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Original Paper

Differential Responsiveness of Proliferation and Cytokeratin Release to Stripped Serum and Oestrogen in the Human Breast Cancer Cell Line, MCF-7

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***In vitro* research into hormone sensitivity and the relation to proliferation of cytokeratin release from cancer cells is scarce. Therefore, we examined the stimulation of proliferation and the release of cytokeratins in a breast cancer cell culture model. Cell growth was stimulated by 17β-oestradiol (10^{-11} M), stripped serum (10%) and by the two together. Cytokeratin release was stimulated only by stripped serum, oestradiol having no effect. After long incubation periods (>12 h), cytokeratin release also commenced in the control and oestradiol treatments. Release rate versus time analysis suggested that there are two different release processes. Cytokeratin release was first stimulated at a stripped serum concentration approximately 100 times lower than that which initiated proliferation. Pharmacological alteration of proliferation with cordyceptin resulted in growth changes without alterations in cytokeratin release. We conclude that cytokeratin release in these cells is unrelated to proliferation, independent of oestrogen action and probably in some way related to growth factor receptor function.**

Key words: cytokeratins, TPA, TPS, CYFRA 21.1, oestrogen, hormone, proliferation, breast cancer, MCF-7, growth factors

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INTRODUCTION

In BREAST cancer cells containing oestrogen receptors (ER+) both oestrogens and growth factors can stimulate proliferation, invasion and the production/secretion of a number of proteins, while in ER– cells these growth factors are the primary driving force of these processes [1]. One of the aims of cancer research is to define the relationships that these tumour specific proteins may have with various aspects of tumour presence, type and/or progression in order to facilitate their proper clinical use in diagnosis and/or prognosis.

One class of proteins found to have altered production/secretion are the cytokeratins, numbered 1–20, that are a part of the family of intermediate filaments of the cytoskeleton. Each cytokeratin has a characteristic distribution in diverse types of epithelial tissues which is generally maintained in their corresponding tumour [2, 3]. They can become released into circulation via cell division or necrosy which liberate soluble fragments of cytoskeleton into the extracellular space [4]. The first antibody against members of this family (TPA) was described by Bjorklund [5]. It recognises cytokeratins 8, 18 and 19 and represents the primary clinical tool for the measurement of cytokeratins. Recently, monoclonal antibodies that recognise cytokeratins 18 (TPS) and 19 (CYFRA 21.1) have been developed and are starting to be used clinically [4, 6, 7]

Based on early studies in HeLa cells, where it was observed that TPA was released into growth medium by viable cells during mitosis [8], it was suggested that TPA may represent a quantitative marker of cell proliferation. There is currently some debate as to the relative contribution of proliferation and necrosy to serum cytokeratins. Several clinical studies have shown that, in patients with breast cancer, cytosolic TPA levels were statistically higher in ER+PgR+ than in ER–PgR– cases [9]. High levels of cytosolic TPA were found to correlate with a better prognosis, but not with patients age, tumour size, lymph node status, steroid receptor status and, importantly, the tumour proliferative rate as measured by the Labelling Index [10, 11]. These data have suggested that TPA could be useful as a prognostic parameter to select highly differentiated and less aggressive breast tumours. However, the mechanism behind this remains unknown.

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Beyond the HeLa cell data on cell cycle-dependent release of TPA, there are few *in vitro* studies that have quantified the relationship between cytokeratin release and either proliferation or steroid induced alterations in cellular physiology. Further, these results were equivocal or inconclusive, especially for steroid hormone stimulation, as experimental protocols did not separate the effects of steroid hormones from those of the peptide growth factors [12–14].

In order to elucidate further the biological mechanism(s) behind the release of cytokeratins and clarify the relationship of TPA release with both proliferative activity and hormone-sensitivity in breast-cancer, we conducted complementary *in vitro* (MCF-7 cells) and *in vivo* (breast tumour cytosol and serum from patients) studies. Here we report the *in vitro* results in which we demonstrate that cytokeratin release is not related to either proliferative activity or oestrogen action. We conclude that release is correlated to some aspect of growth factor receptor activity.

MATERIALS AND METHODS

Cells

MCF-7 human breast cancer cells [15], obtained from the National Cancer Institute of Milan, were routinely maintained as a monolayer in a humified atmosphere of 95% air, 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM)/HAMS F-12 with 50 U/ml penicillin, 50 μg/ml streptomycin, 300 mg/l L-glutamate and 10% fetal calf serum (FCS). Preliminary experiments demonstrated that, in our hands, the presence of phenol red in the culture medium had no effect on growth, the levels of oestrogen receptor or production of ER sensitive proteins (pS2 and cathepsin-D). Phenol red was subsequently included in the culture medium. Culture media was refreshed every 2 days and cells were serially passaged every 3–5 days by releasing the cells with DMEM/HAMS F-12 plus 0.05% trypsin, 0.02% EDTA and seeded at 10^6 cells per 75 cm^2 flask.

Growth and marker release assays

Stock cultures of exponentially growing MCF-7 monolayers were washed with sterile phosphate-buffered saline (PBS), treated with 0.05% trypsin and 0.02% EDTA in PBS to produce a cell suspension. Aliquots of 4000 MCF-7 cells in 200 μl were plated in wells of 96-well plates (GIBCO) in complete medium for 24 h. Cells were then washed once with PBS and incubated for 24 h in serum-free medium (serum starvation). Preliminary experiments established that, after this 24 h incubation in the absence of serum, the cells stopped growing and were metabolically blocked. For the time course experiments, cells were then treated either with the simple growth medium used for starvation (–S), 10^{-11} M E2 (+E2), 10% FCS stripped of steroids (+SS), or 10% FCS stripped of steroids plus 10^{-11} M E2 (+E2, +SS). FCS was stripped of steroids by dextran-coated charcoal treatment as per Johnson and associates [16]. Whole serum oestrogen concentrations, as measured by a chemiluminesce immunoassay (Amersham), were reduced from approximately 10^{-11} to 10^{-14} M by charcoal treatment.

At time 0 and various subsequent times starting at 30 min and finishing at 2 days, the cells were used to measure growth and the conditioned medium used for the measurement of cytokeratins. In some experiments, conditioned medium was also collected at 10 and 20 min for the measurement of cytokeratin release.

Kinetic measurements

After serum starvation, the cells were treated with concentrations of either E2 or SS of 0–10^{-9} M or 0–10%, respectively. At 6 h incubation, an aliquot of growth medium was removed for marker release assay. At 24 h the cells were assayed for proliferation and another aliquot of medium was taken for marker release analysis.

Cordyceptin treatment

Cordyceptin (3'-deoxyadenosine) is a specific inhibitor of the polyadenylation necessary for the maturation of mRNA from hnRNA and prevents the appearance of mRNA in the cytoplasm [17]. Stock solution was prepared in 10% ethanol at 1 mg/ml and working solutions were serially diluted in growth medium. After serum starvation, cells were treated with either simple medium or 10% SS plus 0–10 μg/ml cordyceptin. Samples of media were collected and proliferation measured as described above.

Assays

Proliferation was simultaneously measured in each sample using three independent techniques that assay completely different cellular processes: (1) cells were released by trypsination as described above and their number counted with the use of a Burker Chamber. (2) [^{3}H]thymidine (^{3}H-dT) incorporation assay [2] was performed at various incubation times in culture depending upon the specific experimental design. Briefly, 0.5 μCi[^{3}H]thymidine (82 Ci/mmol, Amersham) was added to each culture well. After the incubation time, indicated in each figure legend, the cells were washed twice with PBS and the cells were released into 100 μl of DMEM media containing 0.05% trypsin. The cells of each well were then put into 5 ml scintillation vials containing 3 ml of aqueous counting scintillant (Packard®). Blanks were wells without cells and were treated identically as above. Samples were counted for activity with a Packard (Minaxi Tri-carb 4000®) liquid scintillation counter. All assays were performed in quadruplicate. (3) MTT test was performed according to Etievant and associates [18] with modification. This assay measures the rate of metabolic hydrolysis by the mitochondria of the compound, 3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT; Sigma). At indicated incubation times, the culture medium was collected and replaced with 100 μl MTT at 1 mg/ml in PBS. The plates were incubated for the times indicated in each figure legend at 37°C and then the solution was carefully aspirated and replaced with 200 μl of dimethylsulphoxide (DMSO). The microplate was shaken and the O.D. of each well was read at a test wavelength of 570 nm and a reference wavelength of 630 nm [19]. All the assays were performed in triplicate.

During the time course, lactate dehydrogenase (LDH) activity in the culture medium and trypan blue exclusion were monitored to determined the contribution of necrosis to the differences in cell number between treatments. LDH activity was measured using the kit supplied by Promega (Madison, Wisconsin, U.S.A.) and trypan blue obtained from Sigma.

Markers

The levels of TPA, TPS and CYFRA released into the culture medium were measured by commercially available two site immunoradiometric (IRMA) kits. The kit for TPA (polyclonal antibody) was obtained from Sangtec Medical (Bromma, Sweden) and recognises cytokeratins 8, 18 and 19.

TPS was measured with a kit purchased from Beki Diagnostics AB (Bromma, Sweden) that uses a monoclonal antibody (M3) that reacts with a fragment of cytokeratin 18. CYFRA was measured with two monoclonal antibodies against cytokeratin 19 in a kit obtained from CIS Bio International (Gif-Sur-Yvette, France).

Kinetic analysis

The stripped serum-dependent cytokeratin release and proliferative kinetic coefficients were estimated by the non-linear curve-fitting regression utilising the iterative Marquardt procedure on the Kalidiograph® (Abelbeck Software) program. The data were fitted to the Michaelis–Menten equation: $R = (R_{max}\ [SS])/(K_s + [SS])$ in which R is the rate of either cytokeratin release or proliferation, [SS] is the experimental concentration of stripped serum, R_{max} is the maximum rate of these processes and K_s is the stripped serum concentration that yields one-half R_{max}.

RESULTS

Stimulation of proliferation by growth factors (SS) and oestradiol

Figure 1 shows the time course of stimulation of MCF-7 cell proliferation by steroid hormone (oestradiol, E2) or stripped serum (SS) as measured simultaneously by three independent methods at both short (30 min to 12 h) and long (intervals of 24 h) term incubation. It can be seen from Figure 1 that proliferation of this MCF-7 cell line was stimulated by either oestradiol alone (10^{-11} M) or by stripped serum alone (10%) and that simultaneous addition of both resulted in further stimulation of proliferation.

Proliferation, resulting from stimulation after the metabolic block, was in equilibrium at the long time periods in that the results from the three independent measurements (cell number, ^{3}H-dT and MTT) gave equivalent results. Short incubation times demonstrated that the different techniques did not have the same time development kinetics. Cell number did not increase significantly until approximately 6 h after treatment, while increases in ^{3}H-dT incorporation and MTT hydrolysis were first measurable at 1 and 2 h, respectively. Importantly, the relationships observed between treatments at long time periods were maintained in all three methods at short time periods. During the time-course, lactate dehydrogenase (LDH) activity in the culture medium and trypan blue exclusion were monitored to determined the contribution of necrosis to the observed differences in cell number between treatments; no increases above initial values in either parameter were observed in any treatment (data not shown).

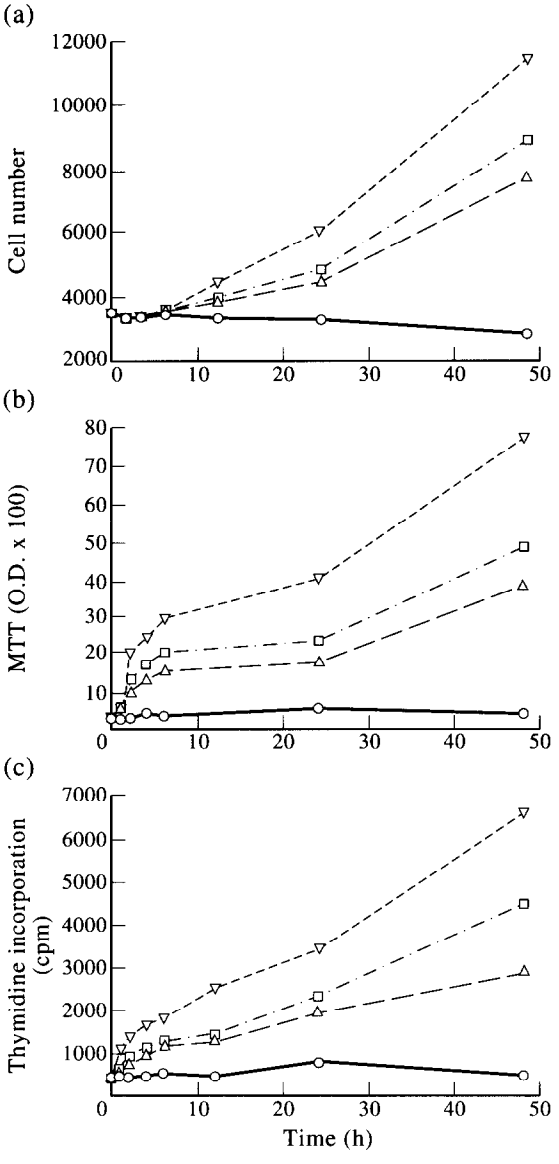

Figure 1. Time course of effect of treatment on MCF-7 proliferation. Monolayer cultures of MCF-7 cells were starved for 24 h and then subjected to the same control conditions (○), oestradiol (10^{-11} M, △), 10% stripped serum (□) or a combination of the two (▽). At the indicated times proliferation was determined as a change in the number of cells (a), the metabolism of MTT (b) and the incorporation of ^{3}H-thymidine (c).

Time course of cytokeratin release

Figure 2 shows the accumulation of TPA, TPS and CYFRA in the culture medium during a representative proliferation experiment. As can be seen, there was a rapid, measurable release of all three cytokeratins only upon stimulation of the cells by stripped serum. Oestradiol addition had no effect on release either alone or in the presence of added stripped serum. The stripped serum-dependent cytokeratin release could be observed at incubation times as short as 10 min (data not shown), and the differences between release in control (basal culture media) or oestradiol treatments and release in the presence of stripped serum were dramatic at short incubation times for all three cytokeratins (accumulation at 6 h was 18 ± 6-, 9 ± 4- and 13 ± 3-fold for TPA, TPS and CYFRA, respectively). Only at long incubation times were differences observed between the release of the three cytokeratins in relation to control versus stripped serum. TPA and CYFRA accumulation stimulated by 10% stripped serum continued to be greater than control, while for TPS accumulation from the starved cells and cells stimulated by only oestradiol approached levels close to those of stripped serum treatments already at 24 h. However, for TPA and CYFRA large amounts also start to be accumulated after approximately 12 h.

In order to observe time-dependent changes in cytokeratin secretion, the accumulation data from these experiments were converted to rate of release versus time (Figure 3). Stripped serum stimulated release of all three cytokeratins started at very high rates that immediately and rapidly declined in a

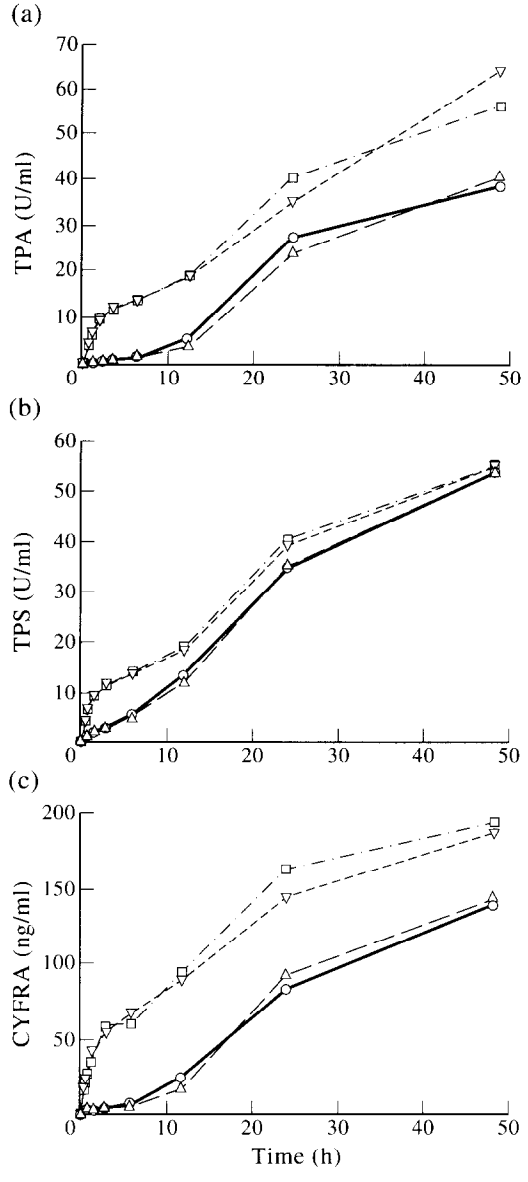

Figure 2. Time course of the accumulation of cytokeratins in growth medium. Monolayer cultures of MCF-7 cells were subjected to the same conditions as in Figure 1 and, at the indicated times, growth medium was collected for TPA (a), TPS (b) or CYFRA (c) measurement.

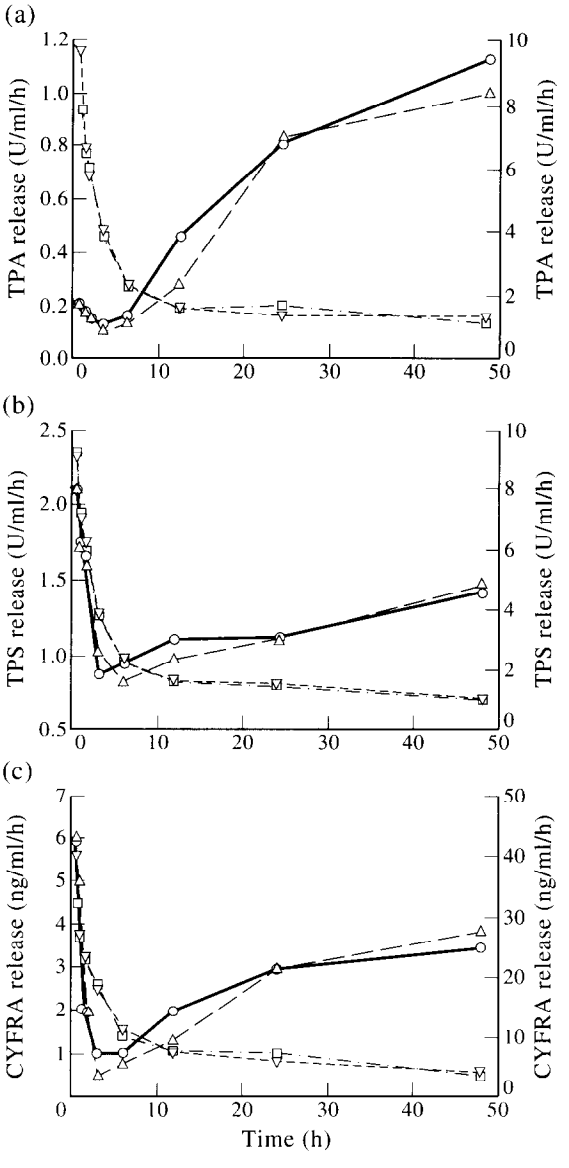

Figure 3. Time course of cytokeratin release rate. The time-dependent rate of TPA (a), TPS (b) or CYFRA (c) release from monolayer cultures of MCF-7 cells subjected to the same conditions as in Figure 1.

hyperbolic manner to a very low rate and remained low for the rest of the experiment. In the control and oestradiol treatments, release initially followed this pattern and then at 6–12 h after treatment, release restarted and increased continually throughout the rest of the time course experiment. The initial component of TPA secretion was much smaller than that of TPS, although their final secretory rate was equal. By 48 h after treatment, the rate of unstimulated secretion in the control and oestrogen treatments was approximately the same as in the two stimulated treatments for all three cytokeratins.

To further validate this pattern, TPA accumulation was measured in the medium after 1 h of stimulation. At 24 h after stimulation, the medium was removed, the cells washed quickly with fresh medium plus the treatment and then incubated a further hour in fresh medium plus the treatment (1 h at 24 h). It can be seen from Table 1 that, in fact, the 1 h release in the stripped serum treatments at 24 h after treatment was much less than in the first hour while TPA release increased after 24 h in the treatments without stripped serum. These data support the calculated relationships in release observed in Figure 3.

Dose–response kinetics of stripped serum effects on proliferation and cytokeratin release

The above time course data suggest that cytokeratin release in these breast cancer cells is not related to stimulation of proliferation, at least in respect of oestrogen-dependent processes. To gain further insight into the possible relationship between growth factor stimulated proliferation and cytokeratin release of the cells, the kinetics of stripped serum-dependent stimulation of both proliferation and cytokeratin release was compared. Figure 4 shows that the dose effect of stripped

Table 1. Accumulation in the growth medium of TPA at 1 h of stimulation and for 1 h after 24 h of stimulation in MCF-7 cells

	1 h	1 h at 24 h
(−) serum	218	380
(+) E2 (10^{-11} M)	195	384
(+) SS (10%)	5985	486
(+) SS (+) E2	6546	560

Values are in U/ml TPA in cell culture medium.

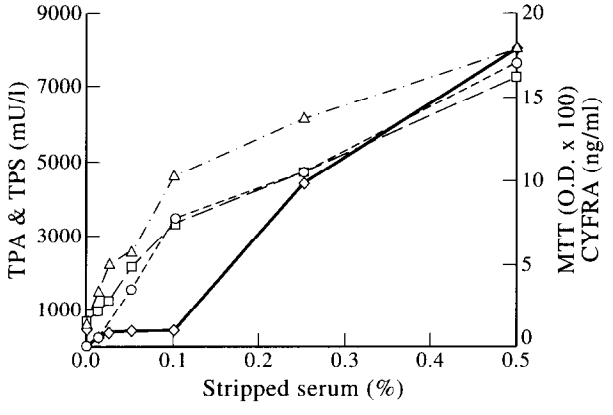

Figure 4. Dose–effect of stripped serum on proliferation and cytokeratin release. Monolayer cultures of MCF-7 cells were starved for 24 h and then subjected to various concentrations of stripped serum. The release of TPA (△) and TPS (□) were measured at 6 h incubation while the rate of MTT metabolism (◇) and CYFRA (○) release were measured after 24 h incubation.

Table 2. Kinetic parameters for the effect of stripped serum on cytokeratin release and proliferation in MCF-7 cells

	[Stripped serum] that initiated release or proliferation	K_d
TPA	<0.012	0.23 ± 0.058
TPS	<0.012	0.22 ± 0.044
CYFRA	<0.012	0.35 ± 0.067
MTT	>0.12 <0.25	1.17 ± 0.19
^{3}H-dT	>0.12 <0.25	1.24 ± 0.35

Concentrations are in per cent stripped serum added to the medium. K_d is the concentration of stripped serum at which release or growth had reached half the maximum response ± S.E.

serum on proliferation was extremely different than its effect on cytokeratin release in MCF-7 cells. Cell proliferation was not stimulated by stripped serum until the concentration was between 0.1 and 0.25% whereas the release of all three cytokeratins was already stimulated at stripped serum concentrations as low as 0.0125%. Thus, the release of cytokeratins were initiated at a stripped serum concentration about 100 times lower than that at which proliferation was initiated. Michaelis–Menten kinetic analysis for half-maximum stripped serum stimulation values (K_s) of cytokeratin release and proliferation revealed that the stripped serum concentrations that produced half the maximum cytokeratin release rate were approximately 5 times smaller than the same parameters for the stimulation of either thymidine incorporation or MTT (Table 2).

Although proliferation was stimulated in a dose dependent manner by oestradiol addition (0–10^{-9} M, data not shown), cytokeratin release was unaffected over the entire range (mean TPA value = 418 ± 22, n = 12).

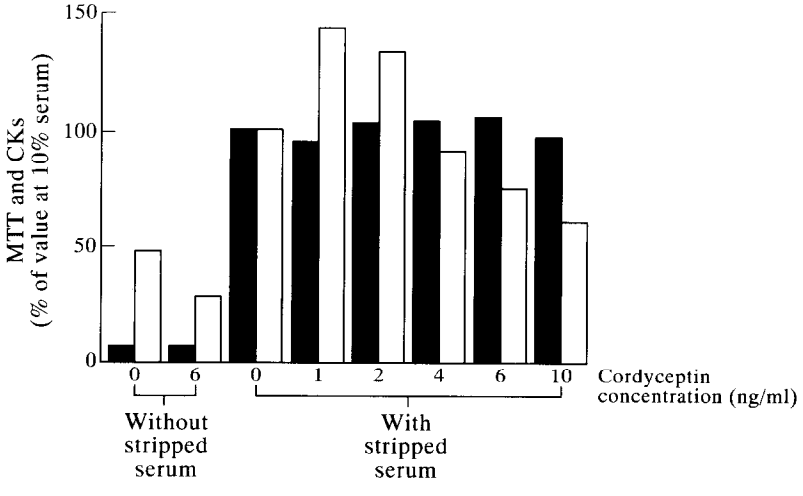

Figure 5. Dose–effect of cordyceptin on proliferation and cytokeratin release. Monolayer cultures of MCF-7 cells were starved for 24 h and then incubated with or without 10% stripped serum and 0 to 10 ng/ml cordyceptin. The release of the cytokeratins (■) and rate of MTT metabolism (□) were measured as in Figure 4. Results are expressed as the percentage of release or metabolism measured at 10% stripped serum and 0 cordyceptin. The cytokeratins were averaged together.

Dose–response effect of cordyceptin on stripped serum-stimulated proliferation and cytokeratin release

In order to further examine and clarify this seeming lack of a relationship between cytokeratin release and proliferation, the proliferative rate of these cells was pharmacologically modified by various concentrations of cordyception, a specific inhibitor of maturation of mRNA from hnRNA (polyadenylation). In Figure 5, the effect of 10% stripped serum plus 0–10 μg/ml cordyceptin on proliferation as measured by MTT and cytokeratin release is demonstrated. The results are presented as the percentage of the rate (MTT) or release (cytokeratins) at 10% stripped serum and no cordyceptin. The percentage release of all three cytokeratins was averaged together for clarity. As can be seen, incubation in the presence of cordyceptin greatly modified growth: stripped serum (i.e. growth factor)-dependent proliferation was further stimulated by the lowest cordyceptin concentration (1 μg/ml, 1.45-fold the stimulation of proliferation of control) while further increases in cordyceptin resulted in concomitant decreases in proliferation, such that at 10 μg/ml cordyceptin the proliferative rate was approximately 45%, lower than that observed in the cells stimulated by only 10% stripped serum. In contrast, the release of all three cytokeratins was stimulated by 10% stripped serum while cordyceptin had no effect on their release at any concentration.

Ratio of release of the three different cytokeratins

To further analyse the relationship between the release of the three cytokeratins, the ratio of their release was calculated for each time point with the various treatments (Table 3). In both the control and the oestradiol treatments, the TPA:TPS ratio started very low and rapidly increased to approximately equal levels between 24 and 48 h, while in the treatments containing stripped serum, their ratio was always approximately 1. The ratio of TPA to CYFRA also started very low in the treatments without stripped serum and increased with time, while the TPS to CYFRA ratio seemed to be random in these treatments. Interestingly, in all the treatments containing stripped serum, the ratio of both TPA and TPS to CYFRA was approximately 250 at all time points.

Table 3. Ratios of cytokeratin release in time course and dose–response experiments

Time (h)	TPA:TPS	TPA:CYFRA	TPS:CYFRA
(–) serum			
0.5	0.085	30	352
1	0.12	105	870
6	0.17	187	1121
12	0.38	192	511
24	0.78	285	406
48	0.72	276	394
(+) E2			
0.5	0.11	46	435
1	0.24	80	321
6	0.15	150	997
12	0.31	211	672
24	0.63	240	380
48	0.74	393	610
(+) SS			
0.5	0.91	272	286
1	0.80	215	270
6	0.97	225	231
12	0.9	203	205
24	1.0	250	247
48	1.1	310	286
		(246 ± 15)	(254 ± 12)
(+) SS (+) E2			
0.5	1.1	241	231
1	0.90	243	270
6	0.94	205	231
12	1.0	212	207
24	1.2	250	250
48	1.4	342	238
		(249 ± 18)	(239 ± 11)

Values are the ratios of the cytokeratin release observed in Figure 2. The values in parentheses are the mean ± S.E. for the ratios of a particular time course.

DISCUSSION

In this study, we utilised the MCF-7 human breast cancer cell line to determine: (1) the hormone sensitivity of cytokeratin release and (2) the relationship of cytokeratin release to alterations in proliferation. This particular MCF-7 line was chosen because, in our hands, its growth can be regulated by oestrogen or peptide growth factors (here added as serum charcoal stripped of steroids; see [20]) and, in this study, a combination of both further stimulated growth (Figure 1). As can be seen in Figure 1, a concentration close to physiological levels of oestrogen (10^{-11} M) resulted in a doubling time of a little more than a day. This is in contrast with other studies on different MCF-7 lines in which concentrations in the range of 10^{-9} to 10^{-7} M stimulated growth at rates of about 1/3 to 1/5-times this value [20], while our cells responded more like those reported by Pagliacci and associates [21]. The cell cycle distribution of the cells after 24 h serum starvation was also similar to theirs (data not shown). These different measures of proliferation, i.e. DNA synthesis, mitochondrial activity and cell division, all had different time courses for recommencing proliferation after the (re)addition of the 'growth' factors (stripped serum and/or E2). Importantly, each independent assay had the same relationship between treatments in the short-term measurements as in the long-term (i.e. one full cell cycle or more). It can therefore be concluded that each of these assays gives a valid measure of proliferation in either short- or long-term experiments. Further, this strong concordance between physiological measures of proliferation and increases in cell number excludes the possibility that necrosis is contributing in these experiments to cytokine appearance in the growth media, a hypothesis corroborated by the lack of LDH activity in the medium over time.

That the growth of these cells was sensitive to both steroid and growth factor addition permitted us to separate the cellular processes stimulated by these different types of hormones; an experimental manoeuvre that previously has not been exploited in the *in vitro* analysis of the biological processes underlying cytokeratin release. Although the stimulation of both cell proliferation and the release of cytokeratin in response to whole serum addition has been characterised in *in vitro* studies with MCF-7 cells [12, 13] and in prostate tumour cell culture [14], experimental evidence is not available on the relative contribution of steroid hormones and growth factors to these processes. Our results clearly show that only the components of stripped serum were able to activate cytokeratin release from these cells. These data suggest that, in these

cells, the release of cytokeratins is stimulated only by activation of growth factor receptors although oestradiol also stimulated growth (Figure 1). Oestradiol is known to increase invasive potential [22] and the synthesis of a number of key proteins such as progesterone receptor [23], tissue plasminogen activator [24], pS2 [25], cathepsin D [26] and a number of growth factors [1]. These experiments demonstrate that cytokeratin release is not related in any way to oestrogen stimulation of proliferative activity and, therefore, cannot be used as a measure of this activity. This inability of oestrogen to stimulate the release of cytokeratins from MCF-7 cells is a fundamental finding of this study.

As cytokeratin release was found to be unrelated to oestrogen-stimulated proliferation, it became important to determine if cytokeratin release was related to or followed alterations in proliferation driven by other factors. This is especially important in the growth factor receptor driven changes as it relates to growth factor control of carcinogenesis, but is also important in tumour proliferation controlled by (non-receptor mediated) factors other than hormones. The stripped serum dose–response curves seen in Figure 4 demonstrate that growth factors affected the processes of proliferation and cytokeratin release very differently. The initiation of cytokeratin release occurred at concentrations much lower than those stimulating proliferation and progressed with very different concentration-dependent kinetics (see Table 2). These data eliminate any direct relationship between growth factor stimulated proliferation and growth factor stimulated cytokeratin release. This suggests that there is only a casual relationship between these two processes, occurring only because both processes are controlled by growth factor receptor activation. The growth factor receptor-dependent intracellular regulatory mechanisms for these two processes and where they deviate have yet to be elucidated.

In order to uncover more clearly any possible relationship between cytokeratin release and proliferative activity, we also studied the interaction between proliferation and cytokeratin release when proliferation was pharmacologically modified (Figure 5). We chose to utilise cordyceptin since it modifies cell growth at a step very close to DNA synthesis, and functions directly on the transcription process without the intervention of any receptor process [17]. The cells were first stimulated with a maximum dose of stripped serum to see if proliferation could be further stimulated and, secondly, to allow sufficient proliferative 'space' to see large inhibitions in growth. Stripped serum-stimulated proliferation was greatly altered by these pharmacological manoeuvres, while the stripped serum-stimulated secretion of cytokeratins was unaffected. The lack of concomitant alterations in cytokeratin release with non-receptor related manipulation of proliferation provides definitive evidence of a lack of a direct relationship between growth and cytokeratin release.

The results presented in this study suggest that the three cytokeratins measured (TPA: 8, 18, 19; TPS: 18; CYFRA: 19) are secreted by at least two discrete mechanisms (Figure 3, Table 1). The first secretory mechanism is stimulated by growth factor receptor activation, rapidly releasing the accumulated intracellular stores, which diminishes after 6–12 h to a maintained level of secretion that may reflect the basal cytokeratin production. That this reduction in rate of secretion in the stripped serum stimulated treatments is not due to growth factor depletion is evident from the results shown in Table 1, in which replenishment of stripped serum at 24 h did not restimulate release. These data also suggest that secretion is not affected by the cytokeratin concentration gradient from the cell to the medium. The second mechanism was evident in the treatments lacking growth factors (without stripped serum, SS–), which seems to commence between 6 and 12 h after the start of the experiment. The ratio data of release of the different cytokeratins (Table 3) supports this hypothesis of two different mechanisms due to the completely different behaviour in the treatments with and without stripped serum.

In conclusion, the results of this study provide insight into the mechanisms behind the release of cytokeratins from tumour cells. The results reported here demonstrate that cytokeratin release was insensitive to oestrogen and stimulated only by stripped serum (i.e. growth factors). Secondly, the presented data also provide evidence that growth factor stimulation of cytokeratin release is not directly related to growth factor stimulation of proliferation nor, in fact, to proliferation at all.

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