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Changes in the phosphorylation status of the 27 kDa heat shock protein (HSP27) associated with the modulation of growth and/or differentiation in MCF-7 cells

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Abstract. We have used human mammary cells of the MCF-7 strain, which constitutively express high levels of the small heat shock protein HSP27 and we have compared the changes in the phosphorylation status of this protein together with changes in cell growth and/or morphology induced by the action of one of the following agents: (1) TPA (12-*O*-tetradecanoylphorbol-13-acetate), known as a differentiation inducer in MCF-7 cells; (2) OH-TAM (hydroxytamoxifen), which exerts a cytostatic and cytotoxic action; or (3) TNFα (tumour necrosis factor), which induces apoptotic cell death in this cell line. Our data show that TPA and TNF stimulate an immediate and massive phosphorylation of HSP27, whereas OH-TAM affect the phosphorylation status of the protein only after a 3 day delay. In the case of TPA, high levels of HSP27 phosphorylation were maintained for at least 4 days, along with growth inhibition and acquisition by the cells of a secretory phenotype. TPA and OH-TAM exerted similar immediated effects on cell growth, despite the different time course of their action on HSP27 phosphorylation. This excludes the possibility that the latter is a necessary consequence of, or an absolute requisite to, growth inhibition. With OH-TAM and TNF the increase in HSP27 phosphorylation was concomitant with the appearance of apoptosis, not observed with TPA. This indicates that increased phosphorylation of HSP27 is not specifically associated with the triggering or the execution of apoptosis in these cells. Altogether, our data support the concept that phosphorylated HSP27 is involved (and might then be rate limiting in some instances) in the execution of vital cell programmes (including resistance to stress, proliferation and differentiation), as well as in that of cell death. This is consistent with its role in actin polymerization and its position downstream of the p38/RK-type MAPkinase, itself a point of convergence for diverse signal transduction pathways.

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Evidence has accumulated in recent years to support the view that, besides its role in cell resistance to stress (Landry *et al.* 1989), the small heat shock protein of 27 kDa (and its murine analogue HSP25) may exert regulatory functions in other cellular processes (Edwards *et al.* 1981, Landry *et al.* 1989, Zantema *et al.* 1989, Fuqua *et al.* 1989, Huot *et al.* 1991, Bitar *et al.* 1991, Mendelsohn *et al.* 1991, Oesterreich *et al.* 1993, Faucher *et al.* 1993). This includes the demonstration that rapid phosphorylation of the protein (hereafter designated HSP27) is stimulated not only by chemical or physical stress, but also by agents as diverse as serum (Welch 1985), mitogens (Saklatvala *et al.* 1991), TNF and other cytokines (Hepburn *et al.* 1988, Kaur *et al.* 1989, Schutze *et al.* 1989, Arrigo 1990, Saklatvala *et al.* 1991), calcium ionophores (Welch 1985), phorbol esters (Welch 1985, Regazzi *et al.* 1988, Faucher *et al.* 1993) and differentiation inducers (Mishishita *et al.* 1991, Shakoori *et al.* 1992, Spector *et al.* 1993, Spector *et al.* 1994). The enzyme responsible for HSP27 phosphorylation is the MAPKAPkinase2 (mitogen-activated protein kinase-activated protein kinase2) (Stockoe *et al.* 1992), located downstream to the p38/RK-type of MAPkinase cascade involved in IL-1, stress and heat shock signalling (Freshney *et al.* 1994, Rouse *et al.* 1994). De-phosphorylation of HSP27 is accomplished in intact cells by protein phosphatase 2A (Cairns *et al.* 1994), which undergoes inactivating phosphorylation under the action of TNF or IL-1 (Guy *et al.* 1995).

Together, these data point to MAPKAP kinase 2 and its substrate HSP27 as pivotal elements in cell regulation by their position at the convergence of a wide diversity of signalling pathways.

The present work was aimed at investigating the effects on HSP27 phosphorylation of agents which diversely affect the growth and/or differentiation of MCF-7 cells, of human mammary carcinoma origin, which, independently of oestrogenic stimulation (Dunn *et al.* 1993), constitutively express high levels of this protein.

The agents used were (1) TPA, a differentiation inducer in MCF-7 cells (Valette *et al.* 1987, Guilbaud *et al.* 1990); (2) hydroxytamoxifen (OH-TAM), an anti-oestrogen acting on MCF-7 as a cytostatic and cytotoxic agent (Bardon *et al.* 1987, Kyprianou *et al.* 1991); and (3) TNFα, which induces apoptosis in our cells (Horman & Mairesse, unpublished results).

The data suggest the concept that phosphorylation of HSP27 is involved in the execution of cellular programmes as diverse as proliferation, differentiation and active cell death, by acting on a key rate-limiting factor that is common to the three pathways, rather than by specifying the cell's orientation in one of them. This is consistent with the position of HSP27, downstream of the p38/RK-type MAPkinase cascade of signal transduction and as a modulator of actin polymerization, itself implicated in the three pathways.

MATERIALS AND METHODS

Cell culture and treatment

The breast cancer cell line MCF-7 was obtained from Dr Leclercq (Bordet Institute, Brussels). Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, glutamine (3.2 nM), penicillin, streptomycin and amphotericin B (fungizone) (Gibco, Paisley, UK). Before OH-TAM treatment, cells were maintained for a minimum of 5 days in the same medium supplemented with dextran-coated charcoal (DCC)-treated serum, in order to adsorb oestrogen present in the bovine serum. OH-TAM treatment was performed in the same conditions. Cells were treated for different times with TPA (25 nM), TNF (30 ng/ml), OH-TAM (1 μM) or the solvent alone. For [^{32}P]-labelling, cells were pre-incubated for 3 h in DMEM-phosphate free medium made 10^{-5} M in phosphate and supplemented with

7.4 MBq/ml $H_3{}^{32}PO_4$ (specific activity 55.56 TBq/mmol; Amersham, UK) then further incubated for 30 min in presence of the different agents. For long-term incubation, cells were grown as described above and [^{32}P] labelling was performed during the last 3 h of the treatment. After incubation, cells were directly solubilized *in situ* either in sodium dodecyl sulphate buffer for one-dimensional (1-D) SDS-PAGE (Laemmli 1970) or in O'Farrell lysis buffer for 2-D-SDS PAGE (Towbin *et al.* 1979).

For radioactivity measurements, 5 μl samples were precipitated in 10% trichloroacetic acid, the precipitates were then washed successively with 5% thrichloroacetic acid, alcohol and acetone and counted in a liquid scintillation counter (TRI-CARB 2100 TR, Packard, Belgium).

Protein electrophoresis

For 1-D SDS-PAGE, samples were electrophoretically analysed on a 10% to 17% SDS-polyacrylamide linear gradient slab gel, in Tris-buffered saline solution (Laemmli 1970).

For 2-D electrophoresis, the separation consisted in a first separation by isoelectrofocusing on an ampholyte gel with a pH 5–7 gradient, followed by separation on an SDS-polyacrylamide gel in the second dimension, according to the O'Farrell method (O'Farrell 1975) (the first gel was loaded with 10^6 cpm (counts per minute) in all cases). Gels were silver-stained, dried and autoradiographed at −80°C on Hyperfilms MP (Amersham, UK) with an intensifying screen. The position of HSP27 isoforms in silver-stained electrophoregrams and their corresponding autoradiograms was determined by superimposition with the [^{32}P] pattern obtained from a 2-D immunoblot.

Immunoblotting

After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose sheets as previously described (Towbin *et al.* 1979). Briefly, the blots were subsequently incubated for 2 h in 10% (wt/vol) of fat-free dry milk in TBS (Tris 20 mM, NaCl 500 mM) and for 4 h in the presence of a monoclonal anti human HSP27 antibody (Stress Gen, Victoria, Canada), at a dilution of 1:500 in the same solution. After extensive washing, immune complexes were incubated with a biotinylated anti-mouse Ig at a dilution of 1:500, serving as a binding bridge to biotin–streptavidin peroxidase preformed complexes (Amersham Ltd, UK) used at the same dilution. Peroxidase activity was revealed by the mixed chromogenic substrate method (Young 1989).

Growth analysis

Cell growth was determined by measuring the DNA content per culture using the ethidium bromide fluorostaining (Boer 1975). Briefly, 5×10^4 cells were plated per Petri dish (3-cm diameter), in DMEM supplemented with total or DCC-treated fetal bovine serum as described above. After 18–24 h the test agent or the corresponding solvent was added to the medium. Cells were harvested at the chosen time by treatment with pronase (20 μg/ml in distilled water) and DNA content was evaluated as described previously (Boer 1975) using a Perkin-Elmer LS-3 spectrofluorometer. All assays were performed in triplicate and the data were presented as average $\pm$SEM value.

RESULTS

Effects on cell growth and morphology

Figures 1 and 2, respectively, show the time course effects of TPA (25 nM), OH-TAM (1 μM) or TNF (30 ng/ml) proliferation and associated morphology of MCF-7 cells, as compared to the control.

TPA treatment (Figure 1a)

In cells incubated with TPA, the growth rate between day 1 and day 4 of treatment was about three times lower than in the controls (doubling time: 70 and 25 h, respectively).

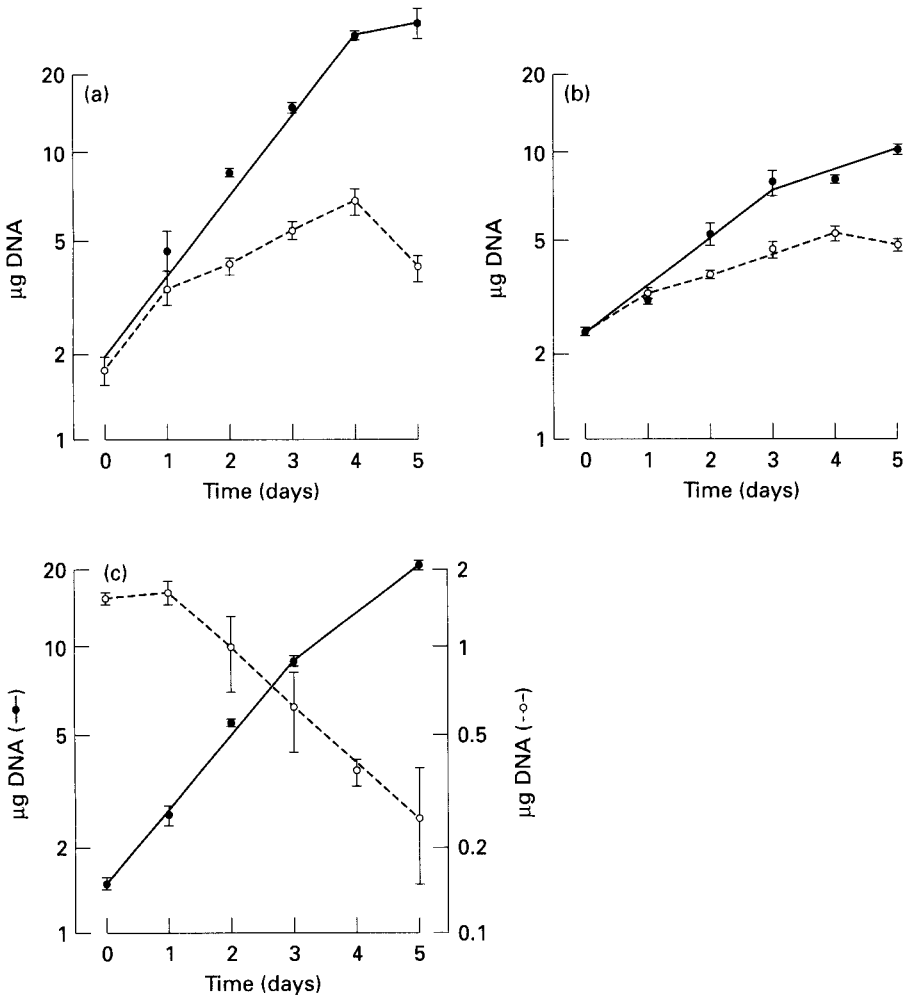

Figure 1. Growth of MCF-7 cells treated with (a) 25 nM TPA; (b) 1 μM OH-TAM or (c) 30 ng/ml TNF (——○——). Control cultures (——●——) were seeded with the same cell number in all cases and received the solvent only. (Note that the scale for the TNF treatment was adjusted vertically in (c), but is the same as in all other cases.) For OH-TAM treatment and the corresponding control, stripped (i.e. DCC adsorbed) serum was used (see 'Methods') which might explain the slower growth of control cells in that case, compared to the other controls, grown in presence of total serum. (DNA assays performed in triplicate; data are average ±SEM value.)

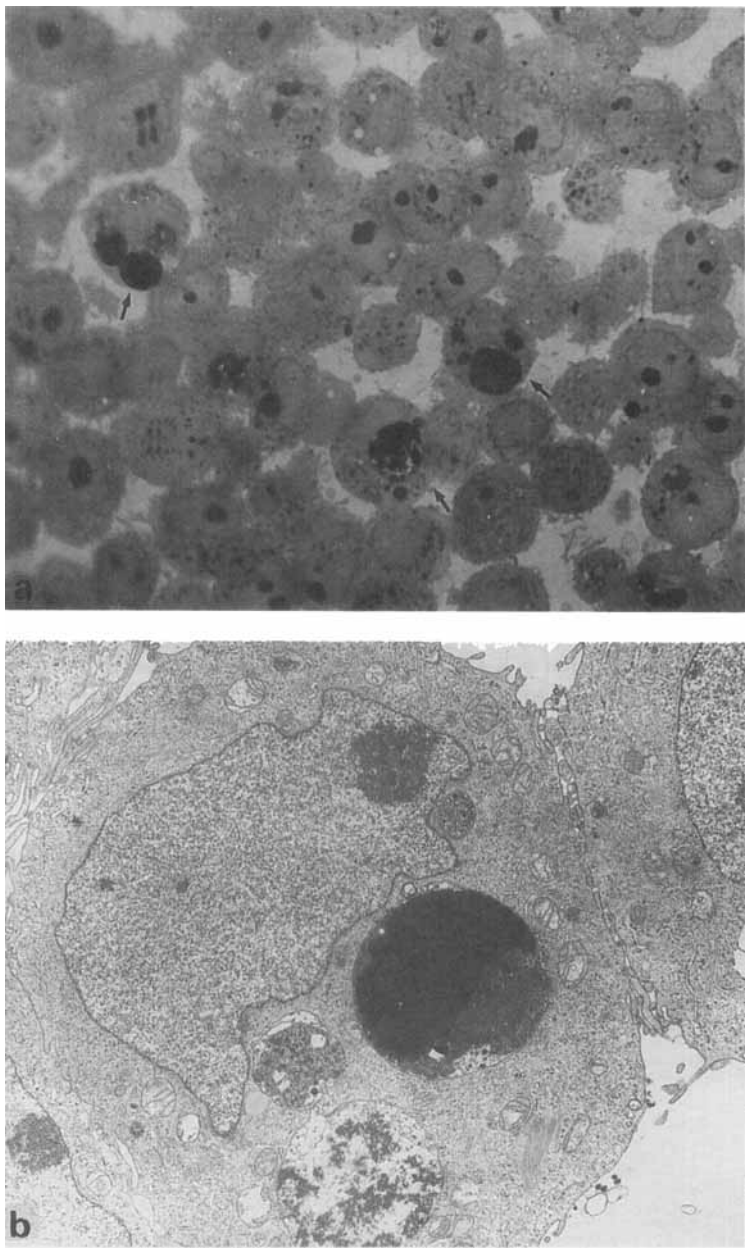

Figure 2. (a) Sections (1-μm thick) of MCF-7 cells pellets embedded in Epon 812 resin after fixation in 2.5% cocadylate buffered glutaraldehyde and post fixation in 2% cocadylate buffered osmium tetroxide. The picture shows apoptosis or apoptotic bodies (arrows) in MCF-7 cells exposed to TNF (30 ng/ml) for 24 h (magnification $\times 580$). (b) Electron microscopical aspect of apoptotic cells seen in the same culture (magnification $\times 6300$).

After 4 days of treatment, the cultures ceased to proliferate and cells started to die, showing signs of necrotic death, not of apoptosis.

From day 2 of treatment the cells started to exhibit changes in cellular morphology corresponding to the acquisition of a mature secretory phenotype, i.e. hypertrophy and the presence of lipid droplets and of intracellular vesicles enclosing granular material (already shown by Mairesse *et al.* 1996). Similar but less pronounced effects developed more slowly with 2.5 nM TPA (not shown).

OH-TAM treatment (Figure 1b)

During the first 4 days of treatment with 1 μM OH-TAM a threefold decrease in growth rate was also observed in treated cells, compared to their controls (doubling time: 120 and 38 h, respectively). On day 5 the cells stopped growing and 5% of the cell population presented classical cytological signs of programmed cell death, not observed at earlier times (not illustrated). Thus, the cytostatic or cytotoxic effect of this compound on MCF-7 cells, noted by other authors (Osborne *et al.* 1983, Bardon *et al.* 1987) appears as a late response to the anti-oestrogen and seems secondary to a prolonged growth inhibition.

TNF treatment (Figures 1c & 2)

TNF treatment induced an immediate growth arrest followed, after 24 h, by a dramatic exponential decrease in cell number. This treatment induced apoptotic cell death, as clearly defined by classical morphological criteria (see Figures 2a & b). Three per cent of the cells were apoptotic, as early as 8 h after treatment, and this incidence increased to 12–15% after 24 h (Figure 2). Induction of apoptosis in MCF-7 cells under TNF action was never reported before.

Maintenance of a plateau in the growth curve during the first day of treatment with this agent (Figure 1c) implies that cell production by divisions still exactly balanced cell loss by apoptosis during that period. At later times, total arrest of cellular divisions and persistance of a constant rate of cell death would then account for the observed exponential decrease in cell number.

HSP27 phosphorylation

The total amount of HSP27 products, relatively to total proteins remained the same at all times whatever the treatment, except for TPA treatment, where an increase was observed at day 5 (Figure 3).

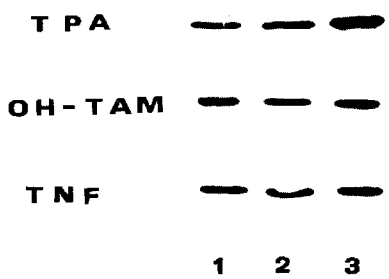

Figure 3. One-dimensional Western blot analysis of HSP27 content in MCF-7 cells treated with TPA or OH-TAM for 24 (lane 2) and 120 h (lane 3), or with TNF for 24 (lane 2) and 48 h (lane 3). Lane 1 shows the basal level of HSP27 in untreated cells. In each case, 5 μg of proteins were loaded on the gel.

Phosphorylation of the protein was evaluated from the labelling intensity of the spots corresponding to HSP27 isoforms in the autoradiograms from 2-D electrophoregrams, compared to the respective control. In parallel, net changes in the phosphorylation status of the protein were also evaluated by changes in the relative contents of the different HSP27 isoforms as demonstrated by silver staining of the corresponding 2-D gels.

The results of this analysis are as follows.

TPA treatment (Figure 4)

Within 30 min of TPA treatment, enhanced HSP27 phosphorylation was clearly demonstrated by a dramatic increase in [^{32}P] labelling intensity of the monophosphorylated isoform '*b*' (pI 5.9) and the detection of the label in the position that corresponds to the hyperphosphorylated isoform '*c*' (pI 5.6), never seen in extracts from control cells. These effects of TPA were maintained for up to day 5, the longest time investigated (Figure 4, left). A further increase in [^{32}P] labelling intensity of isoform '*b*' and appearance of form '*c*', seen at day 5 of treatment may be accounted for by the increase in the content in HSP27 observed at that time with this treatment (Figure 3).

The corresponding silver-stained electrophoregrams (Figure 4, right) accordingly showed that under basal conditions the majority of HSP27 was under the most basic, unphosphorylated isoform '*a*' (pI 6.2) and that TPA treatment induced the early conversion of about 50%

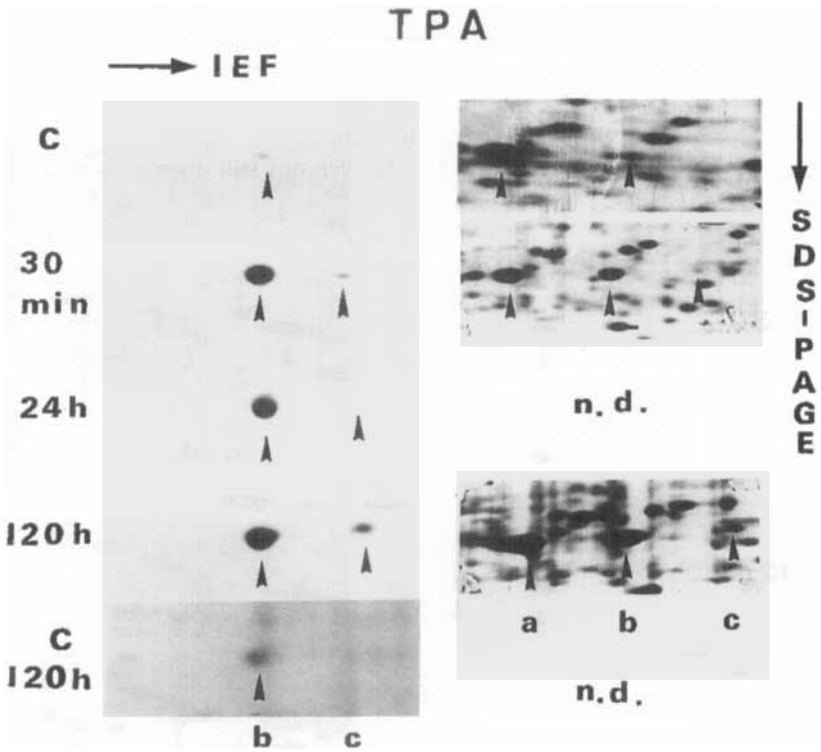

Figure 4. Two-dimensional silver-stained electrophoregrams (right) and corresponding autoradiograms (left) of ^{32}P-labelled MCF-7 cells, untreated (c) or treated with 25 nM TPA for different times. Only portions of the gels corresponding to the HSP27 isoforms are shown. '*a*' is the non-phosphorylated isoform; '*b*' and '*c*' are the mono- and biphosphorylated ones, respectively. n.d.=not determined.

of this isoform to the phosphoisoform '*b*'; the quantity in '*c*' isoform remained insufficient to be detected by this technique. These proportions remained roughly constant for up to day 5.

OH-TAM treatment (Figure 5)

With OH-TAM treatment (Figure 5, left) the level of HSP27 phosphorylation remained unchanged during the first 3 days, but significantly increased at days 4–5, i.e. when growth had ceased (Figure 1b) and signs of apoptotic death started to be detected. There was an indication that the control in this case might have exhibited slightly higher levels of phosphorylation of HSP27 than in the other conditions. This might relate to the use here of stripped serum, which also resulted in slower cell growth.

However, in these experiments (Figures 4–5), long-term culture with the solvent did not result in any significant change the relative proportions of the HSP27 isoforms.

TNF treatment (Figure 6)

After TNF treatment, an increase in HSP27 phosphorylation was detected within 30 min in the autoradiograms, labelled spots '*b*' and '*c*' becoming readily detectable. Silver staining of corresponding electrophoregrams showed that 50% of isoform '*a*' had already converted to isoforms '*b*', '*c*' and even '*d*' (Figure 6, right). After 6 and 24 h of treatment the phosphorylation level decreased, isoform '*c*' again becoming undetectable and the balance in the relative

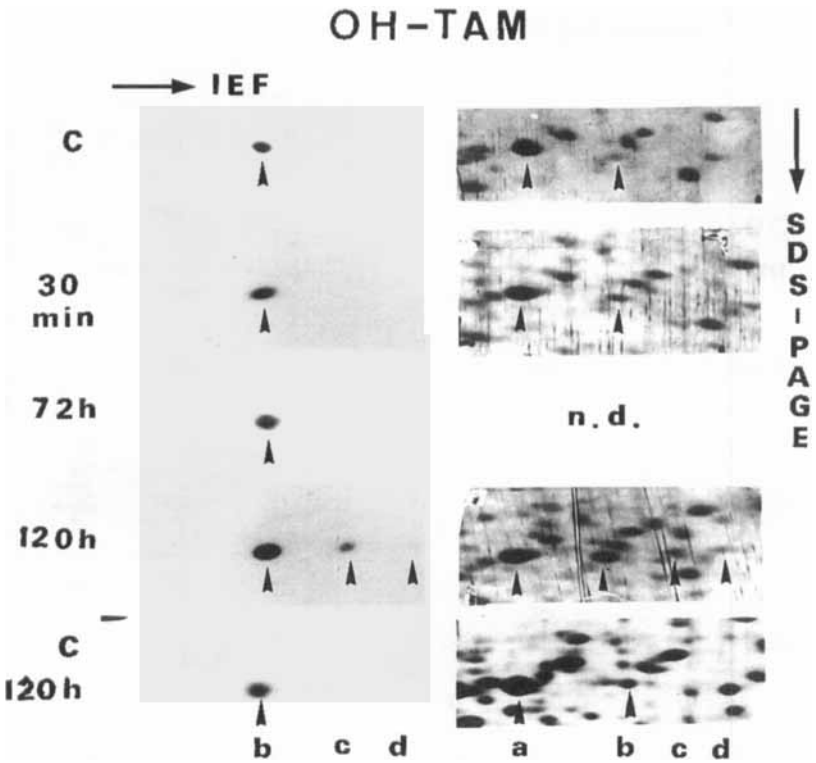

Figure 5. Two-dimensional silver-stained electrophoregrams and autoradiograms as in Figure 4, but with cells exposed to 1 μM OH-TAM.

levels of the different silver-stained isoforms shifting back to predominance of the non-phosphorylated form '*a*' (Figure 6, right). As mentioned above, massive cell death occurred after 24 h of treatment, thus terminating the experiment.

DISCUSSION

Our results show that both TNF and TPA trigger the immediate and massive phosphorylation of HSP27 in MCF-7 cells, whereas OH-TAM affects the phosphorylation status of the protein with a 3-day delay.

Our observation that TNF induced a rapid increase in HSP27 phosphorylation fits with reports establishing that TNF, apparently via its effects to generate reactive oxygen intermediates, activates MAPKAPkinase2 (Huot *et al.* 1995), the enzyme for which this protein is a substrate (Stokoe *et al.* 1992).

Our data with TPA, agree with the previously reported early induction of HSP27 phosphorylation by this agent in different cell types (Welch 1985, Regazzi *et al.* 1988, Issandou *et al.* 1988, Faucher *et al.* 1993), including MCF-7 cells. They further show that a second increase in the cell content in phosphorylated HSP27 took place at day 5 of treatment, corresponding at this time to an increased amount of the protein.

The mechanism of the TPA-induced HSP27 phosphorylation remains unclear. Phorbol esters are indeed recognized as poor activators of p38/RK (Raingeaud *et al.* 1995) and of

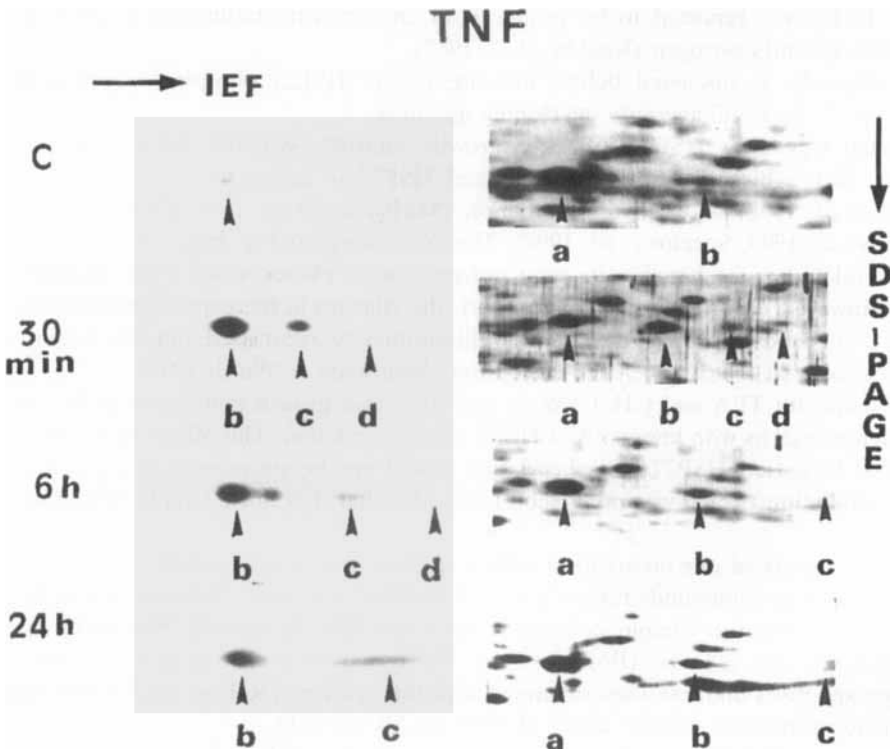

Figure 6. Two-dimensional silver-stained electrophoregrams and autoradiograms as in Figure 4, but with cells exposed to TNF (30 ng/ml).

MAPKAPkinase2 (Guesdon *et al.* 1993). A reasonable hypothesis would then be that another kinase intervenes at the level of PPase2A, for which HSP27 is a substrate, and which is inactivated by phosphorylation (Guy *et al.* 1995). On the other hand, despite that HSP27 is a poor substrate for protein kinase C (PKC) (Zhou *et al.* 1993), its phosphorylation under TPA stimulation in MCF7 cells was cancelled in the presence of a PKC inhibitor (Faucher *et al.* 1993). If so, considering that phosphorylation of HSP27 was sustained at maximal level during at least 5 days, PKC δ appears as a possible candidate for this role. Indeed, contrary to PKC α, which is rapidly downregulated under prolonged activation by phorbol esters (reviewed in Hug & Sarre 1993), this recently discovered member of the PKC family remains persistently upregulated in those conditions (Assert *et al.* 1996).

Concerning OH-TAM, the absence of negative effects of the anti-oestrogen on HSP27 content, as well as the lack of positive effects of oestrogen (Dunn *et al.* 1993 and our own unpublished work) do not fit with an earlier suggestion that HSP27 would be an oestrogen-related protein (Edwards *et al.* 1981, Fuqua *et al.* 1989, Faucher *et al.* 1993). The discrepancy between our observations and previous reports in this respect can be explained by the fact that we are dealing with a variant cell line in which HSP27 is constitutively overexpressed compared to other sub-lines. Indeed, in MCF-7/MG cells which also constitutively overexpress the protein, HSP27 synthesis was not stimulated by oestradiol or heat shock (Fuqua *et al.* 1994).

Being late, the effect of OH-TAM on HSP27 phosphorylation probably reflects the intervention of (a) secondary auto/paracrine intracellular factor(s). A likely candidate would then be TGFβ. Indeed an effective inducer of HSP27 phosphorylation (Shibanuma *et al.* 1992), TGFβ was reported to be produced in an autocrine fashion as a consequence of treatment by anti-oestrogen (Knabbe *et al.* 1987).

Alternatively, as discussed below, the late rise in HSP27 phosphorylation might be a reflection of the simultaneously developing apoptosis.

In what way does HSP27 influence growth control? Available data in the literature suggests that a high level of phosphorylated HSP27 is associated with growth inhibition (Knauf *et al.* 1992) and cell differentiation (Michishita *et al.* 1991, Shakoori *et al.* 1992, Spector *et al.* 1993, Spector *et al.* 1994). This was interpreted as reflecting a causal role of HSP27, related to its function in actin polymerization (Miron *et al.* 1991, Benndorf *et al.* 1994). However, according to a recent report, the changes in the expression/phosphorylation of HSP27 depend on the cell type and the differentiating agent used and, at least *in vitro*, do not necessarily accompany cell differentiation (Minowada & Welch 1995).

Our data with TPA and OH-TAM, in fact show that growth inhibition can be associated with high as well as with low levels of HSP27 phosphorylation. This allows us to conclude not only that increased HSP27 phosphorylation would not be necessarily required to achieve growth inhibition (which was predictable), but also that it is not a simple side effect of the latter.

Another aspect of our observation is that in these experiments, apoptosis developed with two of the three compounds tested, namely OH-TAM and TNF. Whereas it was previously reported that sensitive tumour cells can respond to TNF by necrotic lysis or by apoptosis, depending on the cell line (Bortner *et al.* 1995), and even that normal endothelial cells undergo apoptosis under *in vitro* treatment with this cytokine (Robaye *et al.* 1991), this is the first demonstration of such an effect of TNF on MCF-7 cells.

Concerning OH-TAM treatment, it was previously shown that the anti-oestrogen induced cell death in an oestrogen receptor dependent way in breast cancer cell lines (Valette *et al.* 1987). Our data support the suggestion made by the authors that this might involve

apoptosis. Apoptosis with this treatment might have been provoked by the maintenance of a situation of prolonged deprivation of hormonal stimulation and/or to the resulting decrease in auto/paracrine production of growth factors. This would be similar to what occurs in the endometrium of ovariectomized animals (Nawaz *et al.* 1987), or in the case of tumours developed from MCF-7 cells in oestrogen-implanted, ovariectomized nude mice, and which regressed after removal of the implant, this being associated with apoptosis (Kyprianou *et al.* 1991).

In our experiments, apoptosis, whether as an early effect of TNF or a late effect of OH-TAM, was associated with increased HSP27 phosphorylation, thus raising the question of the relationship between the two cellular responses. Early phosphorylation of constitutively expressed HSP27 in cells treated with TNF has been reported (Robaye *et al.* 1989). It was also recently shown that stimulation of the RK (p38) transduction cascade, resulting in HSP27 phosphorylation (Freshney *et al.* 1994, Rouse *et al.* 1994), precedes the apoptotic cell death that is induced in rat PC-12 phenochromocytoma cells by withdrawal of nerve growth factor (Xia *et al.* 1995). This was taken to suggest that activation of this kinase may contribute to the induction of this type of cell death (Xia *et al.* 1995). If so, phosphorylation of the heat shock protein would not be a sufficient factor, as in our experiments with TPA treatment very high levels of phosphorylated HSP27 were observed very early on but were associated with decreased proliferation rate and differentiation, not with cell death.

A possibility already considered in the above cited work (Xia *et al.* 1995), is that what determines whether a cell will survive or undergo apoptosis is the dynamic balance between growth factor activated pathways (ERK in the above example) and stress-activated JNK-p38(RK) pathways.

On the other hand, increased HSP27 phosphorylation, rather than being related to induction or execution of apoptosis, might correspond to a more or less successful attempt from the cell to resist against agents which, like reactive oxygen intermediates, mediate this induction. This could be extended to the oxidative stress associated to metabolic activation (Buttke & Sandstrom 1994) and might thus apply also the situation in TPA stimulated cells in our experiments.

However, the specific inhibitor of p38 kinase, and thus of HSP27 phosphorylation, SB203580 (Cuenda *et al.* 1995), did not affect TNF-induced cytotoxicity in L929 cells (Beyaert *et al.* 1996, Mairesse *et al.* unpublished results). Conflicting results were also obtained in this respect in experiments with phosphorylation deficient hsp27 gene (Arata *et al.* 1995, Knauf *et al.* 1994, Huot *et al.* 1996). This might perhaps be attributable to the fact that the different cell lines used in those experiments had very different basal expression of HSP27 and exhibited also different basal sensitivities towards the stress conditions tested. In WEHI-S tumour cells, the overexpression of the protein failed to alter susceptibility to TNF, yet protected the cells against apoptotic death induced by different drugs (Jäättelä *et al.* 1992). The least that can be concluded is that the effect of HSP27 in TNF action may vary with the cell type.

A third possibility can therefore be considered, which we favour as a working hypothesis, as it would integrate all our above observations and the fact that HSP27 phosphorylation also increases under the action of growth factors (Saklatvala *et al.* 1991). If we assume that the common point to all the situations we examined resides in the fact that the cellular functions in which HSP27 is involved play a crucial role in the execution of the different cellular programmes that the cell may undergo, namely cell differentiation, proliferation and death by apoptosis, then, under certain conditions (cell status, extracellular conditions, etc.), they may be rate limiting to these processes.

In this view, modulation of HSP27 expression or phosphorylation would not specify which of these programmes the cell would follow, but might or might not be permissive to their execution and, as such, would be controlled as part of the specific signalling pathways that regulate such decisions, ensuring fulfillment of the requirement. This concept fits with HSP27 position downstream to MAKPAPkinase2, itself a point of convergence of a variety of signalling pathways. It also fits with its role in controlling actin polymerization, the latter indeed playing a role in the three cellular programmes. Experiments using transfection of antisense or a 'phosphorylation deficient' hsp27 gene are in progress to clarify this issue.

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