring about the same range of gene expression changes as 300 μM (albeit at a lower level), were employed at pH 4, as this dose produced little cytotoxicity. Real-time data obtained from the analysis of IL-8 expression in cells exposed to 100 μM DCA at pH 4 and 7 is shown in

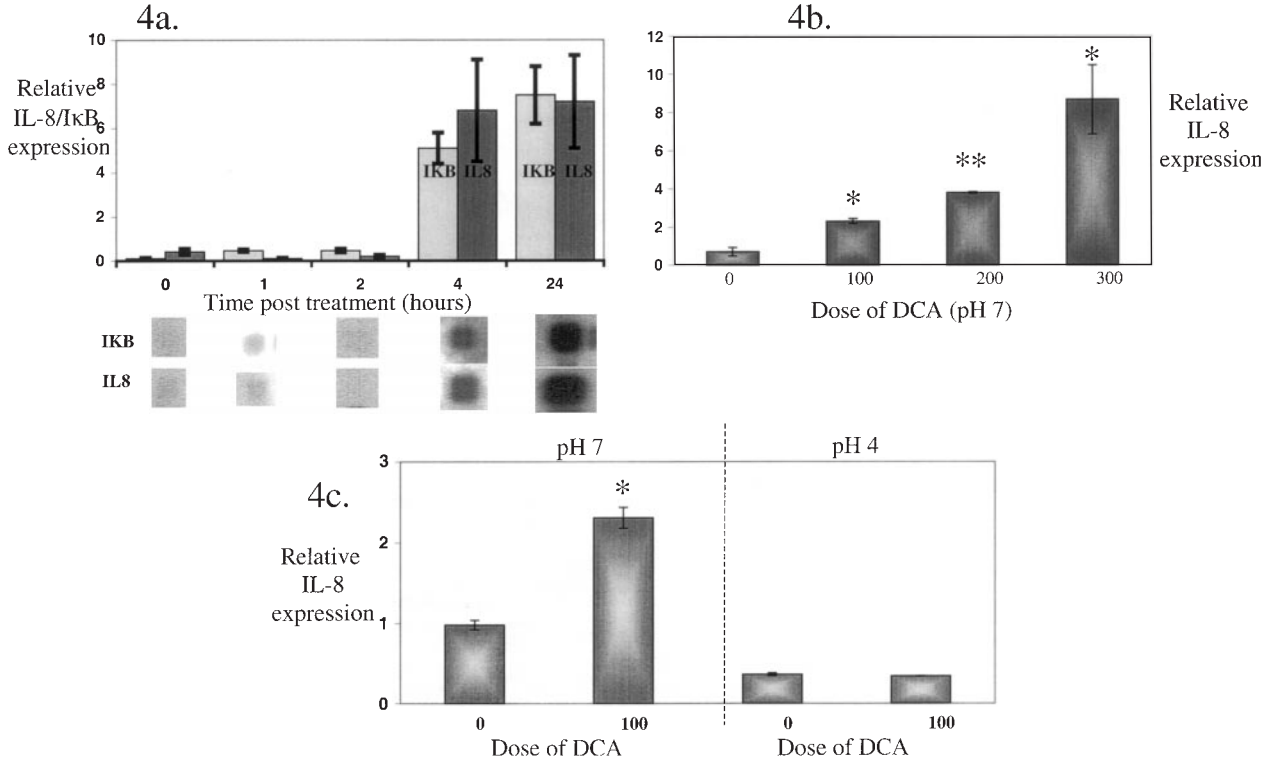

Fig. 4. Real-time PCR was used to confirm and quantify gene expression changes induced by DCA. (**a**) Real-time PCR analysis shows that IκB and IL-8 expression first occur 4 h post-exposure to DCA. Below the graph are the corresponding array signals from example membrane arrays for IκB and IL-8. (**b**) Even the lowest dose of DCA (100 μM) induces a significant increase in IL-8 expression ($P < 0.05$). (**c**) This figure shows that DCA's activation of NF-κB (reflected in IL-8 expression) only occurs at neutral pH, not at acid pH. The left hand part of the graph shows the effect of 100 μM DCA at neutral pH, whereas the right hand part shows the effect at acidic pH.

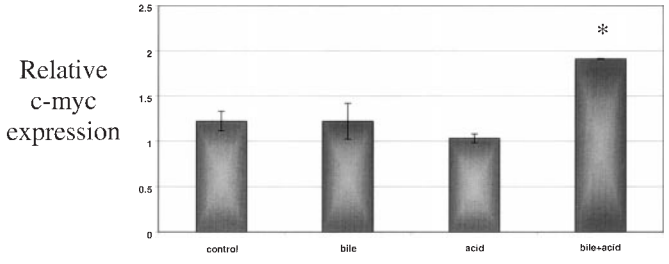

Fig. 5. c-myc expression after acidic bile exposure. Real-time PCR expression levels of c-myc after exposure to acid, bile and acidic bile, demonstrating that only the latter is capable of up-regulating c-myc expression. These data are consistent with those of Tselepis *et al*. (15).

Figure 4c. This demonstrates the fact that DCA is only capable of activating NF-κB (as seen by IL-8 expression) at neutral pH and not at acid pH. The absence of NF-κB activation (IκB and IL-8 expression) at acid pH was also noted when using the cDNA arrays (results not shown). However, the cDNA arrays did show that the oncogene c-myc is up-regulated after acidic bile exposure (but not neutral bile exposure) as established previously (15). Figure 5 shows the real-time PCR analysis of c-myc expression 4 h after exposure to acidic and neutral bile, demonstrating the statistically significant up-regulation of this gene after acidic bile exposure, but not after exposure to acid or bile alone. These data are consistent with that of Tselepis *et al*. (15), who also showed that c-myc up-regulation peaked at 24 h post-exposure to DCA. Hence DCA is capable of activating two different oncogenic transcription factors depending upon the pH of the media at the time of exposure.

Discussion

This study analysed the effects that physiological dose levels of DCA had on gene expression in oesophageal cells *in vitro*. We have shown that DCA specifically activates NF-κB, resulting in substantial up-regulation of NF-κB regulated genes (particularly IκB and IL-8). This is the first report of bile induced NF-κB activation in oesophageal cells; however, NF-κB has been shown previously to play a major role in carcinogenesis in other tissue types (22,31–34). Furthermore, NF-κB activation has been shown to be correlated with chemoresistance (35,36), indeed inhibition of NF-κB improves tumour shrinkage by chemotherapy (22,37), which may be an effective avenue for cancer therapy (38). Inhibiting NF-κB activation has been shown to result in increased apoptosis (27) and blocking of the neoplastic process (39).

The fact that the bile acid DCA is capable of inducing widespread gene expression abnormalities, particularly involving genes related to cell survival, proliferation and invasion (Table I), adds weight to the argument that bile reflux is important in cancer development in Barrett's patients. Importantly, DCA caused this at neutral pH only; this is in contrast to reports that bile induces c-myc expression only at acidic pH (15). Our data also show c-myc up-regulation with acidic bile, but show that neutral bile exposures are important in carcinogenesis in the oesophagus. The fact that we have shown DCA to be active at neutral pH may have widespread implication for patients with non-acid reflux, particularly those taking acid suppressive drugs (e.g. PPIs). Although PPIs reduce oesophageal exposure to both acid and duodenal contents (40), they may also increase the relative concentrations of unconjugated

bile acids (41) such as DCA, in the refluxate. This neutral pH refluxate may then interfere with cell function through the activation of molecules such as NF-κB. Hence it is possible that the current practise of prescribing PPIs for reflux symptoms may be promoting carcinogenesis in these patients via NF-κB activation. This is an area that warrants urgent further investigation.

The bile acid DCA was shown here to cause gene expression abnormalities involving proteins regulating anti-apoptosis, invasion, cell proliferation and cell adhesion (Table I). Due to space limitations, not all can be discussed here, but some of these gene expression changes have been documented previously and are known to be caused by either NF-κB or bile acids. As mentioned earlier, the activation of NF-κB by bile has been demonstrated previously in colon cancer cells (26,27) and hepatocytes (28). IL-8 has also been shown to be a transcriptional target of NF-κB (29) and is known to be up-regulated by NF-κB in gastric cells exposed to *Helicobacter pylori* (33). IL-8 is known to be linked to angiogenesis initiation (42), metastasis (43) and is capable of suppressing apoptosis (44). Other genes shown here to be up-regulated by bile (Table I) have also been identified in previous studies. Bile has been shown to induce the expression of the invasion factors Plau and PlauR (27). Corresponding increases in Plau and PlauR gene expression have also been identified in Barrett's metaplasia and oesophageal cancer (45,46). EGFR and VEGF have been identified previously as NF-κB transcriptional targets (reviewed in 22) and are commonly over-expressed in Barrett's tissues (47,48). The anti-apoptotic genes Flip (49) and A20 (50) have also been shown to be up-regulated by NF-κB, an anti-apoptotic agent itself. Finally, the adhesion gene Icam has been shown to be up-regulated by NF-κB (51), while intregrin adhesion proteins have been shown previously to be up-regulated in Barrett's metaplasia (47). Increases in cell adhesion are consistent with increased inflammation, as elevated adhesion allows neutrophil attachment to epithelial cells (52). The pattern of gene expression changes identified here suggests that neutral bile exposure leads to gene expression alterations that are fundamental to almost every stage of carcinogenesis.

Previous cytogenetic studies in our laboratory have shown that NF-κB and IL-8's chromosome (chromosome 4) is specifically amplified during cancer progression in Barrett's patients (18,19). This selective expansion of cells bearing extra copies of chromosome 4, may now be interpreted in light of the potential mechanism of NF-κB mediated cell survival. Bile has been demonstrated previously to select for apoptosis resistant, NF-κB active clones, in colon cancer cells *in vitro* (27). Hence, we speculate that in bile exposed Barrett's tissues, cells that gain one extra copy (or more) of chromosome 4, proliferate in the presence of bile at the expense of cells with a normal karyotype, which are forced into apoptosis. This survival is due specifically to the elevated NF-κB levels. Moreover, an elevated inflammatory response in neighbouring cells is induced by the NF-κB mediated up-regulation of IL-8, leading to even further increases in apoptosis in the surrounding tissue. In this model it is also interesting to note that a whole family of IL-8 related chemokines (the CXC chemokines) are located on chromosome 4 (53). Indeed Gro 1, a member of this family is shown here to be up-regulated by exposure to bile (Table I).

In conclusion, we have found that the unconjugated bile acid DCA activates NF-κB, a survival factor known to be amplified at the chromosome level in Barrett's progression. Interestingly, bile activated NF-κB here at neutral pH only suggesting that neutral bile reflux may play an important role in cancer development in Barrett's patients.

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References

1. Phillips,R.W. and Wong,R.K. (1991) Barrett's esophagus. Natural history, incidence, etiology and complications. *Gastroenterol. Clin. North Am.*, **20**, 791–816.
2. Jenkins,G.J.S., Doak,S.H., Parry,J.M., D'Souza,F.R., Griffiths,A.P. and Baxter,J.N. (2002) The genetic pathways involved in the progression of Barrett's metaplasia to adenocarcinoma. *Br. J. Surg.*, **89**, 824–837.
3. Jankowski,J.A., Wright,N.A., Meltzer,S.J., Triadafilopoulos,G., Geboes,K., Casson,A.G., Kerr,D. and Young,L.S. (1999) Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. *Am. J. Pathol.*, **154**, 965–973.
4. Barrett,M.T., Sanchez,C.A., Prevo,L.J., Wong,D.J., Galipeau,P.C., Paulson,T.G., Rabinovitch,P.S. and Reid,B.J. (1999) Evolution of neoplastic cell lineages in Barrett oesophagus. *Nature Genet.*, **22**, 106–109.
5. Attwood,S.E., Smyrk,T.C., DeMeester,T.R., Mirvish,S.S., Stein,H.J. and Hinder,R.A. (1992) Duodenoesophageal reflux and the development of esophageal adenocarcinoma in rats. *Surgery*, **111**, 503–510.
6. Stamp,D.H. (2002) Three hypotheses linking bile to carcinogenesis in the gastrointestinal tract: certain bile salts have properties that may be used to complement chemotherapy. *Med. Hypotheses*, **59**, 398–405.
7. Gillen,P., Keeling,P., Byrne,P.J., Healy,M., O'Moore,R.R. and Hennessy,T.P.J. (1988) Implication of duodenogastric reflux in the pathogenesis of Barrett's oesophagus. *Br. J. Surg.*, **75**, 540–543.
8. Nehra,D., Howell,P., Pye,J.K. and Beynon,J. (1998) Assessment of combined bile acid and pH profiles using an automated sampling device in gastro-oesophageal reflux disease. *Br. J. Surg.*, **85**, 134–137.
9. Stein,H.J., Kauer,W.K.D., Feussner,H. and Siewert,J.R. (1998) Bile reflux in benign and malignant Barrett's esophagus: effect of medical acid suppression and nissen fundoplication. *J. Gastrointest. Surg.*, **2**, 333–341.
10. Jankowski,J., Hopwood,D., Pringle,R. and Wormsley,K.G. (1993) Increased expression of epidermal growth factor receptors in Barrett's esophagus associated with alkaline reflux: a putative model for carcinogenesis. *Am. J. Gastroenterol.*, **88**, 402–408.
11. Kauer,W.K.H., Peters,J.H., DeMeester,T.R., Ireland,A.P., Bremner,C.G. and Hagen,J.A. (1995) Mixed reflux of gastric and duodenal juices is more harmful to the esophagus than gastric juice alone. The need for surgical therapy re-emphasized. *Ann. Surg.*, **222**, 525–531.
12. Triadafilopoulos,G. (2001) Acid and bile reflux in Barrett's esophagus: a tale of two evils. *Gastroenterology*, **121**, 1502–1506.
13. Venturi,M., Hambly,R.J., Glinghammar,B., Rafter,J.J. and Rowland,I.R. (1997) Genotoxic activity in human faecal water and the role of bile acids: a study using the alkaline comet assay *Carcinogenesis*, **18**, 2353–2359.
14. D'Souza,F.R., Jenkins,G.J., Parry,E.M., Baxter,J.N. and Parry,J.M. (2003) Bile acids: do they play a role in Barrett's oesophagus. *Gut*, **52** (suppl. 1), 171.
15. Tselepis,C., Morris,C.D., Wakelin,D., Hardy,R., Perry,I., Luong,Q.T., Harper,E., Harrison,R., Attwood,S.E.A and Jankowski,J.A.Z. (2003) Upregulation of the oncogene c-myc in Barrett's adenocarcinoma: induction of c-myc by acidified bile *in vitro*. *Gut*, **52**, 174–180.
16. Kaur,B.S., Omary,M.B. and Triadafilopoulos,G. (2000) Bile salts-induced PGE (2) release, PKCE and COX-2 expression, parallel the increased cell proliferation in an *ex vivo* model of Barrett's esophagus. *Gastroenterology*, **118**, Part 1 (suppl. 2), 1351.
17. Lapre,J.A., Termont,D.S.M.L., Groen,A.K. and Van der Meer,R. (1992) Lytic effects of mixed micelles of fatty acids and bile acids. *Am. J. Physiol.*, **263**, G333–337.
18. Croft,J., Parry,E.M., Jenkins,G.J.S., Doak,S.H., Baxter,J.N., Griffiths,A.P., Brown,T.H. and Parry,J.M. (2002) Analysis of the pre-malignant stages of Barrett's oesophagus through to adenocarcinoma by comparative genomic hybridisation. *Eur. J. Gastroenterol. Hepatol.*, **14**, 1179–1186.

19. Doak,S.H., Jenkins,G.J.S., Parry,E.M., D'Souza,F.R., Griffiths,A.P., Toffazal,N., Shah,V., Baxter,J.N. and Parry,J.M. (2003) Chromosome 4 hyperploidy represents an early genetic aberration in premalignant Barrett's oesophagus. *Gut*, **52**, 623–628.
20. Walch,A.K., Zitzelsberger,H.F. and Bruch,J. *et al*. (2000) Chromosomal imbalances in Barrett's adenocarcinoma and the metaplasia-dysplasia-carcinoma sequence. *Am. J. Pathol*., **156**, 555–566.
21. Reigman,P.H.J., Vissers,K.J., Alers,J.C., Geelen,E., Hop,W.C.J., Tilanus,H.W. and van Dekken,H. (2001) Genomic alterations in malignant transformation of Barrett's esophagus. *Cancer Res*., **61**, 3164–3170.
22. Rayet,B. and Gelinas,C. (1999) Aberrant rel/nfkb genes and activity in human cancer. *Oncogene*, **18**, 6938–6947.
23. O'Riordan,J.M., Abdel-Latif,M.M., McNamara,D., Ravi,N., Kelleher,D., Keeling,P.W.N. and Reynolds,J.V. (2003) Cytokine and NF-kappaB profiles in the metaplasia-dysaplasia-adenocarinoma sequence in the oesophaus *Br. J. Surg*., **90**, 611–612.
24. Abdel Latif,M.M., Windle,H.J., Carton,E., Kelleher,D. and Reynolds,J.V. (2002) Nuclear factor-kappaB: relationship to Barrett's adenocarcinoma, stage and treatment response. *Br. J. Surg*., **89**, 646–647.
25. Tselpepis,C., Perry,I., Dawson,C., Hardy,R., Darnton,S.J., McConkey,C., Stuart,R.C., Wright,N., Harrison,R. and Jankowski,J.A.Z. (2002) Tumour necrosis factor-α in Barrett's oesophagus: a potential novel mechanism of action. *Oncogene*, **21**, 6071–6081.
26. Payne,C.M., Crowley,C., Washo-Stultz,D., Briehl,M., Bernstein,H., Bernstein,C., Beard,S., Holubec,H. and Warneke,J. (1998) The stress-response proteins poly (ADP-ribose) polymerase and NF-kappaB protect against bile salt-induced apoptosis. *Cell Death Differ*., **5**, 623–636.
27. Crowley-Weber,C.L., Payne,C.M., Gleason-Guzman,M. *et al*. (2002) Development and molecular characterisation of HCT-116 cell lines resistant to the tumor promoter and multiple stress-inducer, deoxycholate. *Carcinogenesis*, **23**, 2063–2080.
28. Hellerbrand,C., Hoeger,S., Muelbauer,M., Thasler,W., Lock,G. and Schoelmerich,J. (2000) Bile acids induce proinflammatory cytokine expression in activated hepatic stellate cells via NFKB activation. *Hepatology*, **32**, Part 2, 156.
29. Kim,J.M., Oh,Y.K., Kim,Y.J., Oh,H.B. and Cho,Y.J. (2001) Polarized secretion of CXC chemokines by human intestinal epithelial cells in response to *Bacteroides fragilis* enterotoxin: NF-kappaB plays a major role in the regulation of IL-8 expression. *Clin. Exp. Immunol*., **123**, 421–427.
30. Chiao,P.J., Miyamoto,S. and Verma,I.M. (1994) Autoregulation of I kappa B alpha activity. *Proc. Natl Acad. Sci. USA*, **91**, 28–32.
31. Hinz,M., Loser,P., Mathas,S., Krappmann,D., Dorken,B. and Scheidereit,C. (2001) Constitutive NF-kappaB maintains high expression of a characteristic gene network, including CD40, CD86 and a set of antiapoptotic genes in Hodgkin/Reed-Sternberg cells. *Blood*, **97**, 2798–2807.
32. Oya,M., Takayanagi,A., Horiguchi,A., Mizuno,R., Ohtsubo,M., Marumo,K., Shimizu,N. and Murai,M. (2003) Increased nuclear factor-kappaB activation is related to the tumor development of renal cell carcinoma. *Carcinogenesis*, **24**, 377–384.
33. Maeda,S., Otsuka,M., Hirata,Y., Mitsuno,Y., Yoshida,H., Shiratori,Y., Masuho,Y., Muramatsu,M., Seki,N. and Omata,M. (2001) cDNA micro-array analysis of *Helicobacter pylori*-mediated alteration of gene expression in gastric cancer cells. *Biochem. Biophys. Res. Commun*., **284**, 443–449.
34. Ma,H.J., Parker,K.M., Altamirano,C.V., Mikail,A.E., Glowalla,A., Vescio,R. and Berenson,J.R. (2000) Frequent alteration of the IkB gene in multiple myeloma (MM) patients. *Blood*, **96**, Part 1, 2918.
35. Boland,M.P. (2001) DNA damage signalling and NF-kappaB: implications for survival and death in mammalian cells. *Biochem. Soc. Trans*., **29**, 674–678.
36. Wang,W. and Cassidy,J. (2003) Constitutive nuclar factor-kappaB mRNA, protein overexpression and enhanced DNA-binding activity in thymidylate synthase inhibitor-resistant tumour cells. *Br. J. Cancer*, **88**, 624–629.
37. Johnstone,R.W., Ruefli,A.A. and Lowe,S.W. (2002). Apoptosis: a link between cancer genetics and chemotherapy. *Cell*, **108**, 153–164.
38. Darnell,J.E.,Jr (2002) Transcription factors as targets for cancer therapy. *Nature Rev. Cancer*, **2**, 740–749.
39. Li,J.J., Westergaard,C., Ghosh,P. and Colburn,N.H. (1997) Inhibitors of both nuclear factor-kappaB and activator protein 1 activation block the neoplastic transformation response. *Cancer Res*., **57**, 3569–3576.
40. Marshall,R.E.K., Anggiansah,A., Manifold,D.K., Owen,W.A. and Owen,W.J. (1998) Effect of omeprazole 20 mg twice daily on duodenogastric and gastro-oesophageal bile reflux in Barrett's patients. *Gut*, **43**, 603–606.
41. Theisen,J., Nehra,D., Citron,D., Johansson,J., Hagen,J.A., Crookes,P.F., DeMeester,S.R., Bremner,C.G., DeMeester,T.R. and Peters,J.H. (2000) Suppression of gastric acid secretion in patients with gastroesophageal reflux disease results in gastric bacterial overgrowth and deconjugation of bile acids. *J. Gastrointest. Surg*., **4**, 50–54.
42. Huang,S., Mills,L., Tellez,C., McCarty,M., Yang,X.D., Gudas,J.M. and Bar-Eli,M. (2002) Fully humanized neutralizing antibodies to interleukin-8 (ABX-IL8) inhibit angiogenesis, tumor growth and metastasis of human melanoma. *Am. J. Pathol*., **161**, 125–134.
43. Bar-Eli,M. (1999) Role of interleukin-8 in tumor growth and metastasis of human melanoma. *Pathobiology*, **67**, 12–18.
44. Grutkoski,P.S., Graeber,C.T., Ayala,A. and Simms,H.H. (2002) Paracrine suppression of apoptosis by cytokine-stimulated neutrophils involves divergent regulation of NF-kappaB, BCL-X (L) and BAK. *Shock*, **17**, 47–54.
45. Barrett,M.T., Yeung,K.Y., Ruzzo,W.L., Hsu,L., Blount,P.L., Sullivan,R., Zarbl,H., Delrow,J., Rabinovitch,P.S. and Reid,B.J. (2002) Transcriptional analyses of Barrett's metaplasia and normal upper GI mucosae. *Neoplasia*, **4**, 121–128.
46. Kan,T., Shimada,Y., Sato,F., Maeda,M., Kawabe,A., Kaganoi,J., Itami,A., Yamasaki,S. and Imamura,M. (2001) Gene expression profiling in human esophageal cancers using cDNA microarray. *Biochem. Biophys. Res. Commun*., **286**, 792–801.
47. Al-kasspooles,M., Moore,J.H., Orringer,M.B. and Beer,D.G. (1993) Amplification and over-expression of the EGFR and erbB-2 genes in human esophageal adenocarcinomas. *Int. J. Cancer*, **54**, 213–219.
48. Couvelard,A., Paraf,F., Gratio,V., Scoazec,J.Y., Henin,D., Degott,C. and Flejou,J.F. (2000) Angiogenesis in the neoplastic sequence of Barrett's oesophagus. Correlation with VEGF expression. *J. Pathol*., **192**, 14–18.
49. Kreuz,S., Siegmund,D., Scheurich,P. and Wajant,H. (2001) NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. *Mol. Cell. Biol*., **21**, 3964–3973.
50. Krikos,A., Laherty,C.D. and Dixit,V.M. (1992) Transcriptional activation of the tumor necrosis factor alpha inducible zinc finger protein, a20, is mediated by kappa B elements. *J. Biol. Chem*., **267**, 17971–17976.
51. Rahman,A., Anwar,K.N., Uddin,S., Xu,N., Ye,R.D., Platanias,L.C. and Malik,A.B. (2001) Protein kinase C-delta regulates thrombin-induced ICAM-1 gene expression in endothelial cells via activation of p38 mitogen-activated protein kinase. *Mol. Cell. Biol*., **21**, 5554–5565.
52. Coussens,L.M. and Werb,Z. (2002) Inflammation and cancer. *Nature*, **420**, 860–867.
53. Modi,W.S. and Chen,Z.Q. (1998) Localization of the human CXC chemokine subfamily on the long arm of chromosome 4 using radiation hybrids. *Genomics*, **47**, 136–139.

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