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Relevance of mitogen activated protein kinase (MAPK) and phosphotidylinositol-3-kinase/protein kinase B (PI3K/PKB) pathways to induction of apoptosis by curcumin in breast cells

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Abstract

Following observations that curcumin inhibited proliferation (IC_{50} = 1–5 μM), invasiveness and progression through S/G2/M phases of the cell cycle in the non-tumourigenic HBL100 and tumourigenic MDA-MB-468 human breast cell lines, it was noted that apoptosis was much more pronounced in the tumour line. Therefore, the ability of curcumin to modulate signalling pathways which might contribute to cell survival was investigated. After pre-treatment of cells for 20 min, curcumin (40 μM) inhibited EGF-stimulated phosphorylation of the EGFR in MDA-MB-468 cells and phosphorylation of extracellular signal regulated kinases (ERKs) 1 and 2, as well as ERK activity and levels of nuclear c-*fos* in both cell lines. At a lower dose (10 μM), it also inhibited the ability of anisomycin to activate JNK, resulting in decreased c-*jun* phosphorylation, although it did not inhibit JNK activity directly. In contrast, the activation of p38 mitogen activated protein kinase (MAPK) by anisomycin was not inhibited. Curcumin inhibited basal phosphorylation of Akt/protein kinase B (PKB) in both cell lines, but more consistently and to a greater extent in the MDA-MB-468 cells. The MAPK kinase (MKK) inhibitor U0126 (10 μM), while preventing ERK phosphorylation in MDA-MB-468 cells, did not induce apoptosis. The PI3K inhibitor LY294002 (50 μM) inhibited PKB phosphorylation in both cells lines, but only induced apoptosis in the MDA-MB-468 line. These results suggest that while curcumin has several different molecular targets within the MAPK and PI3K/PKB signalling pathways that could contribute to inhibition of proliferation and induction of apoptosis, inhibition of basal activity of Akt/PKB, but not ERK, may facilitate apoptosis in the tumour cell line.

Keywords: Breast cells; Curcumin; MAP kinases; Cell cycle; JNK; Akt/PKB

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Abbreviations: AP-1, activator protein 1; DMSO, dimethyl sulphoxide; DTT, dithiothreitol; EGF(R), epidermal growth factor (receptor); ER, oestrogen receptor; ERK, extracellular signal regulated kinase; FITC, fluorescein-isothiocyanate; GSK, glycogen synthase kinase; JNK, c-*jun* N-terminal kinase; MAPK, mitogen activated protein kinase; MKK, MAPK kinase; PI, propidium iodide; PI3K, phosphotidylinositol-3-kinase; PKB, protein kinase B; PMSF, phenylmethylsulfonylfluoride; PTEN, phosphatase and tensin homologue (mutated in advanced cancers 1).

1. Introduction

There is compelling evidence that curcumin, a polyphenol found in the spice turmeric, has cancer chemopreventive properties in a range of animal models of chemical carcinogenesis [1–8], including those resulting in tumours of the mammary gland [5,6,9,10]. The compound has antioxidative and anti-inflammatory properties, and several mechanisms have been proposed by which it might block initiation and progression of cancer (reviewed in [11]). Curcumin has been reported to inhibit the growth of a range of breast cell lines and to induce G2 or G2/S arrest [12–14]. However, while apoptosis was observed in one study using

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a cancer line, MCF-7/TH, with changes in the expression of a number of relevant genes including Ki67, PCNA and p53 [14], a second study, using BT20, T47D, SK-BR3 and adriamycin-resistant MCF-7 cells, reported no apoptosis and no changes in the expression of apoptosis-related genes, Bcl-2, p53, cyclin B or transglutaminase [13]. Indeed, many studies suggest that chemopreventive agents can exhibit tissue specificity and may even behave differently in cell lines derived from the same tissue, which have different genotypes or phenotypes. Thus, extrapolating data from one cell type to another can be misleading. With regard to particular target molecules involved in breast cell proliferation and survival, curcumin has been shown in AU565 tumour cells to inhibit both the tyrosine kinase activity and the membrane levels of ErbB2, a member of the epidermal growth factor receptor (EGFR) family [12], and a recent report suggests that it may inhibit telomerase activity in MCF-7 and MCF-7/TH, but not MCF10A cells [15]. Pretreatment of MCF-7 cells with curcumin was found to result in an inhibition of tumour necrosis factor α (TNFα)-induced c-*jun* N-terminal kinase (JNK) activation [16]. However, information on exactly how curcumin may exert its chemopreventive activity specifically in breast cells is still very limited.

Signalling through several pathways has been shown to influence the proliferation rate of breast cancer cells. EGF is a potent mitogen, with overexpression of the ligand and its receptor a common feature of breast cancer. Elevated receptor levels can play a role in progression of tumours to hormone independence [17]. A study by Xing and Imagawa [18] in mouse mammary cells found that activation of the MAPK, ERK, was permissive for the proliferative response to EGF, but also that tumour cells had higher basal levels of ERK activity. EGF at high doses (100 ng/mL) could also induce apoptosis in some breast cells, such as MDA-MB-468, which overexpress EGFR, by causing detachment of cells, followed by release of cytochrome *c* and activation of caspases 3, 6 and 7. This effect was mediated through the EGFR [19].

A dominant negative mutant of c-*jun*, the downstream target of another MAPK signalling pathway involving JNK, was found to inhibit MCF-7 colony formation [20]. Subsequently, the same group showed that overexpression of jun in this cell type caused alterations to the composition of activator protein 1 (AP-1), increasing cellular motility, expression of matrix metalloproteinase (MMP9) and tumour formation in nude mice [21]. Their studies also suggested that transformed cells may be less dependent on AP-1-mediated pathways for continued growth, reflecting their lower AP-1 expression and transactivating capacity [22]. Increased expression of c-*jun* also resulted in non-responsiveness to oestrogen and tamoxifen. Increased AP-1 DNA binding and JNK activity was shown to correlate with resistance to tamoxifen in a panel of breast cell lines and tumour-derived samples [23]. Mandelkar *et al*. [24] showed that tamoxifen-induced apoptosis in the oestrogen receptor (ER)-negative breast lines MDA-MB-231 and BT20 was dependent on JNK activation.

In the highly invasive breast cancer line, BT549, inhibition of a third MAPK pathway involving p38α abrogated the ability of these cells to invade matrigel [25], and a role for p38 has also been proposed in regulating adhesion of breast cells to collagen type IV [26]. Furthermore activation of p38 by heregulin, leading to serine phosphorylation and disassembly of paxillin from focal adhesion complexes, appears to be involved in heregulin-induced cell shape alterations and motility [27].

Another key pathway involved in cell survival signals through phosphotidylinositol-3-kinase (PI3K) and Akt/PKB. Expression of the tumour suppressor, PTEN, which negatively regulates this pathway, induced cell-cycle arrest, apoptosis or anoikis in breast cancer cells following transfection [28,29]. These effects of PTEN were attributable at least in part to its ability to alter phosphorylation of the PKB, p70S6 kinase, BAD and glycogen synthase kinase (GSK)-3 apoptotic mediators and expression of p27, a cell-cycle regulator. Breast cancer cells with mutant PTEN were more sensitive to growth inhibitory effects of PI3K inhibitors. We wished to confirm effects of curcumin on proliferation and apoptosis in HBL100 and MDA-MB-468 cells and to identify changes in signalling pathways which could contribute to such modification of growth. We were interested to examine whether curcumin might have beneficial effects in ER-negative cells, since there are already chemopreventive treatments for ER-positive breast cancer [30]. Having observed a growth inhibitory effect in more than one phase of the cell cycle, having shown that apoptosis occurred preferentially in the tumour cells and that curcumin caused modest inhibition of invasiveness, we looked for modulation of key survival pathways in HBL100 and MDA-MB-468 cells. We showed that curcumin is a good inhibitor of JNK signalling in stimulated cells, but also inhibits signalling through EGFR/ERK and PKB, both of which are constitutively phosphorylated in unstimulated cells. Use of inhibitors U0126 and LY294002 indicated that inhibition of ERK phosphorylation induced necrosis in the HBL100 line, while inhibition of PKB/Akt phosphorylation induced apoptosis only in the MDA-MB-468 cell line.

2. Materials and methods

2.1. Materials

Curcumin, obtained from Sigma-Aldrich Company Ltd, was made up as a 20 mM stock solution in DMSO. U0126 (Promega) and LY294002 (Calbiochem-Novabiochem UK Ltd) were made up as 10 and 50 mM stock solutions, respectively, in DMSO. Nocodazole was from CN Biosciences UK (Calbiochem). Aphidicolin was a gift from Dr. Raj Patel, Biochemistry Department, University of

Leicester (available from Sigma). Antibodies against EGFR, phosphotyrosine (PY99), ERK1 (which also reacts with ERK2), phospho-ERKs 1 and 2 (Tyr 204), JNK, phospho-JNK, c-*jun* and c-*fos* were obtained from Santa Cruz Biotechnology Inc.; anti-phospho-c-*jun* (Ser 73) and anti-phospho-Akt (Ser 473) were from New England Biolabs.; anti-GSK-3β was from Transduction Laboratories; anti-p38 and anti-MKK4 polyclonal antibodies were provided by M.D. Antibodies against Akt/PKB (detecting Akt1/PKBα, independent of phosphorylation state), phospho-GSK-3α/β (Ser 21/9) and PTEN were obtained from Cell Signaling Technology. Fluorescein-isothiocyanate (FITC)-conjugated annexin V was obtained from Bender Med Systems. Matrigel, a basement membrane matrix extracted from the Engelbroth–Holm–Swarm (EHS) mouse sarcoma was obtained, batch-tested, from Becton Dickinson, and fibronectin from bovine plasma (F-1141) was from Sigma.

2.2. Cell culture and quantitation of growth

The breast cell lines were HBL100, derived from normal cells in human breast milk [31] and non-tumourigenic, and MDA-MB-468, an ER α-negative/β-positive, tumour-derived line. Both cell lines were negative with regard to mycoplasma contamination. Following concern that ATCC stocks of HBL100 contained an inappropriate Y chromosome, we had DNA profiling carried out on our cultures. A fluorescence-based PCR analysis of the X-Y homologous gene amelogenin [32] established that they did not contain a Y chromosome. Furthermore the stock from which our cultures were derived exhibits many features of the myoepithelial/basal phenotype, as well as some luminal characteristics and in this respect, resembles the intermediate or cap cells of the developing end-bud structures of the rodent breast which are considered to contain the breast stem cell population [33]. Our HBL100 cells express the myoepithelial-related cytokeratin CK14 in addition to the luminal epithelial-related CK18, as well as the basal associated α6β4 integrin [34] and vimentin. In addition, the cells express the desmosomal components Dsg3 and Dsg2, which have recently been shown to be characteristic of normal breast myoepithelial cells [35].

MDA-MB-468 cells were routinely cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS) and 2 mM Glutamax. HBL100 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) containing 10% FCS and 1 mg/mL glucose. Cells (1×10^4) were allowed to adhere prior to incubation in the presence, or absence of the indicated concentrations of chemopreventive agent, or DMSO vehicle control. At the indicated times, cells were trypsinised and their numbers determined using a Coulter ZM electronic cell counter (Beckman Coulter Ltd). Where indicated, cells were serum-starved by growing in serum-free medium overnight and replacing this with fresh serum-free medium for a further hour before treatment.

2.3. Cell-cycle analysis and determination of apoptosis by flow cytometry

For analysis of cell cycle, 5×10^5 or 1×10^6 cells were seeded onto 9-cm plates and treated with curcumin (20–80 μM) for 48 hr. In subsequent experiments, cells (2×10^5 or 5×10^5 seeded on 6-well plates) were synchronised in either G1 or G2/M phase by treatment for 24 hr with aphidicolin (7.5 μM) or nocodazole (0.2 μg/mL HBL100; 0.15 μg/mL MDA-MB-468), respectively. Following synchronisation, monolayers were washed thoroughly with PBS before replenishment with fresh medium containing 40 μM curcumin or DMSO only and cultured for 4, 8, 16, 24 or 32 hr. Cells were harvested by trypsinisation and fixed overnight in 70% ethanol at 4°, then collected by centrifugation and resuspended in PBS containing 0.1 mg/mL RNase and 5 μg/mL propidium iodide (PI) and incubated overnight at 4°. DNA content was analysed using a Becton Dickinson FACScan and Cell Quest software, plotting 5000 events per sample. Subsequent data analysis was performed using ModFit LT software (distributed in the UK by Becton Dickinson).

The number of cells undergoing apoptosis was estimated from binding of annexin V to externalised phosphatidylserine. Cells (1×10^6 seeded onto 9-cm plates) were treated as earlier or with 10–20 μM U0126 or 50 μM LY294002, and harvested at time points from 24 to 48 hr, washed with PBS, and resuspended in annexin V-binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$). FITC-conjugated annexin V was added to a final concentration of 100 ng/mL and cells incubated for 8 min at room temperature. PI (50 μg/mL) was added before flow cytometric analysis. Necrotic cells could be distinguished from apoptotic cells by the combined fluorescence of FITC and PI.

2.4. Invasion assay

Cell invasion was determined using a modification of the assay described by Albini *et al*. [36] and used previously in our laboratory [34]. The lower surface of an 8-μM pore polyethylene terephthalate (PET) track-etched membrane was coated with 10 μg/mL fibronectin, and the upper surface was coated with EHS basement membrane substrate, Matrigel, at a final concentration of 5 μg/mL. The upper membrane was seeded with 5×10^4 cells, and cells were allowed to attach overnight in serum-supplemented medium before replacing with serum-free DMEM. In the lower chamber a 1:1 ratio of conditioned medium from the human foetal fibroblast cell line HFFF2:serum-free medium was used as a chemotactic stimulus. The test assays were supplemented with curcumin at 1 and 5 μM and

controls with an equivalent volume of DMSO. Assays were carried out over 24 hr in a humidified atmosphere of 5% CO_2/95% air at 37°. The assays were set up in duplicate and for each case, one membrane was processed to maintain the cells on the upper surface (i.e. the inoculated cells) and the other to maintain cells on the lower membrane (i.e. invasive cells). Cell counts were performed on haematoxylin and eosin stained membranes and an invasion index calculated from the number of cells on the lower membrane as a percentage of the sum of the number on the upper and lower membranes.

Each assay was repeated, in duplicate, four times. The mean invasion index (MII) and the standard mean error were calculated using the Statistical Package for Social Scientists (SPSS) software package and means compared using the independent means *T* test, having confirmed equality of variances using Levene's test.

2.5. Detection of EGFR, ERK, JNK, PKB and PTEN

To assess the effect of the agent on phosphorylation of both the EGFR and ERKs 1 and 2, cultures, when 70–80% confluent, were washed with PBS and incubated in serum-free medium before treatment. To determine the effect of curcumin on basal levels of phosphorylated PKB, cells were grown in the presence of serum.

For the EGFR assays cells were rinsed twice with ice-cold PBS and lysed in 500 μL Triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 25 mM β-glycerol phosphate, 2 mM sodium pyrophosphate, 2 mM EDTA, 10%(v/v) glycerol, 2 mM benzamidine, 1 mM sodium vanadate, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 μg/mL pepstatin, 5 μg/mL aprotonin, 5 μg/mL leupeptin, 1% (v/v) Triton X-100 (TX-100) and 0.5 mM dithiothreitol (DTT)). Lysates were cleared by centrifugation at 1000 *g* for 5 min at 4°, flash frozen and stored at −80° until required. To detect phospho-EGFR levels, the receptor was precipitated at 4° for 4 hr from 500 μg cell lysate, using 2 μg of polyclonal anti-EGFR antibody bound to 25 μL (equivalent to 1 mg) protein A agarose beads. After centrifugation, the beads were washed twice in PBS supplemented with 350 mM NaCl, 0.2% TX-100, and twice in PBS/0.2% TX-100. Samples were analysed by SDS–PAGE and immunoblotting, using an anti-phosphotyrosine primary antibody (PY99), and horseradish peroxidase-coupled secondary antibody. Bound antibody was visualised with an ECL detection system (Amersham Life Science Ltd).

To detect PKB, PTEN, JNK and total EGFR, cell lysates were prepared essentially as described earlier, while for the ERK assays, treated cells, seeded at 3×10^5 to 5×10^5 per well, were lysed in 200 μL SDS sample buffer, boiled and sonicated for 30 s. An equal amount of protein per lane (20–100 μg) was run on 10% SDS polyacrylamide gels and analysed as above, by immunoblotting with the appropriate antibody. Blots were scanned using a densitometer (Molecular Dynamics) and quantified using the Image Quant program.

2.6. Nuclear protein extraction and western blotting for c-jun and c-fos

Serum-starved cells (3×10^6) were treated as indicated and collected by centrifugation. Nuclear protein was extracted by resuspending cells in 400 μL buffer A (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.4 mM PMSF and 0.2 mM sodium orthovanadate) followed by lysis with the addition of 25 μL buffer B (10% IGEPAL; Sigma-Aldrich Company Ltd). Nuclei were collected by centrifugation and lysed by resuspension in 50 μL buffer C (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, 10% (v/v) glycerol, 0.2 mM NaF and 0.2 mM sodium orthovanadate). Samples were sonicated for 30 s and protein concentration determined using the Biorad protein assay kit (Biorad Laboratories Ltd). Forty micrograms of nuclear protein was resolved on 10% SDS polyacrylamide gels and western blotting performed, as described earlier, for c-*jun*, phosphorylated c-*jun* or c-*fos* levels.

2.7. Immune-complex kinase assays for ERK, JNK, p38 and MKK4

Cells seeded at 1×10^6 were allowed to recover overnight and then cultured for 60 min in medium with the indicated treatments. Lysates were prepared as for EGFR phosphorylation assays. Clarified lysates were incubated with 15 μL (equivalent to 0.6 mg) protein A agarose to which were bound 5 μg of antibodies to ERK, JNK, p38 or MKK4. The precipitates were washed twice with Triton lysis buffer and once with kinase assay buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerol phosphate, 25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM sodium vanadate and 0.5 mM DTT). Pellets were resuspended in 30 μL kinase assay buffer with addition of appropriate substrate (5 μg photo, heat and acid stable protein I (PHAS I; Calbiochem)), for ERK assays; 5 μg of GST-jun, amino acids 1–79 for JNK assays; 5 μg GST-ATF2 for p38 assays, 1 μg GST-JNK and 5 μg GST-jun for MKK4 assays). Reactions were incubated for 30 min at 30°. Assays were terminated by the addition of SDS–PAGE sample buffer. The samples were then subjected to electrophoresis on 10% SDS–PAGE gels. Incorporation of ^{32}P into the substrate was determined by PhosphorImager (Molecular Dynamics) analysis of the dried gels.

2.8. Immune-complex kinase assay for PKB

Cell lysates were prepared using the lysis buffer supplied with the Akt kinase assay kit (Cell Signaling Technology). PKB activity was determined according to the protocol

supplied. PKB was immunoprecipitated from 200 μg cell lysate protein, which gave results within the linear range of the assay. Curcumin was added directly to the assay for 30 min prior to addition of the substrate, GST-GSK-3. Phosphorylation of GST-GSK-3 substrate was measured by SDS–PAGE using the phospho-GSK-3α/β antibody, and detection was by chemiluminescence using the Lumi-glo detection reagent supplied in the kit. No phosphorylation of the substrate was detected in the absence of PKB.

2.9. Statistics

In the case of the growth studies, significance of results was determined by balanced ANOVA, followed by Fisher's least significant difference *post hoc* test. In all other cases, unless otherwise stated, significance was determined by one-way ANOVA, followed by Tukey's *post hoc* test. A P value of <0.05 was considered significant.

3. Results

3.1. Inhibition of cell proliferation and invasiveness by curcumin

Curcumin at a dose of 10 μM completely inhibited the proliferation of both cell lines with a median inhibitory concentration (IC_{50}) of between 1 and 5 μM (Fig. 1a).

To determine whether curcumin could affect the invasive potential of breast cells, an *in vitro* assay was carried out using concentrations around the IC_{50}. There was a small but significant decrease in invasion by both MDA-MB-468 cells in the presence of 1 μM curcumin ($P = 0.001$) and 5 μM curcumin ($P = 0.003$) and by HBL100 cells ($P = 0.005$ and 0.002, respectively). There was no significant difference in invasion in response to the two concentrations of curcumin (Fig. 1b).

Flow cytometric analysis of DNA content of non-synchronous MDA-MB-468 and HBL100 cells stained with PI, showed that 20 μM curcumin (48 hr) caused a decrease in the proportion of cells in G1 phase, together with an increase in cells in mid to late S phase and G2/M (Fig. 2). To differentiate between the apparent S phase and G2/M phase effects, we measured the effect of curcumin (40 μM) on progression of cells through the cell cycle following synchronisation with either aphidicolin or nocodazole (Fig. 3). Curcumin clearly slowed the movement of cells through S phase following release from aphidicolin-induced G1 block and markedly inhibited exit from nocodazole-induced G2/M block in both cell lines. Movement of aphidicolin-synchronised cells through G2/M and back into G1 (2n) was also delayed. These results indicate that curcumin exerts a dual effect on progression of cells through the cell cycle, during S phase and transition through G2 and mitosis.

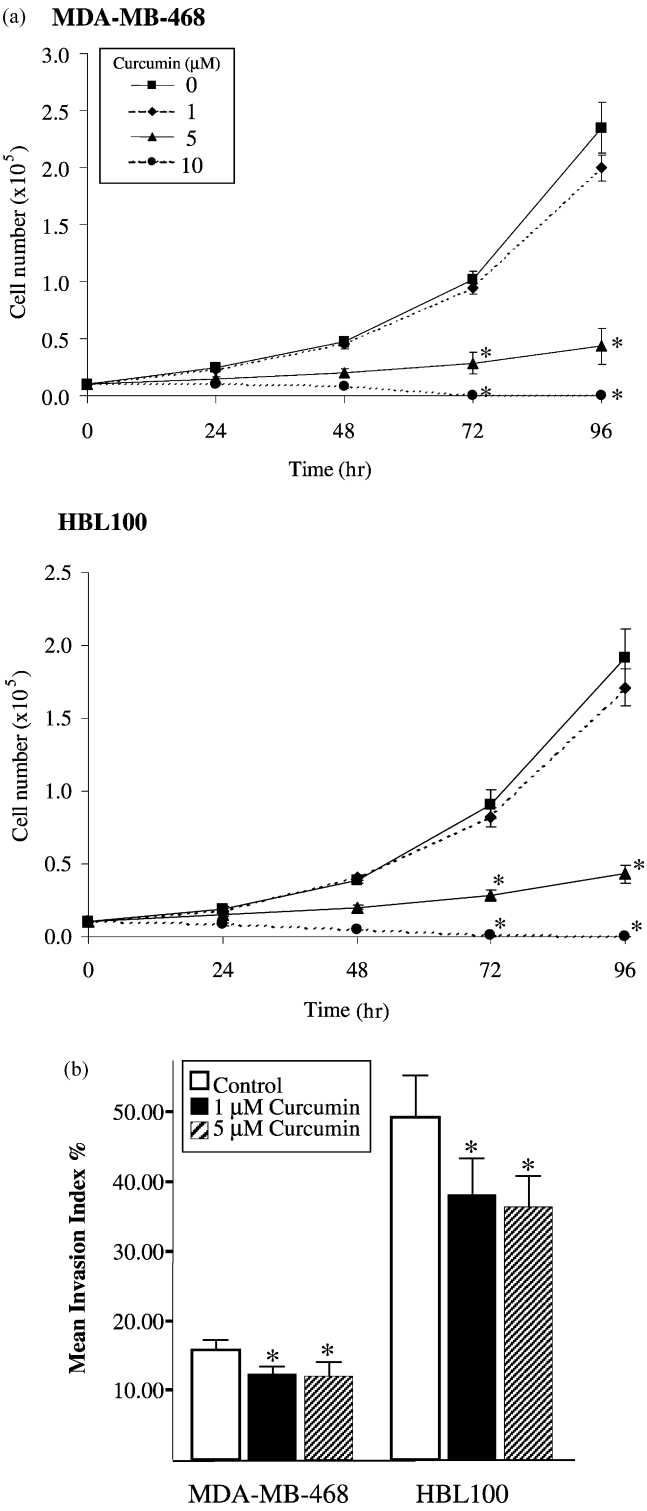

Fig. 1. Inhibition of cell proliferation and invasion by curcumin. (a) Cells were grown in the presence of increasing concentrations or vehicle control (DMSO). Cell numbers were estimated in a Coulter counter following trypsinisation of adherent cells. Each data point represents the mean of three experiments carried out in duplicate (±SD). Statistical significance of changes Cf DMSO control was calculated as described in Section 2. (b) Cells were grown on a Matrigel-coated membrane as described in Section 2 in the presence of 1 or 5 μM curcumin and their capacity to move through the membrane calculated as the MII. Results are the mean of four separate experiments carried out in duplicate. Error bars indicate the 95% confidence intervals of the mean.

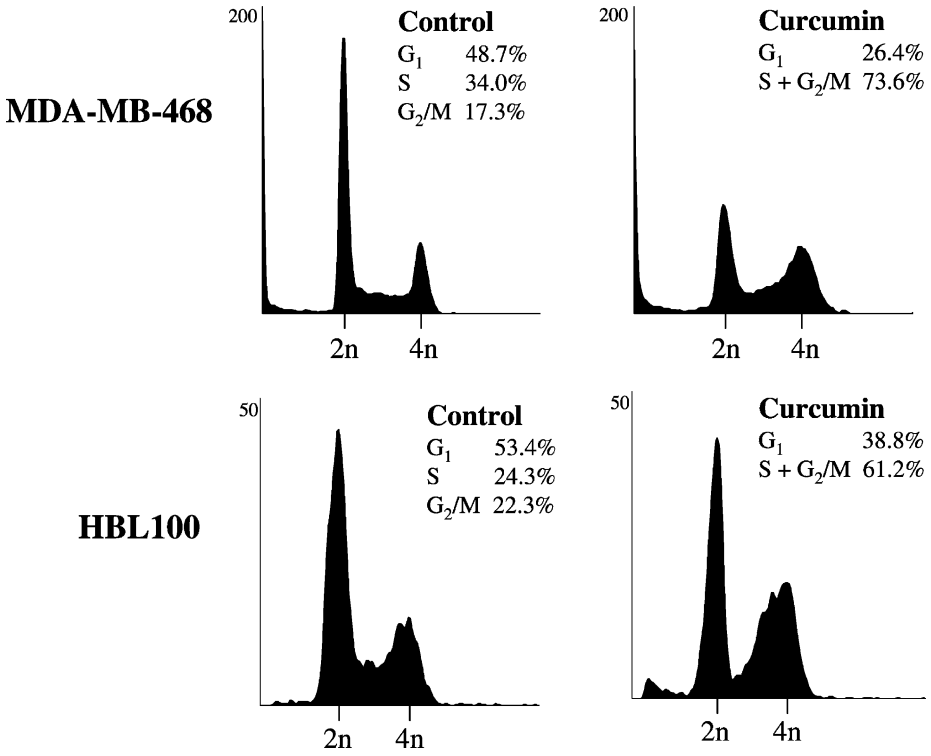

Fig. 2. Accumulation of cells in S/G2/M phase in response to curcumin. MDA-MB-468 and HBL100 cells were cultured for 48 hr in the presence or absence of 20 μM curcumin, harvested and stained with PI, and then analysed by flow cytometry as described in Section 2. DNA content (red fluorescence) is represented on the *x*-axis, and cell counts on the *y*-axis. The histograms presented are representative of at least three separate experiments.

Following release from nocodazole block into fresh medium with no further treatment (Fig. 3: Noc → Medium), both cell lines appeared to undergo endoreduplication (entry into S phase with a 4n DNA content), with approximately 16% MDA-MB-468 and 30% HBL100 becoming polyploid, with a clear 8n DNA population by 24 hr. It is known that microtubule organisation inhibitors, such as nocodazole and colcemid, can induce hyperploidy following prolonged exposure or following release from drug treatment in certain cell lines (see Section 4). Interestingly, this effect appeared to be prevented by curcumin in the HBL100 cell line (Fig. 3: Noc → Cur), with less than 20% cells having DNA content >4n after 24 hr and disappearance of the 8n peak. In the MDA-MB-468 line, polyploid populations developed following curcumin treatment of both aphidicolin- and nocodazole-synchronised cells (Fig. 3: Aph → Cur, 33%; Noc → Cur, 55%), although these had not achieved a DNA content of 8n by 32 hr.

3.2. Induction of apoptosis by curcumin

Following 48-hr treatment with 20 μM curcumin, the number of MDA-MB-468 cells remaining as an adherent monolayer was greatly reduced compared to control cells (data not shown). We, therefore, investigated whether, in addition to perturbation of cell-cycle progression, these cells were undergoing apoptosis in response to curcumin. The proportion of viable, necrotic and apoptotic cells of the combined adherent and detached populations, following treatment with 20, 40 or 80 μM curcumin for 48 hr was determined by flow cytometry using annexin V and PI staining. Curcumin did induce apoptosis in both cell lines, but with the MDA-MB-468 line showing heightened sensitivity compared to the HBL100 line (Fig. 4). At 48 hr, 47% MDA-MB-468 cells were undergoing apoptosis in response to 20 μM curcumin, with a further 49% in necrosis, while over 77% of HBL100 cells remained viable. In response to higher concentrations of curcumin HBL100 cells underwent both apoptosis and necrosis, while the proportion of necrotic MDA-MB-468 cells increased to almost 100%, probably as a result of secondary necrosis following induction of apoptosis. At an earlier time point (24 hr) over 60% of MDA-MB-468 cells were found to be already undergoing apoptosis in response to 20 μM curcumin, compared to only 10% of HBL100 cells (data not shown).

3.3. Inhibition of the EGFR/ERK pathway

The effect of curcumin on total cellular ATP levels was previously determined, to ensure that the compound did not deplete this essential molecule. No significant decrease in ATP content of cells was observed up to 80 μM curcumin.

In agreement with published data, the level of EGFR protein detected in the MDA-MB-468 cells was always at least an order of magnitude greater than that present in the

HBL100 cell line (Fig. 5). In order to explore the effect of the chemopreventive agent on phosphorylation of this receptor, it was first immunoprecipitated, followed by detection of the phosphorylated form with an anti-phosphotyrosine antibody. In the absence of serum virtually no phosphorylated receptor was precipitated from HBL cells (Figs. 5 and 6b). Receptor phosphorylation stimulated by EGF was inhibited by 40 μM curcumin in the MDA-MB-468 cells (Fig. 6a and b), but not significantly by doses up to 80 μM in the HBL100 line (Fig. 6b).

In order to determine the downstream consequences of these effects, the ability of curcumin to inhibit EGF-induced phosphorylation of the MAPKs, ERKs 1 and 2 was examined. After a treatment time of 20 min, curcumin (80 μM) caused inhibition in both cell lines, but at 40 μM caused significant inhibition only in the HBL100 line (Fig. 6c). Significant levels of phosphorylated ERK occurring in both cell lines in the absence of EGF were not influenced by the withdrawal of serum (Fig. 5). To confirm that inhibition of ERK phosphorylation resulted in loss of activity, the ability of immunoprecipitated p42/p44 to phosphorylate the substrate PHAS I was examined. In both cell lines, ERK activity was observed in the absence of EGF stimulation. Significant inhibition of EGF-stimulated activity was observed at 40 μM curcumin (about 50% in MDA and almost complete in HBL; data not shown). Treatment of serum-starved MDA-MB-468 and HBL100 cells with curcumin (20 μM) for a total of 3 hr significantly reduced the levels of c-*fos* expression (Fig. 6d), while at higher doses expression was abolished. Variable levels of c-*fos* were observed in nuclear extracts after serum starvation in untreated cells, with higher levels in nuclei of cells grown in the presence of serum (Figs. 5 and 6d). c-*fos* was difficult to detect in total cell extracts from unstimulated cells (Fig. 6d).

3.4. Inhibition of ERK signalling by U0126 without apoptosis

In order to determine whether inhibition of ERK signalling might contribute to apoptosis, cells were treated with U0126, an inhibitor of MKK1/2. At a concentration of

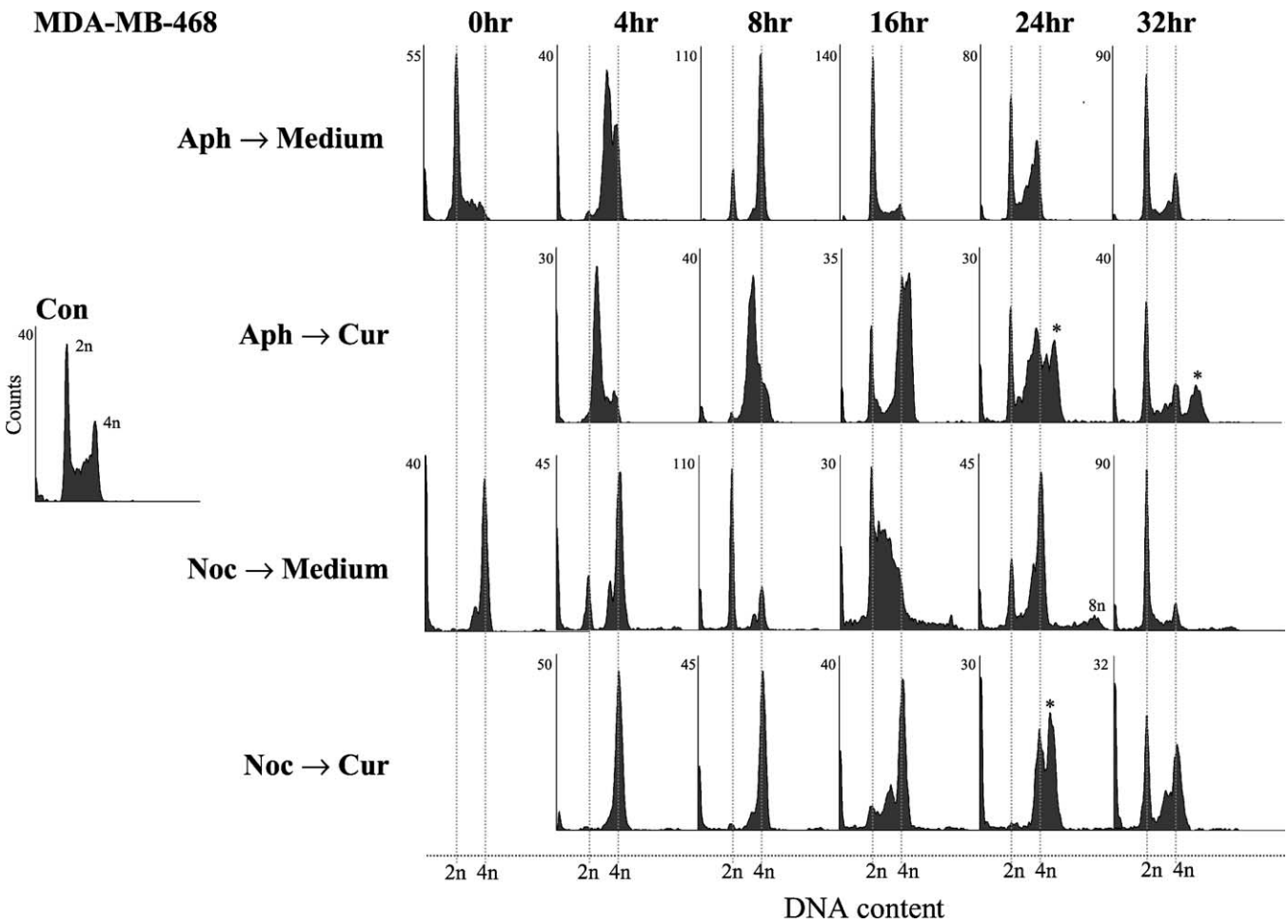

Fig. 3. Prolonged S phase and delayed exit from G2/M in response to curcumin. MDA-MB-468 and HBL100 cells were synchronised by treatment with either aphidicolin or nocodazole for 24 hr before release into fresh medium (Aph → Medium; Noc → Medium) or into medium containing 40 μM curcumin (Aph → Cur; Noc → Cur). Cells were harvested at the times indicated, stained with PI and analysed by flow cytometry. DNA content is represented on the *x*-axis, and cell counts on the *y*-axis.

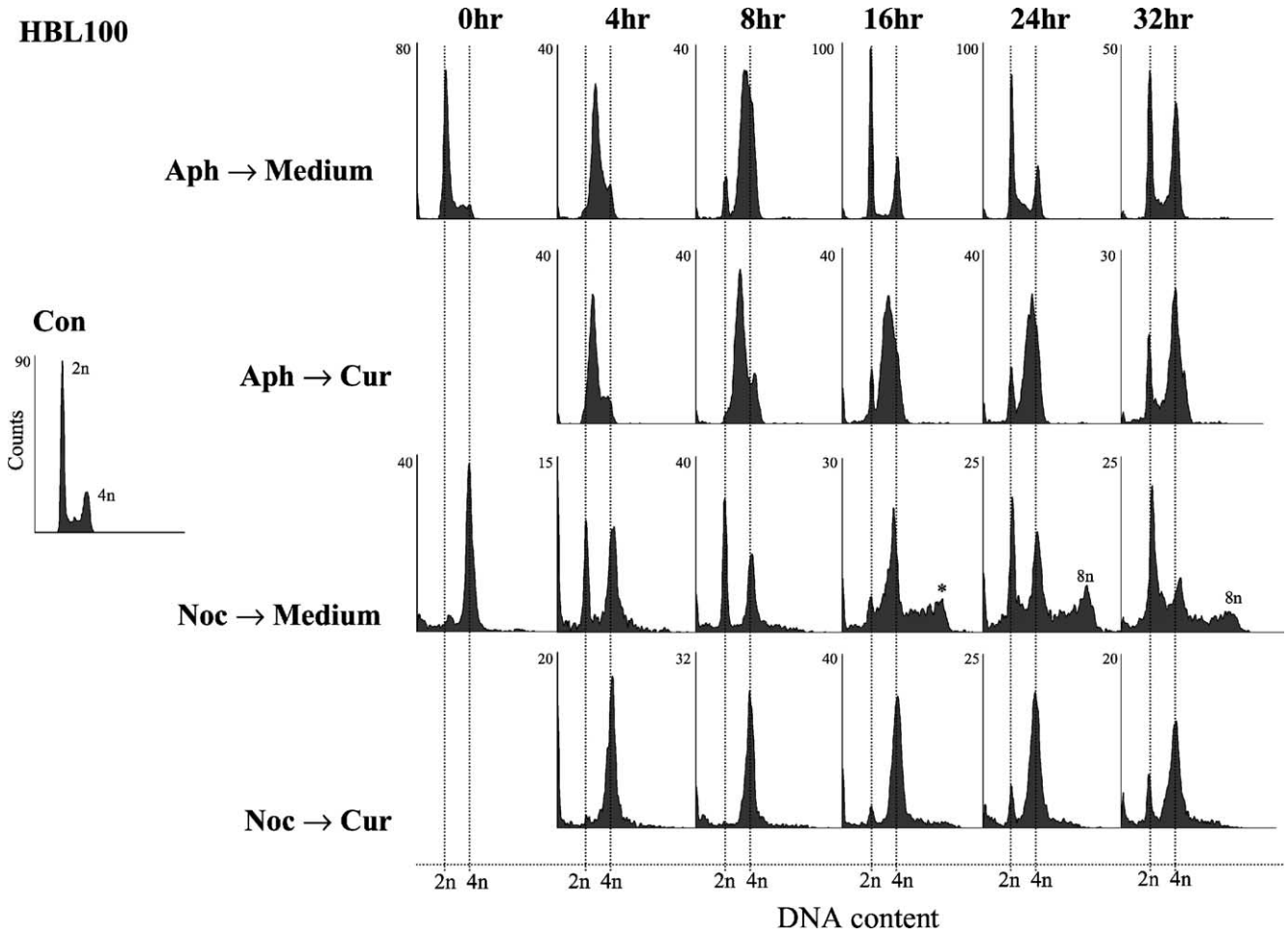

Fig. 3. (*Continued*).

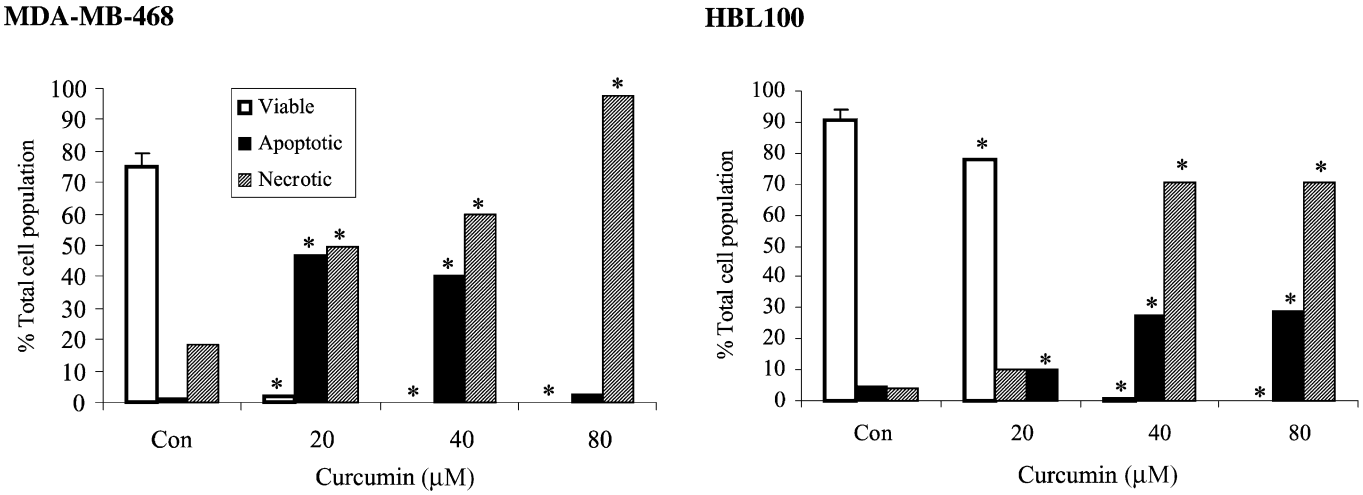

Fig. 4. Induction of apoptosis or necrosis by curcumin. Cells were treated with 20, 40 or 80 μM curcumin for 48 hr prior to collection and staining with annexin V and PI and analysis by flow cytometry. Proportions of live, apoptotic or necrotic cells were determined from the relative intensities of the PI and annexin staining. Results shown are mean ± pooled standard deviation shown on control bar (N = 3 HBL100; N = 4 MDA-MB-468). (∗) Indicates significant difference from the control (DMSO treated) group ($P < 0.05$) as determined by the ANOVA balanced model followed by Fisher's least significant difference *post hoc* test.

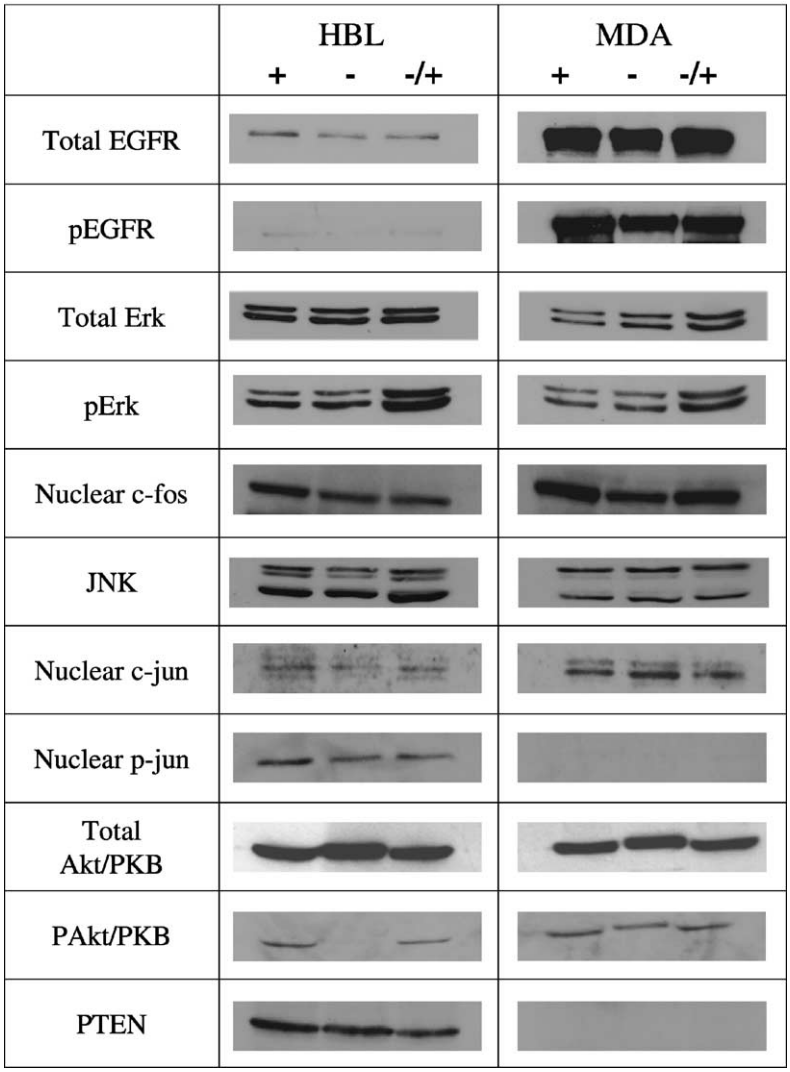

Fig. 5. Basal expression of signalling molecules in the presence or absence of serum. Cells were grown in the presence of serum (+), or serum-starved for 24 hr followed by a further hour in serum-free medium (−). A third sample was prepared where serum-starved cells had serum replaced for 4 hr before harvesting (−/+). Whole cell lysates or nuclear fractions (c-*fos*, c-*jun*, phospho-c-*jun*) were prepared and equal amounts of protein were analysed by western blotting after separation on SDS–PAGE. In the case of phospho-EGFR, equal amounts of protein were used from which to immunoprecipitate the receptor as described in Section 2. Samples from the two cell lines were run on the same gel. No bands were obtained for phospho-JNK in either unstimulated cell line.

10 μM basal phosphorylation of ERKs 1 and 2 was completely inhibited in MDA-MB-468 cells and partially in HBL100 cells after 1 and 5 hr. Activity was returning by 24 hr in both cell lines (data not shown). Flow cytometric analysis using annexin binding revealed no increase in apoptosis at 24 hr in either cell line (Table 1). Treatment with 20 μM U0126 still did not induce significant apoptosis, but caused necrosis in the HBL100 cells, where there was still residual ERK phosphorylation. Similar results were obtained after 48 hr of treatment (data not shown).

3.5. Inhibition of JNK activity by curcumin

In order to examine the effect on JNK, another MAPK, cells were pre-treated with the chemopreventive agent before stimulation with anisomycin. The HBL100 line appeared to contain higher basal levels of JNK 1 (lowest band) (Fig. 5). Significant inhibition of anisomycin-induced JNK activity was observed in the tumour cell line at doses as low as 10 μM curcumin, similar to the growth inhibitory dose in these cell lines. However, in the HBL100 line significant inhibition was only achieved at 40 μM (Fig. 7). Basal levels of phosphorylated JNK were not detected by western blotting in either cell line, and only very low levels of JNK activity were observed in unstimulated cells (Fig. 7).

Curcumin was not inhibitory when added directly to the immune-complex assay (data not shown), suggesting that it acts upstream of JNK. Evidence to support this was obtained from an assay in which curcumin inhibited the activity of MKK4 in pre-treated MDA-MB-468 cells at 20 μM and in HBL100 cells at a dose of 10 μM, but once

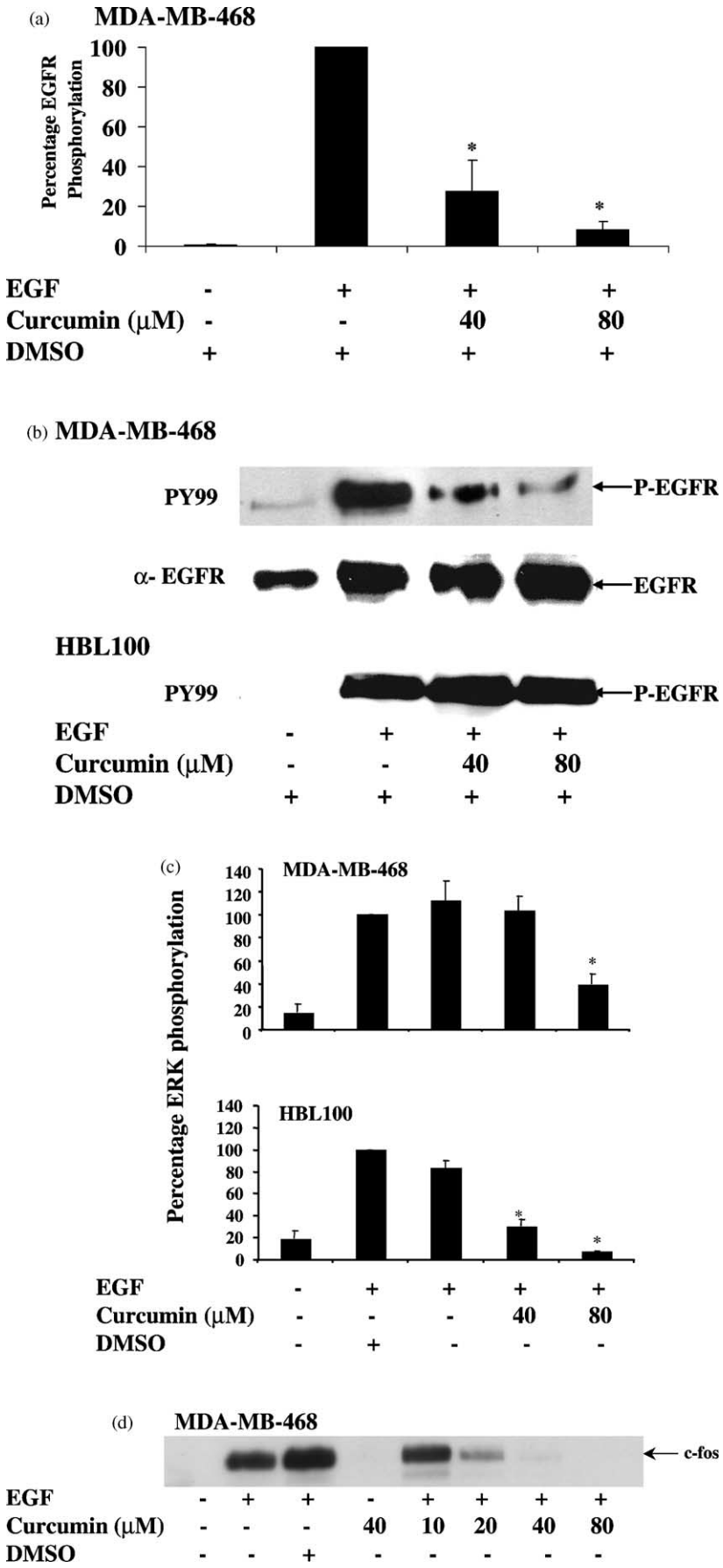

Fig. 6. Inhibition of EGFR and ERKs 1 and 2 phosphorylation and of c-*fos* expression by curcumin. MDA-MB-468 or HBL100 cells were harvested after stimulation with EGF (15 nM for 5 min), with or without pre-treatment for 20 min with 40 or 80 μM curcumin and cell lysates prepared as described in

Table 1
Ability of U0126 and LY294002 to induce apoptosis

	% Viable (LL)	% Apoptotic (UL)	% Necrotic (UR)
HBL100			
Control (with DMSO)	59.7; 69.6	9.6; 7.0	22.7; 17.6
10 μM U0126	70.4; 56.2	5.5; 6.2	19.0; 30.8
20 μM U0126	34.7; 40.8	8.1; 6.7	45.4; 37.9
50 μM LY294002	69.1; 76.1	12.3; 9.9	16.5; 10.9
MDA-MB-468			
Control (with DMSO)	92.2; 91.1	4.8; 5.8	2.5; 2.4
10 μM U0126	92.1; 93.5	5.6; 4.0	2.2; 2.1
20 μM U0126	90.9; 93.6	6.2; 4.2	2.5; 1.9
50 μM LY294002	57.6; 69.3	34.0; 21.2	6.9; 6.6

Cells grown in serum-containing medium were treated with the indicated doses of inhibitor for 24 hr. They were then harvested and prepared for flow cytometric analysis of annexin V binding as described in Section 2. Results of two separate experiments are presented as the percentage of cells in the lower left (LL), upper left (UL) and upper right (UR) quadrants of the scatter plots.

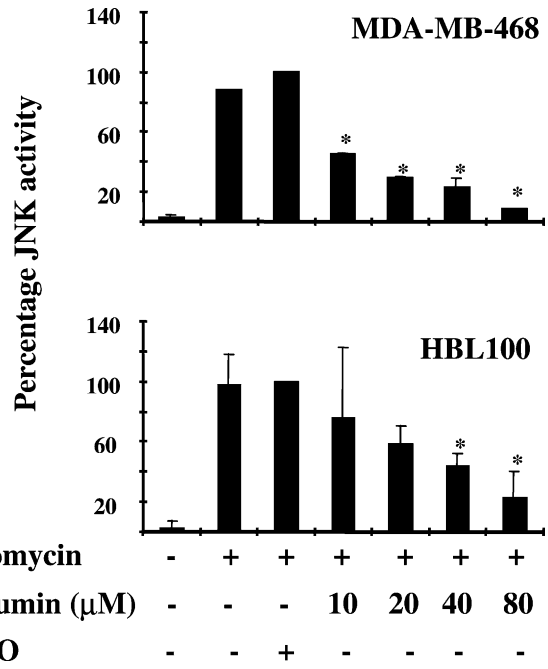

Fig. 7. Inhibition of JNK activity by curcumin. After stimulation of cells with anisomycin (100 nM, 30 min), the activation of JNK was measured using an immune-complex kinase assay. Immunoprecipitated JNK was incubated with substrate GST-jun (αα1–79) in the presence of γ-^{32}P-ATP and phosphorylated GST-jun was detected using a phosphorimager. The effect of pre-treatment (30 min) of cells with increasing concentrations of curcumin on induced JNK activity was examined. Results represent the mean (±SD) of at least three experiments. $^{*}P < 0.05$.

again no inhibition was seen when curcumin was added directly to the assay (Fig. 8). When overexpressed MEKK1 was immunoprecipitated from HEK293 cells and coupled with MKK4 or MKK7 and JNK in an *in vitro* assay, preliminary data suggested that direct addition of curcumin (10 μM) to the assay inhibited phosphorylation of jun (data not shown).

We examined the downstream consequences of JNK inhibition by looking at the phosphorylation of nuclear c-*jun*. HBL100 cells were found to have a significant basal expression of c-*jun* (in agreement with [22]), which was not obviously enhanced by anisomycin (Fig. 9a). On the other hand, low basal levels of the phosphorylated protein were significantly enhanced by anisomycin within 15 min (Fig. 9b), and the increase was inhibited by 20 μM curcumin, while 40 μM appeared to decrease phosphorylated jun to below basal levels (Fig. 9c). Similar results were obtained in MDA-MB-468 cells, although in this case phospho-c-*jun* was barely detectable in unstimulated cells (data not shown and Fig. 5).

3.6. Lack of inhibition of p38 MAPK activity by curcumin

The effect of curcumin on the activity of a third MAPK, p38, was also examined, using anisomycin as the stimulating agent and ATF2 as the substrate in the kinase assay. No inhibition was observed either after pre-treatment of the cells or when curcumin was added directly to the assay, at doses up to 80 μM (data not shown). No p38 activity was observed in unstimulated cells grown in the presence of serum.

3.7. Inhibition of Akt/PKB phosphorylation by curcumin

While HBL100 cells had higher levels of total PKB protein, MDA-MB-468 cells had a higher level of phosphorylated PKB when grown in serum-containing medium (Fig. 5). Curcumin treatment for 30 min at concentrations of 40 μM and above caused inhibition of basal PKB phosphorylation in the tumour cell line (Fig. 10), and although some inhibition was observed at these doses in HBL100 cells, the effect was inconsistent and not statistically significant (data not shown). Treatment with 10 or 20 μM for up to 10 hr did not cause significant inhibition. Addition of curcumin at doses up to 40 μM did not inhibit PKB activity directly. It is of interest to note that while serum withdrawal abolished phosphorylation of PKB in HBL100 cells, very little effect was observed in

Section 2. Panels (a) and (b) EGFR was immunoprecipitated from the lysates and levels of phosphorylated receptor were estimated using an anti-phosphotyrosine antibody (PY99) on western blots. Levels of total receptor were determined using an antibody specific for the EGFR. Levels of phosphorylated ERKs 1 and 2 (Panel c) and total c-*fos* protein (Panel d) were determined from western blots of whole cell lysates using specific antibodies. Results in (a) and (c) are expressed relative to the levels of phosphorylated EGFR/ERK in the EGF/DMSO-treated samples (lane 2) determined by densitometric analysis of the blots. Representative blots are shown in (b) and (d). Data in (a) and (c) represent the mean (±SD) of three separate experiments carried out in duplicate. (∗) Indicates significant difference from the EGF/DMSO-treated sample ($P < 0.05$).

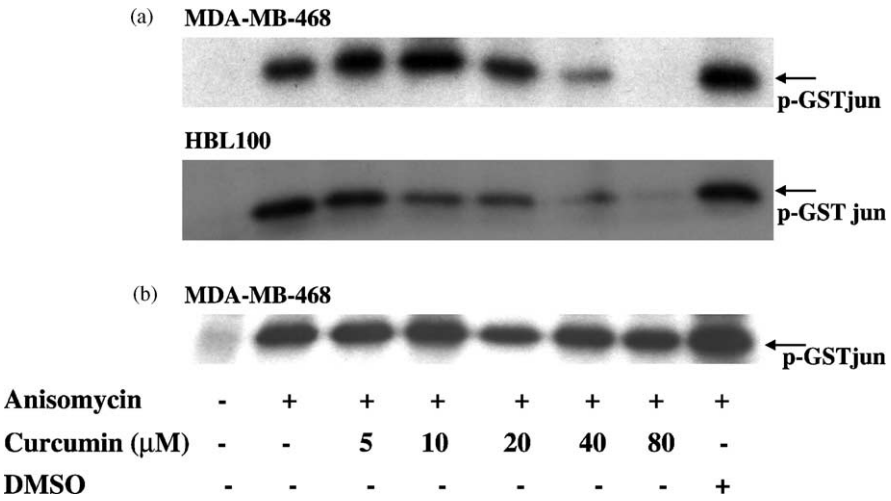

Fig. 8. Inhibition of MKK4 by curcumin. Cells were stimulated with anisomycin (100 nM, 30 min) and MKK4 measured by immune-complex assay. MKK4 activity was assayed by reconstituting the signalling cascade through to c-*jun in vitro*, with phosphorylation of c-*jun* taken as a measure of activity. Panel (a) Cells were pre-treated with increasing concentrations of curcumin, or with the vehicle control (DMSO). Panel (b) Curcumin was added directly to the assay. Representative blots from three separate experiments are shown.

MDA-MB-468 cells (Fig. 5), which do not express the negative regulator of PKB phosphorylation, PTEN (Fig. 5).

3.8. Induction of apoptosis in MDA-MB-468 cells by LY294002

The PI3K inhibitor LY294002 was used to determine whether inhibition of PKB phosphorylation was contributing to the induction of apoptosis. At a dose of 50 μM it completely inhibited PKB phosphorylation in both cell lines after 1 hr, with activity returning by 8 hr. Flow cytometric analysis at 24 hr indicated significant apoptosis occurring only in the MDA-MB-468 cells (Table 1). This result was confirmed by the observation that poly-ADP-ribose polymerase cleavage also occurred only in the MDA-MB-468 cells (data not shown).

4. Discussion

Results from this study clearly show that the minor dietary component, curcumin, can have selective modulatory effects on proliferation and cell signalling pathways. It caused arrest in both cell lines in the S/G2/M phases of the cell cycle, but induced apoptosis much more readily in MDA-MB-468 cells. There was a small, but significant inhibition of invasive capacity by curcumin in both cell lines. It has been reported that a mixture of curcuminoids inhibited the expression of gelatinase B (MMP9) in endothelial cells [37]. MMP9 can be regulated *via* AP-1, PI3K/PKB and GSK-3β [38,39].

Curcumin has previously been shown to cause cell-cycle arrest in G2/S [13], or G2 [14] in other breast cell lines, but these same two studies reported conflicting results on

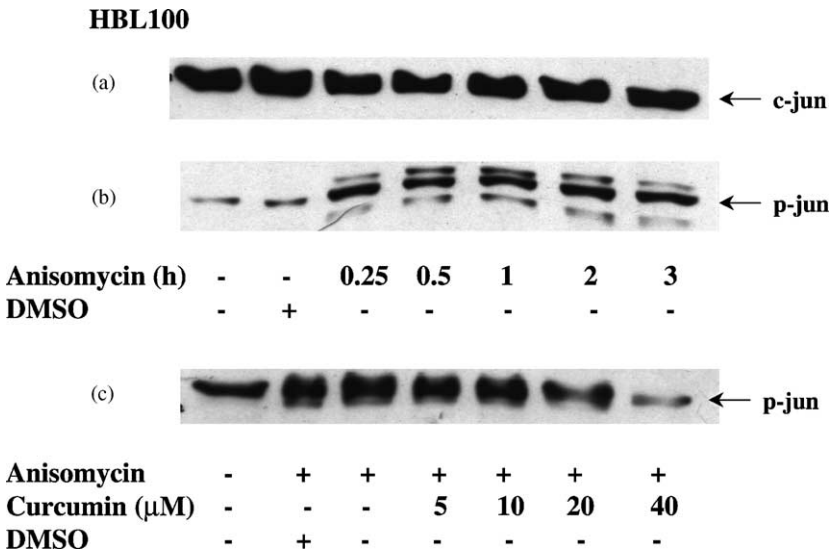

Fig. 9. Effect of anisomycin and curcumin on c-*jun*. Nuclear fractions were prepared from treated cells. The effect of anisomycin (100 nM) for times up to 3 hr on (a) total nuclear c-*jun* and (b) phosphorylated nuclear c-*jun* was determined by western blotting. The effect of pre-treatment of cells with 5–40 μM curcumin (for 30 min) on anisomycin-stimulated phosphorylation of c-*jun* is shown in panel (c). Representative blots for HBL100 cells are shown.

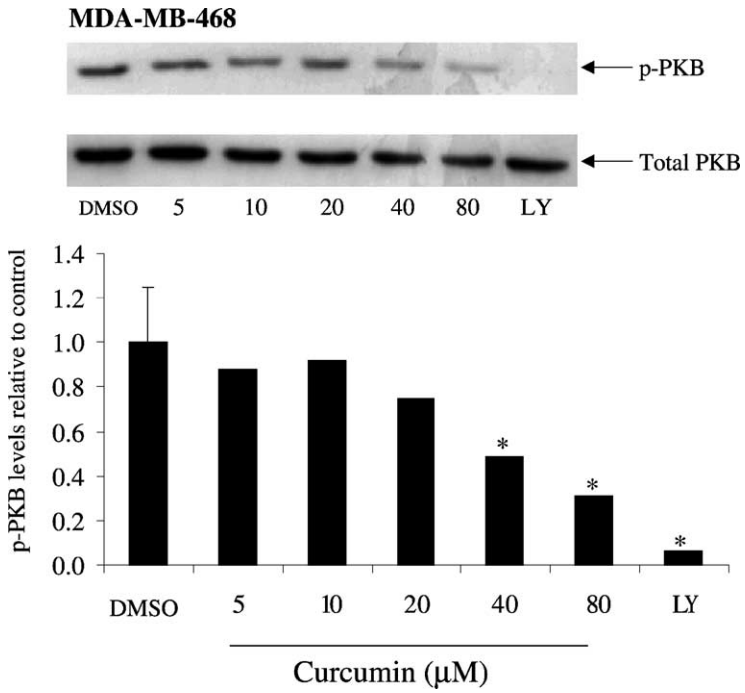

Fig. 10. Inhibition of Akt/PKB phosphorylation by curcumin. MDA-MB-468 cells were treated with increasing concentrations of curcumin (5–80 μM) for 30 min or with LY294002 (50 μM) for 60 min. Phosphorylated Akt/PKB and total Akt/PKB were determined by western blotting. Combined results (N = 5) for the decrease in phospho-Akt/PKB relative to the DMSO control are expressed ±SD. $^{*}P < 0.05$.

induction of apoptosis. Our results suggest that some breast lines may be more susceptible to apoptosis than others. Lack of wild type p53 and compromised G1 checkpoint control have been linked to polyploidy in response to microtubule inhibitors in a number of cell lines, including MDA-MB-468. Recently Motwani *et al*. [40] showed that flavopiridol prevented nocodazole-induced polyploidy in these cells. Flavopiridol decreased cyclin E levels and inhibited cyclin E/cyclin-dependent kinase (Cdk2) kinase activity, required for endoreduplication, and cyclin B1/cell division cycle (cdc2) kinase activity, which is required for entry into mitosis in these cells. Another study by Tsuiki *et al*. [41] showed that staurosporine enhanced hyperploid formation in nocodazole-treated p53 mutant U251MG cells, and demonstrated that this effect was prevented by wild type p53 using LN382 cells which contained temperature-sensitive p53. Induction of p21 also prevented endoreduplication through regulation of cyclin E/Cdk2 activity in HCT116 cells [42]. Curcumin can modulate expression of p53, p21, cyclin E and cdc2 in a variety of cell lines [14,43–45]. The mechanism by which curcumin may enhance endoreduplication in MDA-MB-468 cells, or protect against polyploidy in HBL100 cells, will form the subject of a separate investigation in this laboratory.

The MDA-MB-468 line had higher basal expression of total and phosphorylated EGFR than the HBL line. Although inhibition of receptor phosphorylation by curcumin (up to 80 μM) was only significant in the MDA cells, these were apparently less sensitive than HBL cells to inhibition of ERK phosphorylation, suggesting that after curcumin treatment sufficient phosphorylated receptor still remained to fully activate ERK. However, firstly 10 μM U0126 inhibited ERK phosphorylation completely in MDA-MB-468 cells, but only partially in HBL100 cells, secondly substantial levels of phospho-ERK were present in HBL100 cells even in the absence of phospho-EGFR, and thirdly serum withdrawal did not decrease phosphorylation in either cell line. These results suggest constitutive activation or the involvement of other pathways. ER-negative and invasive breast cancer lines, including MDA-MB-468 have been shown to have constitutively higher ERKs 1 and 2 levels than ER-positive and non-invasive lines. Krueger *et al*. [46] found inhibition of MAPK activation by antisense MKK expression in MDA-MB-468 cells significantly inhibited cell migration and *in vitro* invasion. Inhibition of EGFR phosphorylation by curcumin could also influence PKB activity (see the following discussion).

The importance of the MEK/ERK signalling pathway in survival of a number of different breast cell lines, including MDA-MB-468, was reported in a preliminary study [47]. It appeared that for most of the cell lines, survival and regrowth after prolonged serum withdrawal was correlated with the presence of phospho-ERK and inhibited by U0126. However, the presence of serum allowed MDA-MB-468 cells to survive for longer in the presence of U0126, suggesting an ERK-independent survival pathway in this cell line. This would be consistent with the lack of apoptosis observed by us following treatment of this cell line with U0126.

In the present study substantial inhibition of anisomycin-stimulated JNK activity was achieved at a dose of curcumin approaching the IC_{50}, resulting in a decrease in c-*jun* phosphorylation. The tumour cell line was more sensitive to inhibition of this MAPK than the non-malignant HBL100 cell line. The fact that curcumin did not inhibit JNK when added directly to the assay suggested that it was acting upstream, in agreement with a recent study in Jurkat cells [16]. However, results from our study also imply that it does not affect a common upstream activator of JNK and p38, since curcumin did not inhibit activation of the latter. Our data also show that curcumin indirectly inhibited the activity of MKK4, suggesting that the target is upstream of this molecule. MKK4 can activate both JNK and p38, but it may be a more potent stimulator of JNK, since dominant negative MKK4 inhibits JNK activity more strongly than that of p38. In addition, the upstream activator of MKK4, MEKK1, causes selective activation of the JNK pathway in transfection experiments (reviewed in [48]). Overexpression of upstream components of the JNK pathway in Jurkat cells also suggested that curcumin may be exerting its effect at the MAPK kinase kinase (MAPKKK) level of the cascade [16]. Also consistent with the position of the target upstream of MKK4, expression of a TRE-luciferase construct driven by overexpression of MEKK1 was found to be inhibited by curcumin (but at 100 μM) in HT29 colon tumour cells [49].

While activation of the ERK pathway has generally been linked to proliferation responses, the physiological role of JNK is less well defined. Depending on the circumstances, JNK has been shown to trigger apoptosis, proliferation or differentiation. The consequence of JNK activation (or inhibition) almost certainly depends on cell type, and the combination of signals received by the cell. Clearly cell proliferation is a tightly regulated process so that deregulation of many distinct targets could potentially promote cell-cycle withdrawal and apoptosis. Curcumin appeared to be a good inhibitor of the JNK pathway, but in the absence of stimulation neither HBL100 nor MDA-MB-468 cells exhibited significant JNK activity and nuclear phosphorylated c-*jun* was not detectable in MDA-MB-468 cells when grown in serum-containing medium in the absence of anisomycin, suggesting that inhibition of this pathway might not be relevant to the growth arrest and apoptosis observed.

In the present study, curcumin was found to inhibit basal phosphorylation of PKB more effectively in MDA-MB-468 cells than in HBL100 cells. We showed previously by PARP cleavage and here by flow cytometry that complete inhibition of PKB phosphorylation by LY294002, an inhibitor of PI3K, resulted in apoptosis only in the MDA-MB-468 cells. Taken together, these data suggest that the PKB pathway may be important for survival in the MDA-MB-468 cells, but is not predominantly responsible in the HBL100 cells since treatment with LY294002 does not induce apoptosis in this cell line. However, while LY294002 inhibited phosphorylation of PKB more effectively than curcumin, the percentage of cells undergoing apoptosis in response to curcumin was higher, suggesting that curcumin also inhibits other survival mechanisms. Alternatively, it could be that inhibition by LY294002 is less sustained than that by curcumin. Curcumin did not directly inhibit PKB activity, suggesting that it is acting upstream.

Since stimulation of growth factor receptors is known to activate the PI3K/PKB pathway, it could be that inhibition of EGFR phosphorylation by curcumin contributes to the decreased phosphorylation of PKB. In colon cells, curcumin has been shown to inhibit NF-κB activation (e.g. [50]), another well-recognised survival mechanism. This transcription factor, which can be downstream of PKB, regulates many genes including cyclin D1 [30], cyclooxygenase 2 [50], Bcl-xl and MMPs [39]. The exact mechanism whereby curcumin inhibits PKB phosphorylation and the key downstream molecules responsible for inducing apoptosis are under investigation.

Acknowledgments

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References

[1] Huang MT, Smart RC, Wong CQ, Conney AH. Inhibitory effect of curcumin, chlorogenic acid, caffeic acid, and ferulic acid on tumour promotion in mouse skin by 12-*o*-tetradecanoylphorbol 13 acetate. Cancer Res 1988;48:5941–6.

[2] Huang MT, Wang ZY, Georgiadis CA, Laskin JD, Conney AH. Inhibitory effects of curcumin on tumour initiation by benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene. Carcinogenesis 1992;13:2183–6.

[3] Huang MT, Lou YR, Ma W, Newmark HL, Reuhl KR, Conney AH. Inhibitory effect of dietary curcumin on forestomach, duodenal and colon carcinogenesis in mice. Cancer Res 1994;54:5841–7.

[4] Rao CV, Rivenson A, Simi B, Reddy BS. Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. Cancer Res 1995;55:259–66.

[5] Singletary K, MacDonald C, Wallig M, Fisher C. Inhibition of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumorigenesis and DMBA-DNA adduct formation by curcumin. Cancer Lett 1996;103:137–41.

[6] Pereira MA, Grubbs CJ, Barnes LH, Li H, Olson GR, Eto I, Juliana M, Whitaker LM, Kelloff GJ, Steele VE, Lubet RA. Effects of the phytochemicals, curcumin and quercetin, upon azoxymethane-induced colon cancer and 7,12-dimethylbenz[*a*]anthracene-induced mammary cancer in rats. Carcinogenesis 1996;17:1305–11.

[7] Kawamori T, Lubet R, Steele VE, Kelloff GJ, Kaskey RB, Rao CV, Reddy BS. Chemopreventive effect of curcumin, a naturally occurring

anti-inflammatory agent, during the promotion/progression stages of colon cancer. Cancer Res 1999;59:597–601.
[8] Chuang SE, Kuo ML, Hsu CH, Chen CR, Lin JK, Lai GM, Hseih CY, Cheng AL. Curcumin-containing diet inhibits diethylnitrosamine-induced murine hepatocarcinogenesis. Carcinogenesis 2000;21: 331–5.
[9] Inano H, Onoda M, Inafuku N, Kubota M, Kamada Y, Osawa T, Kobayashi H, Wakabayashi K. Chemoprevention by curcumin during the promotion stage of tumorigenesis of mammary gland in rats irradiated with γ-rays. Carcinogenesis 1999;20:1011–8.
[10] Inano H, Onoda M, Inafuku N, Kubota M, Kamada Y, Osawa T, Kobayashi Y, Wakabayashi K. Potent preventive action of curcumin on radiation-induced initiation of mammary tumorigenesis in rats. Carcinogenesis 2000;21:1835–41.
[11] Stoner GD, Mukhtar H. Polyphenols as cancer chemopreventive agents. J Cell Biochem 1995;S22:169–80.
[12] Hong R-L, Spohn WH, Hung M-C. Curcumin inhibits tyrosine kinase activity of $p185^{neu}$ and also depletes $p185^{neu1}$. Clin Cancer Res 1999;5:1884–91.
[13] Mehta K, Pantazis P, McQueen T, Aggarwal BB. Antiproliferative effect of curcumin (diferuloylmethane) against human breast tumor cell lines. Anticancer Drugs 1997;8:470–81.
[14] Ramachandran C, You W. Differential sensitivity of human mammary epithelial and breast carcinoma cell lines to curcumin. Breast Cancer Res Treat 1999;54:269–78.
[15] Ramachandran C, Melnick SJ, Jhabvala P, Fonseca HB. Inhibition of hTERT expression and telomerase activity by curcumin. Proc Am Assoc Cancer Res 2001;42:575.
[16] Chen Y-R, Tan T-H. Inhibition of the c-jun N-terminal kinase (JNK) signaling pathway by curcumin. Oncogene 1998;17:173–8.
[17] Keinhuis C, Sluyser M, de Goeij C, Koenders P, Benraad T. Epidermal growth factor receptor levels increase but epidermal growth factor receptor ligand levels decrease in mouse mammary tumours during progression from hormone dependence to hormone independence. Breast Cancer Res Treat 1993;26:289–95.
[18] Xing C, Imagawa W. Altered MAP kinase (ERK1,2) regulation in primary cultures of mammary tumor cells: elevated basal activity and sustained response to EGF. Carcinogenesis 1999;20:1201–8.
[19] Kottke TJ, Blajeski AL, Martins M, Mesner PW, Davidson NE, Earnshaw WC, Armstrong DK, Kauffmann SH. Comparison of paclitaxel, 5-fluoro-2′-deoxyuridine- and epidermal growth factor(-EGF)-induced apoptosis. J Biol Chem 1999;274:15927–36.
[20] Chen TK, Smith LM, Gebhardt DK, Birrer MJ, Brown PH. Activation and inhibition of the AP-1 complex in human breast cancer cells. Mol Carcinog 1996;15:215–26.
[21] Smith LM, Wise SC, Hendricks DT, Sabichi AL, Bos T, Reddy P, Brown PH, Birrer MJ. c-Jun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone-resistant phenotype. Oncogene 1999;18:6063–70.
[22] Smith LM, Birrer MJ, Stampfer MR, Brown PH. Breast cancer cells have lower activating protein 1 transcription factor activity than normal mammary epithelial cells. Cancer Res 1997;57:3046–54.
[23] Johnston SRD, Lu B, Scott GK, Kushner PJ, Smith IE, Dowsett M, Benz CC. Increased activator protein-1 DNA binding and c-jun NH2 terminal kinase activity in human breast tumors with acquired tamoxifen resistance. Clin Cancer Res 1999;5:251–6.
[24] Mandelkar S, Yu R, Tan TH, Kong ANT. Activation of caspase 3 and c-jun NH2-terminal kinase-1 signalling pathways in tamoxifen-induced apoptosis of human breast cancer cells. Cancer Res 2000; 60:5995–6000.
[25] Huang S, New L, Pan ZX, Han JH, Nemerow GR. Urokinase plasminogen activator/urokinase-specific surface receptor expression and matrix invasion by breast cancer cells requires constitutive p38 alpha mitogen-activated protein kinase activity. J Biol Chem 2000; 275:12266–72.
[26] Paine E, Palmantier R, Akiyama SK, Olden K, Roberts JD. Arachidonic acid activates mitogen-activated protein (MAP) kinase-activated protein kinase 2 and mediates adhesion of a human breast carcinoma cell line to collagen type IV through a p38 MAP kinase-dependent pathway. J Biol Chem 2000;275:11284–90.
[27] Vadlamudi R, Adam L, Talukder A, Mendelsohn J, Kumar R. Serine phosphorylation of paxillin by heregulin-beta 1: role of p38 mitogen activated protein kinase. Oncogene 1999;18:7253–64.
[28] Lu Y, Lin Y-Z, LaPushin R, Cuevas B, Fang X, Yu SX, Davies MA, Khan H, Furui T, Mao M, Zinner R, Hung M-C, Steck P, Siminovitch K, Mills G. The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. Oncogene 1999;18:7034–45.
[29] Weng L-P, Smith WM, Dahia PLM, Ziebold U, Gil E, Lees JA, Eng C. PTEN suppresses breast cancer cell growth by phosphatase activity-dependent G1 arrest followed by cell death. Cancer Res 1999;59: 5808–14.
[30] Biswas DK, Cruz AP, Gansberger E, Pardee AB. Epidermal growth factor-induced nuclear factor κB activation: a major pathway of cell cycle progression in estrogen-receptor negative breast cancer cells. Proc Natl Acad Sci USA 2000;97:8542–7.
[31] Gaffney EV. A cell line (HBL-100) established from human breast milk. Cell Tissue Res 1982;227:563–8.
[32] Sullivan KM, Mannucci A, Kimpton CP, Gill P. A rapid and quantitative DNA sex test—fluorescence-based PCR analysis of X-Y homologous gene amelogenin. Biotechniques 1993;15:636–8.
[33] Rudland PS. Generation of lobuloalveolar development from isolated rat mammary ducts and endbuds. J Histochem Cytochem 1991;39: 1257–66.
[34] Jones JL, Royall JE, Critchley DR, Walker RA. Modulation of myoepithelial-associated alpha6 beta 4 integrin in a breast cancer cell line alters invasive potential. Exp Cell Res 1997;235:325–33.
[35] Runswick SK, O'Hare MJ, Jones JL, Streuli CH, Garrod DR. Desmosomal adhesion regulates epithelial morphogenesis and cell positioning. Nat Cell Biol 2001;3:823–30.
[36] Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM, McEwan RN. A rapid in-vitro assay for quantitating the invasive potential of tumour cells. Cancer Res 1987;47:3239–45.
[37] Mohan R, Sivak J, Ashton P, Russo LA, Pham BQ, Kasahara N, Raizman MB, Fini ME. Curcuminoids inhibit the angiogenic response stimulated by fibroblast growth factor 2, including expression on matrix mettalloproteinase gelatinase B. J Biol Chem 2000;275: 10405–12.
[38] Troussard AA, Costello P, Yoganathan TN, Kumagai S, Roskelley CD, Dedhar S. The integrin linked kinase (ILK) induces an invasive phenotype via AP-1 transcription factor-dependent upregulation of matrix metalloproteinase 9 (MMP9). Oncogene 2000;19:5444–52.
[39] Kim D, Kim S, Koh H, Yoon S-O, Chung A-S, Cho KS, Chung J. Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase induction. FASEB J 2001;15:1953–62.
[40] Motwani M, Li X-K, Schwartz GK. Flavopiridol, a cyclin-dependent kinase inhibitor, prevents spindle inhibitor-induced endoreduplication in human cancer cells. Clin Cancer Res 2000;6:924–32.
[41] Tsuiki H, Nitta M, Tada M, Inagaki M, Ushio Y, Saya H. Mechanism of hyperploid cell formation induced by microtubule inhibiting drug in glioma cell lines. Oncogene 2001;20:420–9.
[42] Stewart ZA, Leach SD, Pietenpol JA. p21Waf1/Cip1 inhibition of cyclin E/cdk2 activity prevents endoreduplication after mitotic spindle disruption. Mol Cell Biol 1999;19:205–15.
[43] Jee SH, Shen SC, Tseng CR, Chiu HC, Kuo ML. Curcumin induces a p53-dependent apoptosis in human basal cell carcinoma cells. J Invest Dermatol 1998;111:656–61.
[44] Chuang SE, Cheng AL, Lin JK, Kuo ML. Inhibition by curcumin of diethylnitrosamine-induced hepatic hyperplasia, inflammation, cellular gene products and cell-cycle-related proteins in rats. Food Chem Toxicol 2000;38:991–5.

[45] Bech-Otschir D, Kraft R, Huang XH, Henklein R, Kapelari B, Pollmann C, Dubeil W. COP9 signalosome-specific phosphorylation targets p53 to degradation by the ubiquitin system. EMBO J 2001; 20:1630–9.

[46] Krueger JS, Keshamouni VG, Atanaskova N, Reddy KB. Temporal and quantitative regulation of mitogen-activated protein kinase (MAPK) modulates cell motility and invasion. Oncogene 2001;20: 4209–18.

[47] Ripple MO, Eastman A. The importance of MEK to ERK signaling for breast cancer cell survival. Proc Am Assoc Cancer Res 2001; 42:673.

[48] Davis RJ. Signal transduction by the c-jun N-terminal kinase. Biochem Soc Symp 1998;64:1–12.

[49] Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, Brenner DA, Sartor RB. Curcumin blocks cytokine-mediated NF-κB activation and proinflammatory gene expression by inhibiting inhibitory factor I-kB kinase activity. J Immunol 1999;163:3474–83.

[50] Plummer SM, Holloway KA, Manson MM, Munks RJL, Kaptein A, Farrow S, Howells L. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-κB activation via the NIK/IKK signalling complex. Oncogene 1999;18:6013–20.