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Original Paper

Induction of Metalloproteinase (MMP1) Expression by Epidermal Growth Factor (EGF) Receptor Stimulation and Serum Deprivation in Human Breast Tumour Cells

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The levels of the matrix metalloproteinase MMP1 mRNA in three breast tumour cell lines with varying numbers of epidermal growth factor (EGF) receptors, MDA-MB-231, T47D and MCF7, were investigated following treatment with EGF or TGFα in serum-free medium for up to 24 h. A higher level of MMP1 mRNA was found in both control and treated MDA-MB-231 cells compared with the other two cell lines. A 2-fold increase in MMP1 transcripts was observed in MDA-MB-231 cells following a 30 min treatment with EGF and 2 h with TGFα. An increase in MMP1 transcripts following serum deprivation in the absence of growth factor stimulation was also seen. This effect was not evident with the other cell lines. In MDA-MB-231 cells, low concentrations of MMP1 protein were detected in medium from treated cells and was only significantly increased after 24 h but it was inhibited by cycloheximide. The early effect of EGF on MMP1 expression was not inhibited by cycloheximide. Treatment with cycloheximide for longer periods produced increased transcripts of MMP1, TGFα and EGF-receptor, suggesting the activation of processes for tissue breakdown and subsequent repair may occur on prolonged inhibition of protein synthesis. These results confirm a relationship between EGF-receptor stimulation and MMP1 expression in some EGF-receptor positive tumour cells, which, in part, occurs at the transcriptional level, and have implications for the invasive progression of EGF-receptor positive tumours particularly in areas of nutritional deprivation.

Key words: metalloproteinase, growth factors, serum deprivation, MDA-MB-231, MCF7, T47D, cycloheximide, phorbol ester

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INTRODUCTION

BREAST CANCER cell growth is regulated by numerous hormones and growth factors, including oestradiol, epidermal growth factor (EGF) and transforming growth factor α and β (TGFα and TGFβ). Both EGF and TGFα are ligands for the EGF-receptor. A number of studies have shown that, in breast cancer samples, there is an inverse relationship between the presence of oestrogen and EGF receptors, and high levels of EGF-receptor expression are an indicator of poor prognosis [1, 2]. However, the biological basis for this has not been established, but is likely to involve more than altered growth control, since factors increasing tumour invasion and spread must also be involved. This study explores the possibility that EGF may also induce the expression of type I collagenase (MMP1) and so facilitate tumour invasion.

The matrix metalloproteinases, together with other proteases, have been widely implicated in tumour invasion and metastasis [3]. They are members of a unique family of proteolytic enzymes which contain a zinc ion at their active sites and can degrade collagens and other components of the extracellular matrix (ECM). The enzymes require activation from their latent forms, and are additionally regulated by endogenous proteins known as tissue inhibitors of metalloproteinases (TIMPS) [4].

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A study of oesophageal squamous cell carcinoma cell lines [5] has shown the production of a variety of MMPs, and that the production of pro-MMP9 may be regulated by EGF via overexpression of EGF receptors. However, no effect was reported on the levels of MMP1. Type IV collagenases (MMP2 and MMP9) have also been studied in malignant breast disease [6] and bladder cancer [7], where there was a positive correlation between production of these matrix metalloproteinases and malignant disease or tumour grade and invasion. No correlation has been found between MMP levels and tumour grade in ovarian cancer [8]. Other recent studies on metalloproteinase expression have been carried out [9, 10] and demonstrate the importance of the collagenases in the biology of some tumours, and that the independent regulation of the different enzymes and their inhibitors is important in the invasive potential of tumour cells.

In gastrointestinal tract cancers, MMP1 was found to be expressed in stromal cells closely associated with cancer cells, suggesting a pathophysiological role of MMP1 in the invasion and metastasis of cancer cells [11]. A recent study [12] demonstrated expression of MMP1 in 3 of 11 invasive breast ductal carcinomas, in fibroblasts, but not in the 2 fibroadenomas tested or in normal breast structures present in the tissue sections. mRNA encoding MMP1 was also detected in occasional stromal and tumour cells in 2 of 17 breast carcinomas [13]. We have also observed a positive correlation between TGFα and MMP1 expression in human breast cancer samples (Dr J. Lunec, Newcastle-upon-Tyne). This may, in part, explain the poor prognosis of EGF-receptor positive patients. There have been no previous studies on MMP1 regulation by growth factors in human breast tumour cell lines.

MMP1 expression is positively regulated by the activator protein-1 (AP1) transcriptional regulatory complex [14], which is also inducible by EGF [15], suggesting a possible link between EGF-receptor stimulation and MMP1 expression. The present work tests whether a relationship exists between EGF-receptor stimulation (by EGF or TGFα) and MMP1 expression in various breast cancer cell lines with differing EGF and oestrogen receptor status. The cell lines used were the oestrogen receptor (ER) negative and highly EGF-R positive MDA-MB-231 cells, as well as the ER positive T47D and MCF-7 cell lines, the T47D cells having an intermediate level of EGF receptors [16].

The effect of the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA), which also activates the AP1 transcriptional complex and is known to induce the MMP1 gene [14], was also studied for comparison. Cycloheximide, which inhibits protein synthesis, was used to help establish whether the induction of MMP1 observed was a transcriptional effect or was the result of post-translational regulatory mechanisms.

MATERIALS AND METHODS

Cell growth and treatment

Three breast cancer cell lines, T47D, MCF-7 and MDA-MB-231, were routinely grown in Eagle Basal Medium with Earle's salts (EBME) containing 10% fetal bovine serum (FBS) and subcultured weekly. All cell lines were mycoplasma negative.

Cells required for treatment were seeded at 3×10^6 cells in large petri dishes in EBME containing 10% FBS. After 4 days, when the cells were approximately 70% confluent, the cells were washed twice with phosphate buffered saline (PBS) and depleted of serum by addition of EBME containing 0.1% bovine serum albumin (BSA) for 24 h, apart from controls containing 10% FBS. Serum depletion was used to remove all endogenous growth factors from the medium. Fresh medium was then added with 10 ng/ml EGF or TGFα. Controls were given either FBS or BSA in the medium. Following incubation from 30 min to 24 h, the cells were washed with ice-cold PBS, scraped from the plates and centrifuged. The cell pellets were frozen at −70°C prior to RNA extraction.

When cycloheximide was used, this was added to the fresh medium and the cells incubated for an hour before addition of growth factor. Cycloheximide was used at a final concentration of 10 μg/ml.

TPA treatment

The effect of TPA on MMP1 expression in the three cell lines was also investigated. The MDA-MB-231 cells were treated with 50 μM TPA in medium containing 0.1% BSA as previously described. The MCF-7 and T47D cells were grown in phenol-red-free medium containing 10% charcoal-stripped serum before treatment with 50 μM TPA alone or with 10^{-9} M oestradiol for 24 h.

RNA extraction

RNA was extracted from frozen cell pellets by the phenol-guanidinium isothiocyanate method using a commercial reagent mixture RNAzol (Biogenesis), followed by chloroform extraction and isopropanol precipitation. The RNA was dissolved in water, the concentration determined by absorbance at 260 nm and the samples stored at −70°C.

Dot blot and Northern blot analysis

RNA samples (10 μg) were glyoxylated as previously described [17] prior to application to a Nylon membrane (Hybond-N, Amersham, U.K.) for dot blot analysis. For Northern blot analysis, 20 μg samples of RNA were glyoxylated prior to electrophoresis in 1.2% agarose gel in 10 mM phosphate buffer. The gel was stained in ethidium bromide to verify equal loading of samples before blotting the RNA on to the Hybond membrane. After transfer and air-drying, RNA was fixed to the membrane by ultraviolet irradiation crosslinking for 3.5 min using a mid-range transilluminator (Ultra Violet Products, Inc., U.K.).

Probe preparation and hybridisation

Radioactive probes were prepared using the random primer extension method [18]. The MMP1, EGF-R and TGFα probes were prepared from cDNA clone inserts, the probes having been obtained from P. Angel, A. Ullrich [19] and R. Derynk, respectively. The 18S rRNA probe was prepared as previously described [20], using the PCR product generated by oligonucleotide primers –ATGCTCTTAGCTGAGTGTCC– and –AACTACGACGGTATCTGATCG– (residues 864–881 and 1154–1175) and a human cDNA sample.

The filters were prehybridised for 3 h at 65°C and hybridised overnight at 65°C. The prehybridisation and hy-

bridisation solution contained a final concentration of 5 × Denhardts solution, 4 × SET buffer (20 × SET: 3 M NaCl, 0.4 M Tris pH 7.8, 20 mM EDTA), 0.2 M phosphate buffer pH 6.8, 0.1% SDS and 0.1 mg/ml denatured salmon sperm DNA. For hybridisation, the labelled and denatured probe was added to a final radioactivity concentration of 10^6 cpm per ml of hybridisation solution used. Filters were then washed twice in 2 × SSC, 2% SDS (unless otherwise stated) at 65°C for 5 min, and a final wash for 15 min.

Filters were autoradiographed using X-ray film or exposed to a PhosphorImager screen (Molecular Dynamics, U.K.) for direct quantitative autoradiography. Filters were stripped and rehybridised with 18S RNA probes (using 1×10^4 cpm/ml hybridisation solution) to verify equal loading and transfer of RNA on to the filters.

MMP1 protein concentration

Conditioned medium was collected from MDA-MB-231 cells treated with EGF or TGFα for 0.5 and 2 h and EGF for 24 h, with control samples, and stored at −20°C. The media were concentrated 8-fold using Centricon-10 (Amicon) and used for the measurement of total MMP1 with the Biotrak MMP1 ELISA system (Amersham). The assay is based on a two site ELISA sandwich format and MMP1 may be measured in the range 6.25–100 ng/ml. Cells were pelleted and stored as previously described prior to lysis by sonication in 1 ml 50 mM Tris–HCl pH 7.4. The sample was spun for 15 min at 13 000 rpm at 4°C and the supernatant used for protein estimation (Pierce Coomassie Protein Reagent) and measurement of total MMP1 concentration as above.

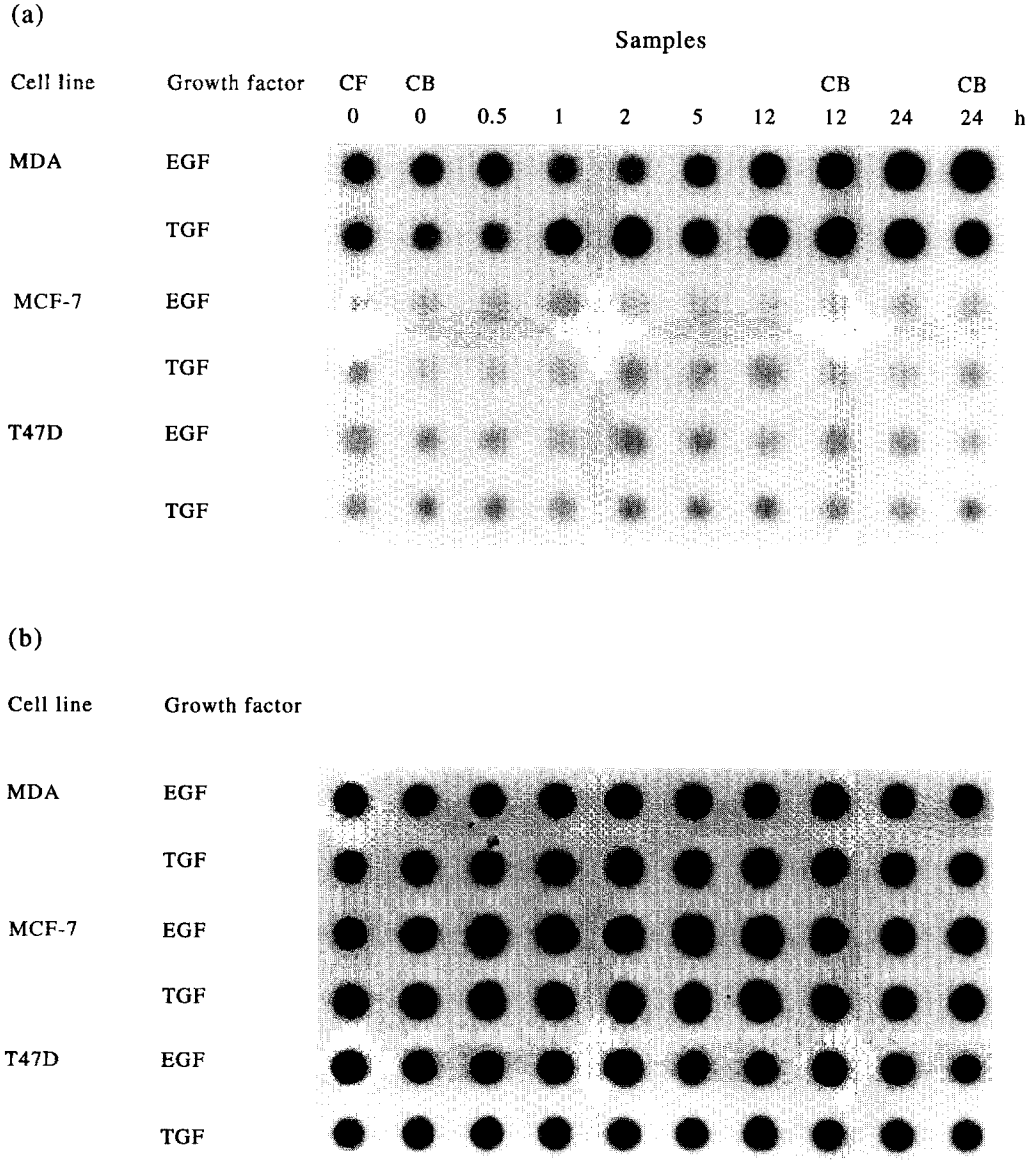

Figure 1. Dot blot analysis using (a) MMP1 probe and (b) 18S rRNA probe to analyse RNA from three breast tumour cell lines treated with EGF or TGFα in serum-free medium for the times indicated. MDA, MDA-MB-231; CF, control in medium containing 10% FBS; CB, control in medium containing 0.1% BSA (serum-free medium).

RESULTS

MMP1 induction in breast cancer cell lines

The effect of EGF and TGFα treatment on MMP1 mRNA levels in the three breast cancer cell lines for varied times of treatment is shown in Figure 1. The MDA-MB-231 cells showed an abundance of MMP1 transcripts in both control and treated samples compared with the other two cell lines. Transient changes in MMP1 transcript levels were observed in response to treatment and the time course of these changes was different for EGF and TGFα. Treatment with EGF produced a 2-fold increase in transcripts following 30 min incubation which decreased to control levels after 1 h, whereas with TGFα an increase was seen after 1 and 2 h which subsequently decreased following 5 h of treatment. An increase was also seen following 12 and 24 h incubation in serum-free medium both in the absence and presence of growth factors.

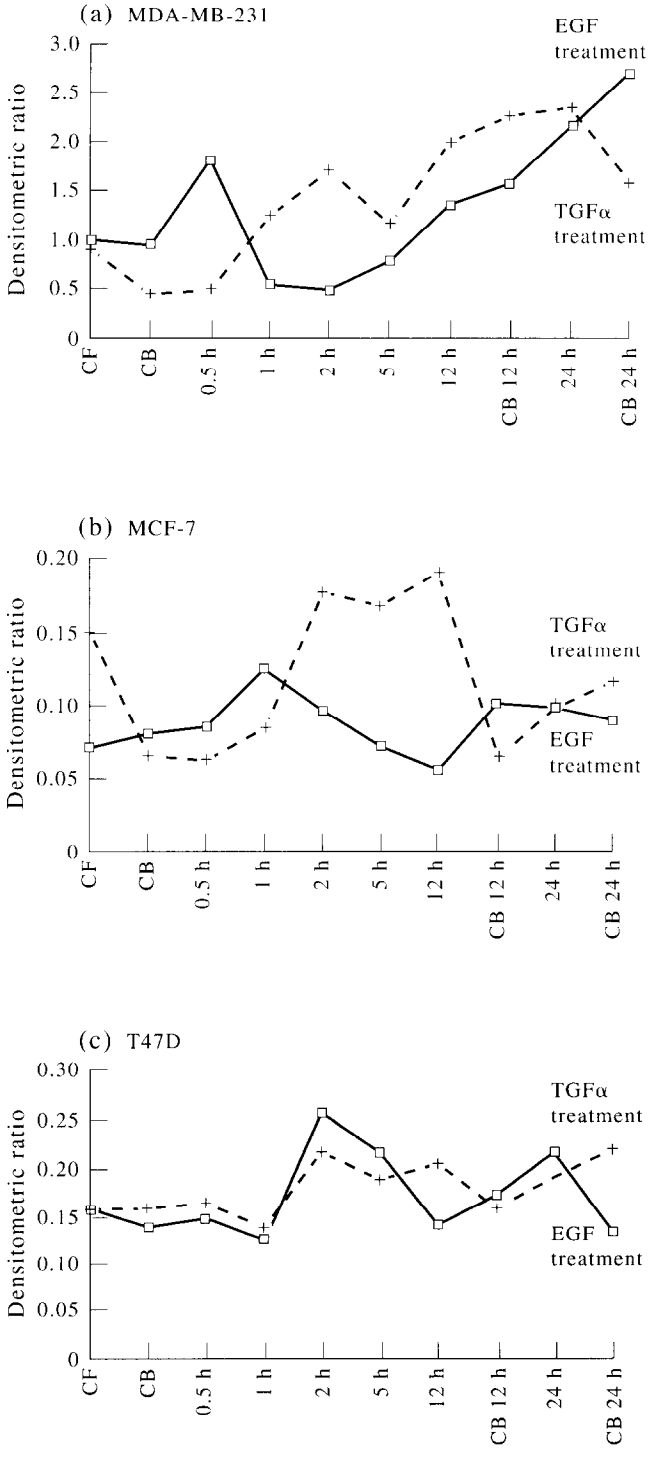

Figure 2. Densitometric ratio of MMP1 mRNA/18S RNA from the dot blot analysis shown in Figure 1 for the three cell lines. CF, control in medium containing 10% FBS; CB, control in medium containing 0.1% BSA (serum-free medium).

An increase in MMP1 transcripts was seen in the MCF-7 cells following EGF treatment for 1 h and TGFα treatment for 2 h. Stimulation for 2 h with either EGF or TGFα produced increased MMP1 mRNA levels in T47D cells. In addition, the MCF7 and T47D cells showed no increase in MMP1 mRNA in response to serum deprivation as seen with the MDA-MB-231 cells. The result of 18S RNA hybridisation (Figure 1b) verified equal loading of the RNA samples. The ratio of MMP1 to 18S mRNA for all three lines obtained by densitometry is shown in Figure 2.

On Northern hybridisation with the MMP1 specific probe, a 1.7 Kb MMP1 transcript was seen in the MDA-MB-231 cell lines, again showing the effects of TGFα at 1 and 2 h and EGF at 30 min and the increase in MMP1 transcripts at 12 and 24 h (Figure 3). No evidence of a 1.7 Kb transcript was detected on Northern hybridisation of samples from MCF-7 (data not shown) or T47D cell lines. However, in some samples of T47D-treated cells, a higher molecular weight transcript was detected (Figure 4). The stained gel showed equal loading of RNA of all the samples.

Effect of serum deprivation on MMP1 induction

The increase in MMP1 transcripts seen in the MDA-MB-231 cells in the 12 h control sample was investigated further. Treatments were performed using an increased number of control sample time points with both FBS and serum-free medium to determine the time course of the effect. An increase in MMP1 expression was seen in the 5 h serum-free BSA control which was further increased at 12 and 24 h in the BSA controls, but not the FBS controls, explaining the increases seen at 5, 12 and 24 h shown in Figures 1–3. The cells incubated in the serum-free medium for this additional 24 h did not change their morphological appearance and remained adhered to the culture dish in a monolayer.

Effect of cycloheximide on mRNA induction in MDA-MB-231 cells

The protein synthesis inhibitor cycloheximide was used to determine whether the observed changes in transcription required *de novo* synthesis of transcription factors or proteins implicated in autocrine signalling changes. RNA extracts from cells treated in the time course with cycloheximide and growth factors were subjected to Northern blot analysis and hybridisation with labelled probes for MMP1, TGFα, EGF-receptor and 18S RNA. The results are shown in Figure 5. Treatment of the cells for 24 h with 10 μg/ml cycloheximide did not cause a decrease in cell numbers (data not shown) and the cells showed no morphological changes. The results for MMP1 show an increase in tran-

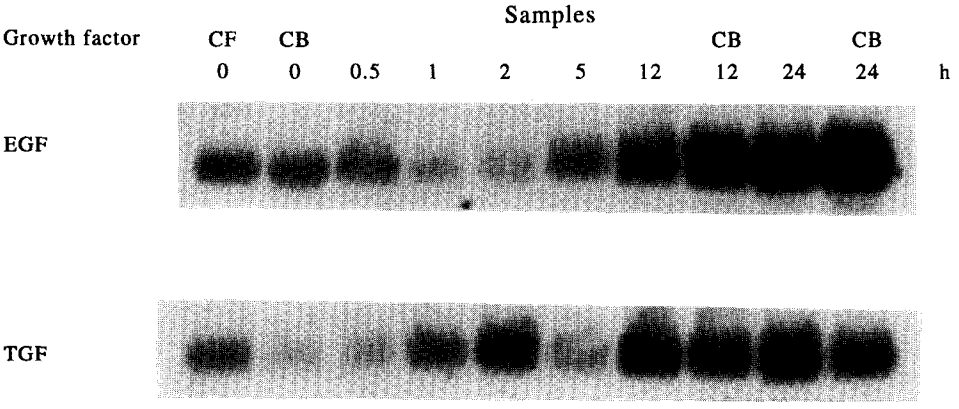

Figure 3. Northern analysis of MDA-MB-231 samples treated with EGF or TGFα as in Figure 1 and probed for MMP1. CF, control in medium containing 10% FBS; CB, control in medium containing 0.1% BSA (serum-free medium).

scripts with 30 min EGF stimulation in the absence and presence of cycloheximide showing that *de novo* protein synthesis was not required for this effect. An increase in MMP1 transcripts was seen following longer term cycloheximide treatment for 24 h. There was also an increase in transcripts for TGFα and EGF-receptor following 5 and 24 h treatment with cycloheximide compared with controls. These observations suggest a mechanism for generalised activation of transcription to increase gene expression in an attempt to maintain protein levels.

The EGF-receptor hybridisation probe showed three bands of 10, 5.6 and 2.9 Kb. Stimulation with EGF produced a transient increase in EGF-receptor mRNA at 30 min and this again was not inhibited by cycloheximide. This increase in the 10 Kb band was at least double the control value as shown in Figure 6, where the ratio of the 10 Kb EGF-R transcript to the 18S transcript was plotted using integration values from the PhosphorImager analysis. Probing for TGFα transcripts showed no effect of treatment by either EGF or TGFα.

Effects of phorbol ester on MMP1 expression

As a positive control, all three cell lines investigated were treated with TPA, a known inducer of MMP1 transcription [14], and the oestrogen-responsive cell lines T47D and MCF-7 were also tested for the effect of oestradiol. The results are shown in Figure 7. TPA induced MMP1 expression in MCF-7 cells and this was enhanced in the presence of oestradiol. However, in T47D cells, no response to TPA was detected. In the ER-negative MDA-MB-231 cells, TPA induced a 10-fold increase in MMP1 expression which was not inhibited by the presence of cycloheximide, showing this was a direct signalling effect not requiring *de novo* protein synthesis. Analysis using the PhosphorImager showed that the MMP1/18S ratio increased 10-fold both in the absence and presence of cycloheximide in the TPA treated cells. This demonstrates the ability of these cells to increase greatly their MMP1 mRNA levels. This level of induction of MMP1 transcripts was considerably greater than that achieved with varying doses of EGF or TGFα from 0.1 ng/ml to 10 ng/ml over a 2 h time course (data not shown).

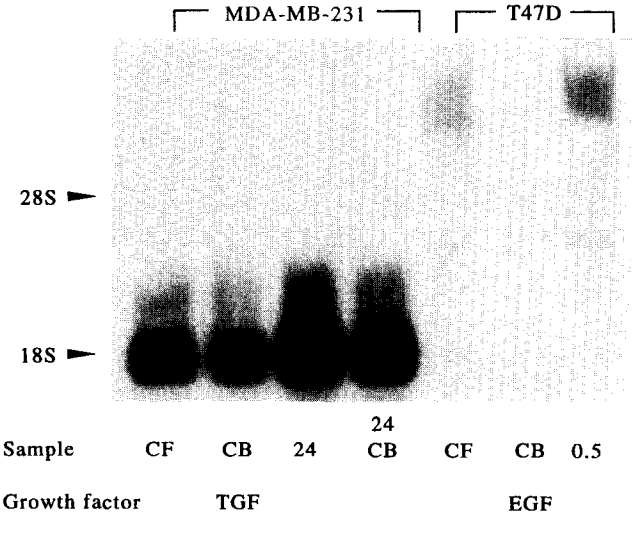

Figure 4. Northern analysis of MMP1 transcripts in MDA-MB-231 and T47D cells (samples as in Figure 1). CF, control in medium containing 10% FBS; CB, control in medium containing 0.1% BSA (serum-free medium).

MMP1 protein concentration

Forty millilitres of medium was removed from the cells used for the lysate preparation. The level of MMP1 protein in unconcentrated conditioned medium was only detectable in the 24 h sample both in the presence and absence of EGF treatment, where levels of MMP1 were 6 and 7 ng/ml, respectively, at the lower end of the MMP1 standard range. Following an 8-fold concentration of the media, levels for the above samples increased to 28 and 30 ng/ml, respectively and low levels of MMP1 (<6 ng/ml) were detected in the other samples. The level of MMP1 in the concentrated medium from cells treated for 24 h with EGF and cycloheximide was similar to that of the initial control BSA sample (both < 6 ng/ml) in comparison with the 24 h EGF treated sample.

The concentration of MMP1 detected in the cell lysate supernatants were all again less than 6 ng/ml and, using a lower standard, were shown to be between 4.0 and 6.0 ng/ml. This suggests that even when the cells were producing MMP1 which was being released into the medium, the level of MMP1 within the cells remained at a low level.

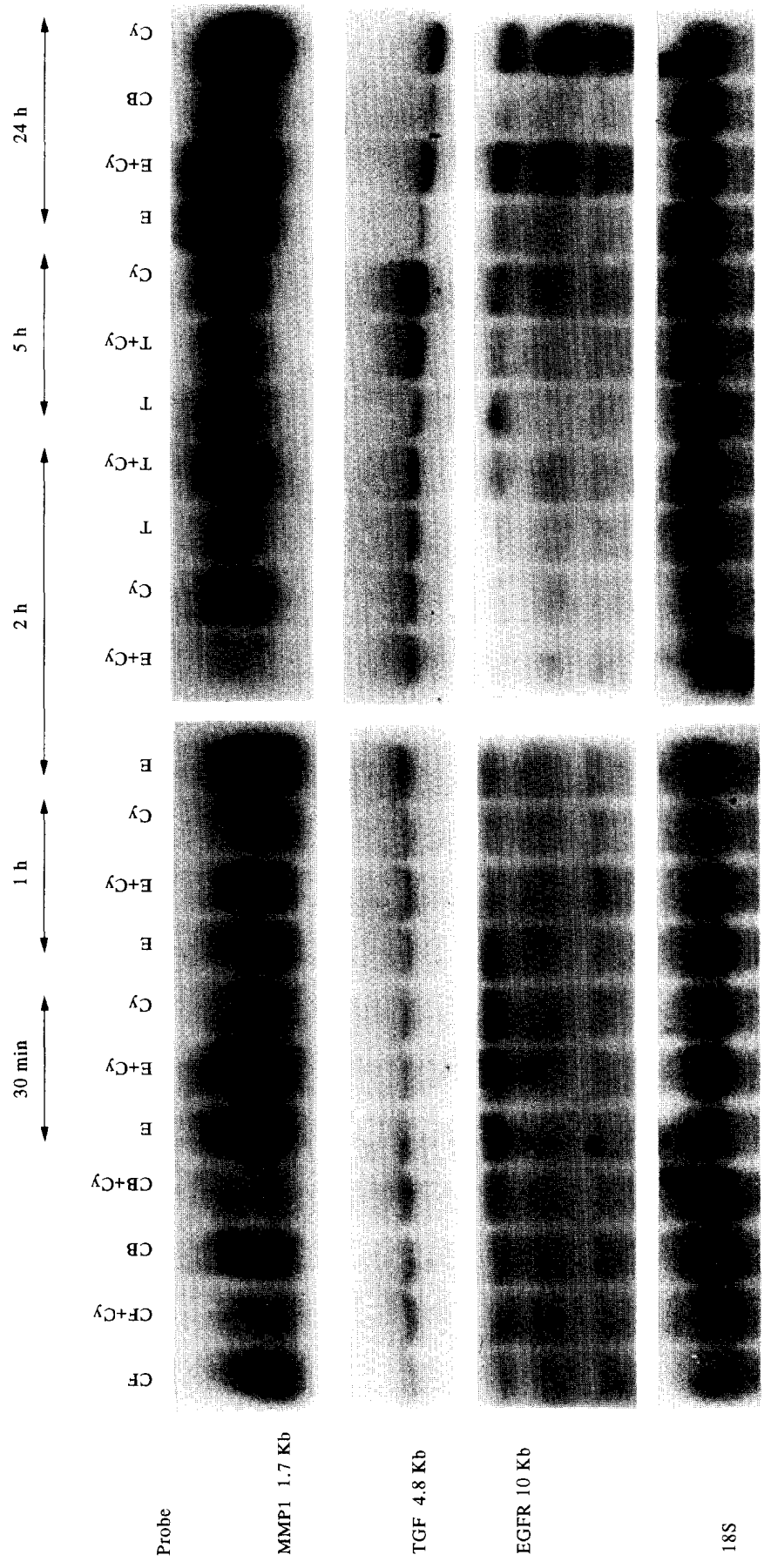

Figure 5. Northern analysis of RNA from MDA-MB-231 cells treated for the indicated times with 10 ng/ml EGF (E) or TGFα (T) and/or 10 μg/ml cycloheximide (Cy). CF, control in medium containing 10% FBS; CB, control in medium containing 0.1% BSA (serum-free medium). EGFR, EGF-receptor.

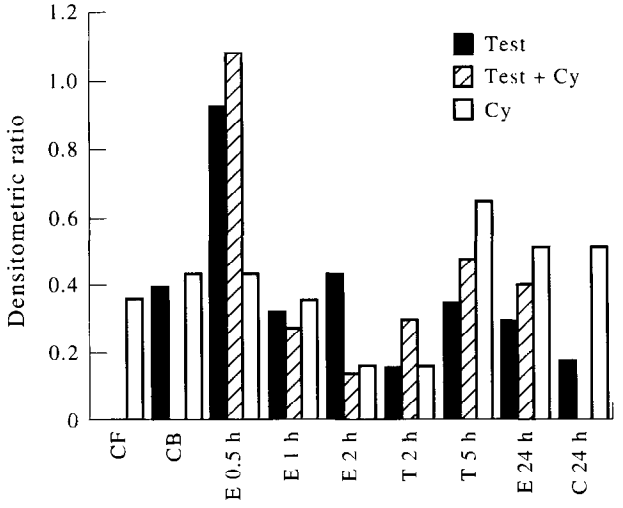

Figure 6. Ratio of the 10 Kb EGF-R mRNA to 18S RNA in MDA-MB-231 cells treated as in Figure 5. C 24 h is the control in 0.1% BSA. Treatment with TGFα is indicated by T and with EGF by E.

DISCUSSION

The effects of EGF on the growth of breast cancer cells vary widely and it has been demonstrated that cells with a relatively low number of EGF receptors respond mitogenically to EGF, whereas cells with higher numbers of EGF receptors may not respond or can be growth inhibited by EGF [16]. The cell lines used in this study vary both in their EGF-receptor numbers and their oestrogen receptor status. The ER-negative, highly positive EGF-receptor cell line, MDA-MB-231, showed the highest constitutive levels of MMP1 transcripts and also the greatest changes in response to EGF and TGFα stimulation. However, EGF-receptor number is not simply related to MMP1 mRNA levels, since very low levels were also seen in the BT20 cell line (data not shown) which has similar numbers of oestrogen and EGF receptors as the MDA-MB-231 cells [16].

Although there was some evidence of low transcript levels in the MCF-7 cell line on dot blot analysis, these were not detectable on Northern analysis with total RNA, possibly because of the lower sensitivity of the latter technique. In T47D cells treated with EGF for short times, a higher molecular weight signal than expected was observed on probing for MMP1. This may represent primary transcripts which have not been processed by splicing to the mature form.

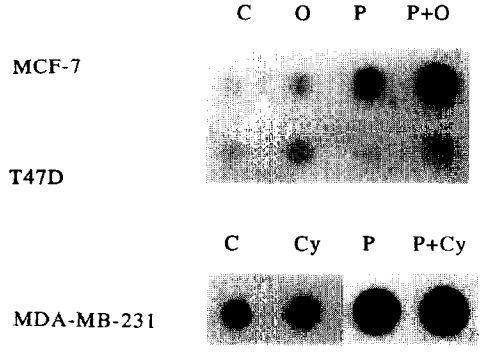

Figure 7. Induction of MMP1 in three breast cancer cell lines with TPA. C, controls; O, 10 nM oestradiol; P, 50 nM TPA; Cy, 10 μg/ml cycloheximide. Treatment was for 24 h.

Alternatively, it may represent cross-reactivity with a different gene, although the signal was maintained even after washing at a higher stringency. This emphasises the need to ensure probe specificity using Northern blot hybridisation in order to interpret the results of dot blot analysis.

Low concentrations of MMP1 protein were detected in the MDA-MB-231 cells and the medium collected following treatment of the cells. Although no increase in MMP1 protein could be detected after short term treatment with EGF or TGFα to parallel the increase detected with the mRNA, a significant increase in protein was seen after 24 h in the serum-free medium both with and without EGF treatment. The low levels of MMP1 present in the conditioned medium would explain why attempts to identify increases of active MMP1 by zymography were unsuccessful, and the BSA in the medium limits the extent to which this can be concentrated.

The increased level of MMP1 found in the MDA-MB-231 cells may be indicative of a higher invasive potential. Studies using this cell line showed that the cells had a greater ability to form invasive penetrating colonies in either the matrigel outgrowth assay or the Boyden chamber chemoinvasion assay [21] compared with the MCF-7 and T47D cells. In a study on a human gastric carcinoma cell line [22], an increase in MMP1 mRNA after 12 h EGF treatment and 3 h TGFα treatment, using incubation in serum-free medium, was demonstrated. Normal fibroblasts also showed an increase in MMP1 mRNA levels following 12–48 h of EGF treatment [23].

The serum deprivation studies demonstrated that prolonged serum deprivation, from 36 h, markedly increased MMP1 expression and protein synthesis in the MDA-MB-231 cells. This contrasts with observations on normal human fibroblasts where serum deprivation has been reported to reduce the level of MMP1 transcripts [14]. Possible related effects of serum deprivation on tumour cells have been noted by several authors. A study using breast tumour cell lines [24] showed that the use of serum-free medium greatly enhanced the degradation of basement membrane, particularly in the MDA-MB-231 cell line. It was suggested this may be due to the removal of naturally occurring enzyme inhibitors present in serum, but our results indicate that the induction of MMP1 gene expression and increased protein synthesis may have been a contributory mechanism. It has also been noted [25] that collagenolytic activity from breast tumour cell lines could only be detected in medium when the cells were grown in the absence of serum. Serum deprivation has also been shown to cause apoptosis (programmed cell death) in a variety of cell lines [26–29]. The effects seen on MMP1 expression may be linked with those seen on the transcripts of various genes which are altered as part of the apoptotic response. The induction of metalloproteinase expression in concert with apoptosis may be associated with the need to break down extracellular matrix for tissue remodelling. In the context of cancer, such a link between serum deprivation, apoptosis and the induction of metalloproteinase activity would clearly have important implications for mechanisms of tumour invasion and metastasis. Hypoxic regions of nutritionally deprived cells occur during tumour progression and an increase in MMP1 expression, such as shown in MDA-MB-231 cells when deprived of serum, may

be an important contributive factor for invasive tumour progression and metastasis. It is noteworthy that serum deprivation should have this pronounced effect particularly in the ER-negative, EGF-receptor positive MDA-MB-231 cells, since this pattern of receptor expression has a well-established association with poor prognosis in human cancer. Extension of these studies to the effect of serum deprivation and hypoxia on other metalloproteinases and their inhibitors would be of interest, together with further exploration of possible links with mechanisms of apoptosis.

The effect of the phorbol ester TPA on MMP1 expression was also seen to vary in the three breast cancer cell lines studied. In the MDA-MB-231 cells, TPA produced a 10-fold increase in MMP1 mRNA, which was not inhibited by cycloheximide. These cells, therefore, have the ability to produce larger increases in MMP1 mRNA than we found with EGF or TGFα stimulation, where only 2-fold increases were observed. In a study of metalloproteinases and their inhibitors in several cell lines [30], it has been previously reported that TPA increased MMP1 mRNA in the MDA-MB-231 cells.

Cycloheximide is an inhibitor of protein synthesis and is used to ascertain whether *de novo* protein synthesis is required in particular processes. The increase in MMP1 mRNA following 30 min treatment with EGF was not inhibited by cycloheximide and, therefore, does not require *de novo* protein synthesis. A significant increase in protein was seen after 24 h in the serum-free medium both with and without EGF treatment. This 6-fold increase was abolished in the presence of cycloheximide, although the mRNA levels remained high. The effect of cycloheximide on the levels of MMP1 mRNA and protein shows that no *de novo* protein synthesis is necessary to induce increased levels of MMP1 mRNA. This may be due to the protein product having an effect on the transcription of the gene or on mRNA stability, or may be due to the serum deprivation or cycloheximide producing a generalised stress response to prolonged inhibition of protein synthesis. The 24 h treatment with cycloheximide also produced an increase in MMP1 transcripts as well as increases in TGFα and EGF-receptor expression, and the cells remained morphologically intact over this period. The effect of cycloheximide on MMP1 expression, coupled with the effect on TGFα and EGF-R expression, may, therefore, be evidence for the induction of an active tissue breakdown and cellular recovery process involving autocrine growth stimulation in response to the long term inhibition of protein synthesis.

The increases found in MMP1 expression as a result of EGF-receptor stimulation provide a possible contributory mechanism to explain, in part, the poor prognosis of patients with ER negative and EGF-receptor positive tumours. The marked increases in MMP1 mRNA and MMP1 protein levels in response to serum deprivation, and increases in MMP1 mRNA in response to inhibition of protein synthesis, may be relevant to conditions which prevail in poorly vascularised regions of tumours, suggesting they may result in the activation of genes which can promote the degradation of extracellular matrix and consequently angiogenesis, tumour invasion and spread. Further work to study the effect of these conditions and stimulation by growth factors on the expression of other metalloproteinases and their inhibitors is required to understand the growth and invasive progression of tumours more fully.

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