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Transient accumulation, phosphorylation and changes in the oligomerization of Hsp27 during retinoic acid-induced differentiation of HL-60 cells: possible role in the control of cellular growth and differentiation

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Abstract Expression of the mammalian small stress protein Hsp27 has been increasingly linked to cell growth regulation and differentiation. Hsp27 is a phosphoprotein which forms oligomers with native sizes ranging between 100 and 800 kDa. We have examined the fate of Hsp27 transiently expressed during the retinoic acid (tRA)-induced granulocytic differentiation of human leukemic HL-60 cells. We show that tRA, in addition to its effects on Hsp27 accumulation and phosphorylation, transiently increased the oligomerization state of this protein. While Hsp27 phosphorylation by tRA is an early phenomenon that takes place before cellular growth is altered, the redistribution of Hsp27 oligomers occurred later and concomitantly with the maximal accumulation of this protein. Hence, complex regulations of Hsp27 are induced by tRA which suggest that this protein plays a role in the pathway through which retinoids exert their effects. To approach Hsp27 function during HL-60 cell differentiation, experiments aimed at reducing the cellular content of this protein were performed by transiently inhibiting Hsp27 mRNA translation by a specific anti-sense oligonucleotide. This process, which decreased the basal level of Hsp27 by about 40%, did not interfere with the growth of undifferentiated HL-60 cells. In contrast, a decreased level of Hsp27 diminished the differentiation-mediated down-regulation of cell growth and altered some morphological changes induced by this retinoid. These results suggest that Hsp27 is a mediator of granulocytic differentiation.

INTRODUCTION

Cellular differentiation limits the clonal expansion of cell populations since, in general, differentiation and cell growth are mutually exclusive. The importance of this process is emphasized by the fact that during hematopoiesis its dysregulation contributes to leukemogenesis (Sachs 1980). One of the prototypic models used to study how cell differentiation is regulated is the promyelocytic leukemic HL-60 cell line. These actively dividing cells are characterized by an incomplete differentiation which is arrested at the promyelocytic stage of myeloid development. This differentiation arrest can be reversed, however, since these cells undergo either granulocytic or monocytic maturation in response to a number of exogenous agents, such as phorbol esters or retinoic acid (Collins 1987).

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Relatively little is known about how cell differentiation is regulated. Consequently, cell differentiation is actually a major field of investigation and proteins that are specifically synthesized during this process are investigated. In this respect, recent studies have revealed that the expression of the small heat shock (or stress) proteins (sHsp) is closely linked to changes in the state of cell differentiation. The expression of these proteins, first reported during the differentiation of *Drosophila* imaginal discs (Ireland et al 1982; Sirotkin et al 1982; Cheney and Shearn 1983; Pauli et al 1990; reviewed in Arrigo and Landry 1994; Arrigo 1995) was also observed in yeast during ascospore formation (Kurtz et al 1986). A similar expression of sHsps was observed during the differentiation of different mammalian cell types, such as embryonal carcinoma, embryonic stem cells (Stahl et al 1992), mouse Ehrlich ascite tumor cells (Benndorf et al 1988; Gaestel et al 1989), normal B cells, B lymphoma (Spector et al 1992), osteoblasts, promyelocytic leukemia cells (Shakoori et al 1992), normal T cells (Hanash 1993) and NB4 promyelocytic cells (Spector et al 1995). We have also recently described a transient accumulation of Hsp27 during the phorbol ester-induced monocytic differentiation of human HL-60 cells (Spector et al 1993) as well as during the all-trans retinoic acid (tRA)-induced granulocytic differentiation of these cells (Spector et al 1994). Moreover, a link between Hsp27 and growth regulation has been recently suggested (Knauf et al 1992).

The mammalian small stress protein Hsp27 (also denoted Hsp28 and in murine cells Hsp25) and sHsp from other species belong to the family of Hsps whose synthesis is induced or stimulated by heat shock and other forms of stress (Arrigo and Landry 1994). These proteins are characterized by a sequence similarity with αA,B-crystallin (Ingolia and Craig 1982). The number of sHsps varies between species; in mammals there are three: Hsp27, Hsp20 and αB-crystallin (Arrigo and Landry 1994; Kato et al 1994a). These proteins are characterized by complex oligomeric properties; depending on the physiology of the cell, they form structures with native molecular masses ranging from 100 to 800 kDa (Arrigo and Welch 1987; Arrigo et al 1988; Arrigo and Landry 1994; Mehlen and Arrigo 1994;). Small stress proteins have been described as modulators of actin architectures (Miron et al 1991) and shown to possess in vitro molecular chaperone activities (Jakob et al 1993) and in vivo protective effects against heat shock and other types of stress (Landry et al 1989; Mehlen et al 1993; Oesterreich et al 1993; Mehlen et al 1995a, b). Recently, we reported that sHsp from different species, such as human Hsp27, *Drosophila* Hsp27 and αB-crystallin share the ability to modulate intracellular redox and that this property is necessary for their protective activity against oxidative stress (Mehlen et al 1996).

Considering that Hsp27 has been increasingly linked to cell growth and differentiation, we sought to determine whether this protein is a growth-regulatory candidate during the tRA-induced granulocytic differentiation of HL-60 cells. tRA is particularly interesting because of its increasing clinical use as a differentiating agent in the treatment of human malignancies, such as promyelocytic leukemia (Huang et al 1988; Castaigne et al 1990). We now show that tRA, in addition to its effects on Hsp27 accumulation and phosphorylation (Spector et al 1994), also transiently increases the oligomerization state of this protein. As an approach to the role played by Hsp27 during tRA differentiation, we took advantage of antisense oligonucleotide technology to reduce the intracellular level of this protein. This process, which did not alter the growth of undifferentiated HL-60 cells, partially inhibited the decreased cellular growth and morphological changes induced by tRA. In contrast, it did not alter the ability of these cells to produce superoxide anion in response to phorbol ester myristate acetate (PMA). Hence, Hsp27 appears as an intermediary in the pathway through which retinoids exert their differentiating effects.

MATERIALS AND METHODS

Cell culture

HL-60 cells, obtained from the American Type Culture Collection, were cultured in RPMI 1640 (L-glutamine, Hepes) medium supplemented with 10% heat-inactivated fetal calf serum (Gibco Laboratories, Eragny, France) in tissue culture flasks (Falcon, Lincoln Park, NJ, USA).

Induction of granulocytic differentiation

HL-60 cells were maintained at logarithmic growth phase at 3×10^5 cells/ml. To induce granulocytic differentiation, cells were treated with tRA (Sigma Chemical, St Louis, MO) at a final concentration of 1 μM. At various time points prior to and following treatment with tRA, ferricytochrome c reduction assay was performed as a marker of granulocytic differentiation (Breitman et al 1990). In brief, cells ($1–3 \times 10^6$) were spun and resuspended in 1 ml of phosphate-buffered saline (PBS) (NaCl/Pi: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) containing 80 μM cytochrome c (Sigma Chemical, St Louis, MO) and 162 nM PMA (Sigma Chemical, St Louis, MO). Incubation in this medium was for 20 min at 37°C. After centrifugation of cells, absorbance of the medium was read at 550 nm. Morphological changes induced by tRA were also recorded. To this end, Wright–Giemsa stains of cytospins (Collins et al 1990) were analyzed by

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phase contrast microscopy using a Zeiss Axioskop photomicroscope using a Planapo 63X (1.3 NA) lens. Images were recorded onto Tri-X Pan (Eastman Kodak Co.).

One- and two-dimensional gel electrophoresis and immunoblot analysis

Gel electrophoresis and immunoblotting were performed as previously described (Arrigo et al 1988). Silver staining of the gels was performed using the silver staining kit of Bio-Rad SA (Ivrys, Seine, France) according to the manufacturer's instructions. For two-dimensional immunoblots, 10^7 cells were used. Immunoblots, probed with either anti-Hsp27 (Arrigo et al 1988) or anti-Hsp90 (Mehlen et al 1995a) antibodies, were developed with the ECL-kit from Amersham Corp. (UK). Autoradiography of membranes was performed in a way that took into account the linear response of the film. Autoradiographs were then scanned with the Bioprofil system (Vilber Lourmat, France).

Sizing chromatography

At various time points prior to and following treatment with tRA, 5×10^8 HL-60 cells were washed in PBS, harvested and lysed in 20 mM Tris, pH 7.4; 5 mM $MgCl_2$; 20 mM NaCl; 0.1% Triton X-100; and 0.1 mM EDTA. Cell lysates were spun at 10 000 x *g* for 10 min and then loaded on a Sepharose 6B column in the same buffer without Triton X-100, as previously described (Arrigo et al 1988; Mehlen and Arrigo 1994; Mehlen et al 1995b). Fractions were analyzed by SDS-PAGE and immunoblotted as described previously. The column was standardized with carbonic anhydrase (29 kDa), alcohol dehydrogenase (150 kDa), apoferritin (440 kDa), thyroglobulin (669 kDa) and blue dextran.

Cellular proliferation and DNA synthesis analysis

At different time points before and following treatment with tRA, cells were counted. For this purpose an hemocytometer chamber and a Nikon TMS inverted photomicroscope equipped with phase-contrast equipment were used. Microcultures were also pulsed with 1 µCi [^{3}H]-thymidine as described by Spector et al (1993, 1994) (Amersham Corp., UK) for 4 h. Dried filters were then counted on a Beckman LS6000 SC scintillation counter.

Hsp27 translational inhibition using anti-sense oligonucleotide strategy

The anti-sense oligonucleotide which corresponded to the first 17 nucleotides of the coding region of human *hsp27* gene (Hickey et al 1986) was used. The sense oligonucleotide was also used as a negative control. The sequence of the anti-sense and sense oligonucleotides were 5'-ACGCGGCGCTCGGTCAT-3' and 5'-ATGACCGAGCGCCGCGT-3', respectively. An anti-sense oligonucleotide of vesicular somatistis virus (VSV): 5'-TTGGGATAACACTTA-3' was also used as a negative control. The non-modified oligonucleotides, synthesized with a 381A DNA synthesizer (Applied Biosystems), were purified by sodium acetate/ethanol precipitation and resuspended in sterile double-distilled water. To introduce the different oligonucleotides into cells, the procedure described by Barry et al (1993) was used. In brief, 5×10^5 HL-60 cells were washed in PBS and resuspended in permeabilization buffer (100 mM piperazine-N-N'-bis [2–ethanesulfonic acid], 1, 4 piperazine diethanesulfonic acid [PIPES], pH 7.4; 137 mM NaCl; 5.6 mM glucose; 2.7 mM KCl; 2.7 mM EGTA; 1 mM ATP; 0.1% bovine serum albumin) containing 0.2 U/ml streptolysin-O (Sigma Chemical, St Louis, MO) and 50 µM (final concentration) of oligonucleotides. Cells were incubated 5 min at room temperature before 5 ml of complete RPMI growth medium (containing 10% fetal calf serum) were added to the mixture to stop the permeabilization process. Cells were then spun and resuspended in 3-day-old conditioned growth medium containing 10 µM oligonucleotide. After 2 days of incubation, cells were replated in fresh medium supplemented with 10% fetal calf serum and, at different times, the mitotic index was determined and aliquots of cells were processed for Hsp27 analysis, ferricytochrome c reduction and morphological analysis.

RESULTS

Following treatment with retinoic acid (1 µM), HL-60 cells undergo specific changes in their morphology and physiological properties that can be used to follow their differentiation. Differentiation of HL-60 cells into growth-inhibited granulocyte-like cells is usually complete in 72 h in the presence of 1 µM of tRA and the peak of accumulation of Hsp27 is observed after 24 h of exposure to this drug (Spector et al 1994). To gain more information concerning the kinetics of tRA-induced transient accumulation and phosphorylation of Hsp27, we took advantage of a cell culture medium, made up with a new batch of serum, that slowed down the differentiation process. Under these conditions, more than 7 days, instead of 72 h, were needed to observe a complete tRA-induced inhibition of cellular growth. It is seen in Figure 1A that after 5 days of growth, the number of HL-60 cells after treatment with tRA was roughly 25% less than the control cells. In addition, a 50% reduction in DNA synthesis was observed after 5 days of treatment with tRA (Fig. 1B). These phenomena reflect the gradual decrease in the growth rate of HL-60 cells treated with tRA. Analysis of

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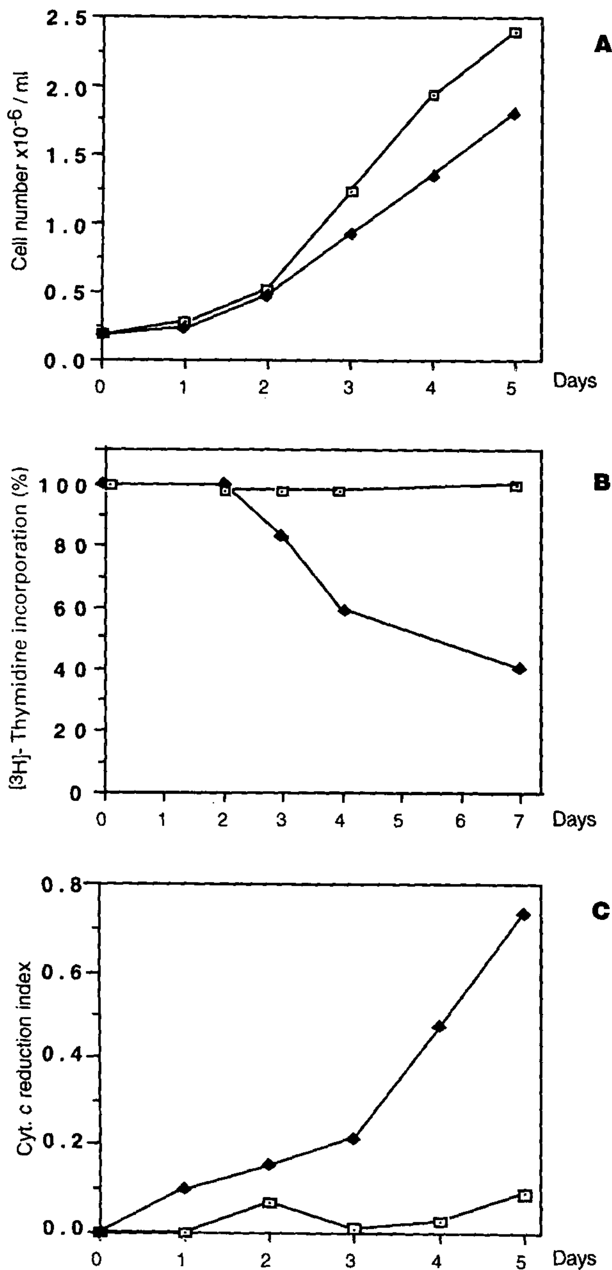

Fig. 1 tRA-induced growth inhibition in HL-60 cells. (A) Analysis of tRA-induced growth inhibition. HL-60 cells (2.10^6 cells/ml), growing in RPMI serum containing 10% serum, were divided into two flasks prior to the experiment. In one flask cells were left untreated while in the second 1 µM tRA was added. The growth of control (open symbol) and tRA-treated (filled symbol) cells was analyzed. A typical experiment is presented. Between experiments, standard deviations were less than 10%. (B) Cellular proliferation level (% of control [^{3}H]-thymidine incorporation) was determined each day following tRA addition by incubating equal number of cells with 1 µCi [^{3}H]-thymidine for 4 h. (C) Determination of the ability of cells to reduce ferricytochrome c in the presence of PMA as a measure of their granulocytic differentiation. This reactive oxygen intermediate was detected in the growth medium by the reduction of ferricytochrome c as described in Materials and Methods. Cytochrome c reduction index represents the ratio between the value measured at a specific time following tRA addition to the number of HL-60 cells analyzed.

the tRA-mediated granulocytic differentiation of HL-60 cells was performed by analyzing the appearance of specific markers. Differentiating HL-60 cells and mature granulocytes are characterized by their ability to produce superoxide anion ($O2^-$) when stimulated by phorbol esters, such as PMA (Breitman 1990). These reactive species can be detected in the growth medium by measuring the reduction of ferricytochrome c (see Materials and Methods). Figure 1C shows that the ability to produce $O2^-$ began to increase after 3 days of incubation with tRA. Morphological changes were also monitored to follow the progression of the tRA-induced myelomonocytic differentiation. This phenomenon is characterized by a decrease in the nuclear/cytoplasmic ratio, condensation of nucleoplasm, indentation, convolution and segmentation of nuclei and loss of multiple nucleoli (Collins et al 1990; Spector et al 1994). HL-60 cells, prior to and at different time periods following treatment with tRA were examined by Wright–Giemsa stain (see Fig. 2). Less than 1% of untreated HL-60 cells exhibited morphological features characteristic of differentiated granulocytes. Significant maturation was observed after 10 days of treatment, at which time about 80% of the cells exhibited the characteristic morphological changes described above. Hence, in our system, growth inhibition preceded the appearance of differentiation markers such as the ability to produce $O2^-$ and morphological changes.

As we previously described (Spector et al 1994), the small stress protein Hsp27 accumulates transiently and then disappears during tRA-mediated HL-60 cell differentiation. A quantitative analysis of this phenomenon is illustrated in Figure 3. This was assessed by determining Hsp27 levels by immunoblot analysis prior to and at different time points following the addition of tRA (see Materials and Methods). It is seen in Figure 3A that the maximal accumulation of Hsp27 occurred at 3 days after the start of tRA exposure. Thereafter, the cellular content of Hsp27 rapidly declined and by 5 days the level of this protein was 30% lower than that of control cells. Taken together with our previous observation (Spector et al 1994), these results indicate that the transient accumulation of Hsp27 occurs during the early phase of the differentiation process concomitantly with the beginning of the down-regulation of cell growth.

Phosphorylation is a characteristic modification of Hsp27. In two-dimensional gels, Hsp27 is resolved as different isoforms 'a, b and c'. The 'b' and 'c' isoforms are phospho-isoforms that display increased numbers of phosphate residues while the 'a' isoform is the non-phosphorylated form of the protein (Gaestel et al 1991; Arrigo and Landry 1994). The level of Hsp27 phosphorylation can, therefore, easily be quantified by two-dimensional immunoblot analysis. As seen in Figure 3B, untreated HL-60 cells contained mainly the unphosphorylated 'a'

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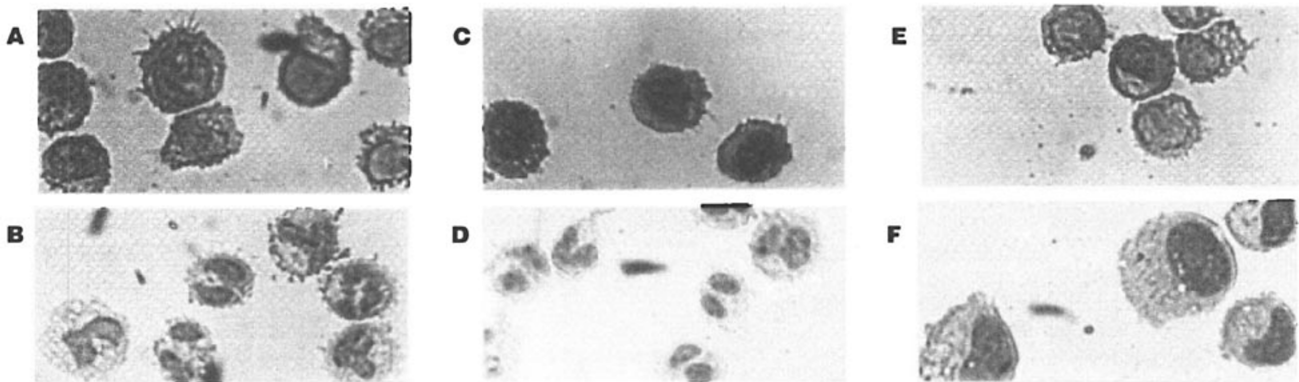

Fig. 2 Decreased Hsp27 levels interfere with the tRA-induced morphological changes of HL-60 cells. Cytospin preparations of streptolysin-O-treated HL-60 suspension cell cultures were stained with Wright–Giemsa before and after 10 days of exposure to 1 μM of tRA. (A, B) HL-60 cells treated with control VSV oligonucleotide exposed (B) or not (A) to tRA. (C, D) same as (A, B) but in this case Hsp27 sense oligonucleotide was used during the streptolysin-O treatment. A similar morphology was observed when cells were either not treated with streptolysin-O or treated with streptolysin-O in the absence of an added oligonucleotide. (E, F) same as (A, B) but cells treated with Hsp27 anti-sense oligonucleotide. Note, that in this case, tRA does not induce the classical morphology of differentiated granulocytes and that several cells display an enlarged size.

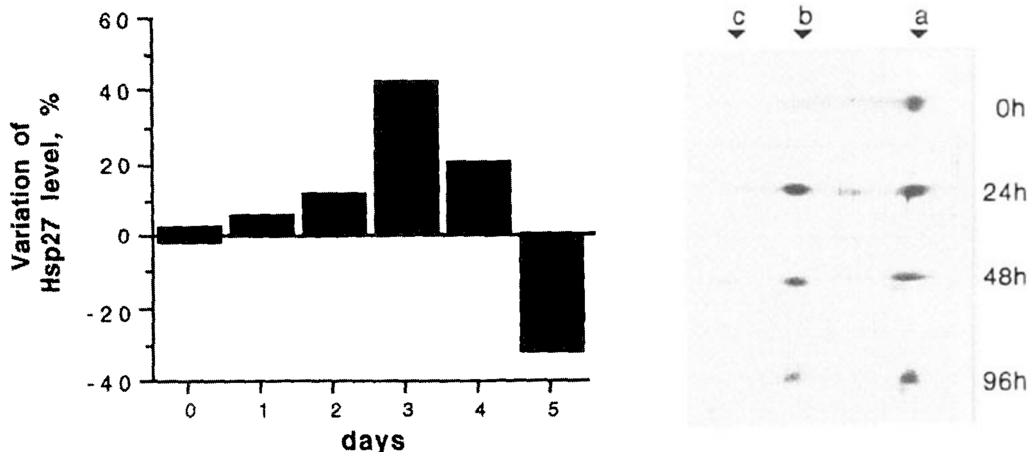

Fig. 3 Transient increase in Hsp27 level and phosphorylation during tRA-induced differentiation of HL-60 cells. (A) Hsp27 steady-state levels examined by immunoblot analysis at various time points prior to and following treatment with tRA (1 μM). At each time point, equal amounts of total cellular proteins were separated by SDS-PAGE and analyzed in immunoblots probed with Hsp27 antiserum. Autoradiographs of ECL-revealed immunoblots were quantified as described in Materials and Methods. Results are presented as percentage of the variation of Hsp27 level during tRA-induced differentiation. The percentage was equal to the level of Hsp27 at a specific time minus Hsp27 level in untreated cells divided by Hsp27 level in untreated cells × 100. Note the maximal level of Hsp27 after 3 days of treatment with tRA. In parallel experiments performed with cells not treated with tRA, Hsp27 level was found to remain constant with time. (B) Two-dimensional immunoblots of total cellular proteins probed with anti-Hsp27 serum were performed as described in Materials and Methods. Autoradiographs of ECL-revealed immunoblots are presented. The acidic end is on the left side of the gel. Arrowheads 'a, b and c' represent the different isoforms of Hsp27. The profile of Hsp27 isoforms at various time points prior to (0) and at 24 h, 48 h and 96 h following treatment with 1 μM tRA. Note the maximal accumulation of the 'b' phosphoisoform after 24 h exposure to tRA. In this experiment the duration of the ECL exposure was different from one sample to the other in order to detect the variations in the level of the different isoforms. This experiment, therefore, does not reflect the absolute level of Hsp27 as a function of the duration of tRA treatment, and was not used for the quantification in part A.

isoform, while 24 h after the addition of tRA, an intense and maximal accumulation of the 'b' phospho-isoform was observed. From then on, the level of the 'b' isoform slowly declined. See Figure 4 for a quantitative analysis of this phenomenon. It can, therefore, be concluded that the phosphorylation of Hsp27 precedes the accumulation of this protein during granulocytic differentiation of HL-60 cells.

In addition to phosphorylation, sHsps also undergo changes in their oligomerization state, particularly following cell exposure to mitogens, cytokines or heat stress (Arrigo and Welch 1987; Arrigo et al 1988; Kato et al 1994b; Mehlen and Arrigo 1994; Mehlen et al 1995b; Lavoie et al 1995). We sought to determine whether tRA-induced differentiation was also associated with changes in Hsp27 oligomerization. To this end, equal numbers of

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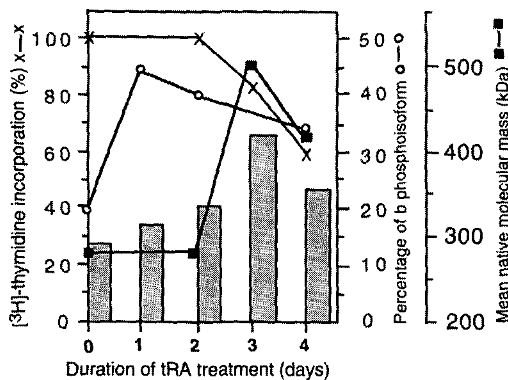

Fig. 4 Changes in Hsp27 phosphorylation, oligomerization and cellular content as a function of the duration of tRA treatment and [^{3}H]-thymidine incorporation. Data from Figures 1, 3 and 5 were quantified as described in Materials and Methods and presented as a function of the duration of tRA treatment. Gray plots represent the accumulation of Hsp27. The level of oligomerization of this protein is illustrated as Hsp27 mean native molecular weight. This was calculated by using the following formula: Hsp27 mean native molecular weight = Σ (Mi. Xi) / Σ Xi. The native molecular weight and the intensity of the corresponding protein band (in the Western blots) are represented by M and X, respectively, while i represents the individual column fractions. The intensity of Hsp27 phosphorylation is illustrated as the percentage of the b phosphoisoform versus the total isoforms of this protein.

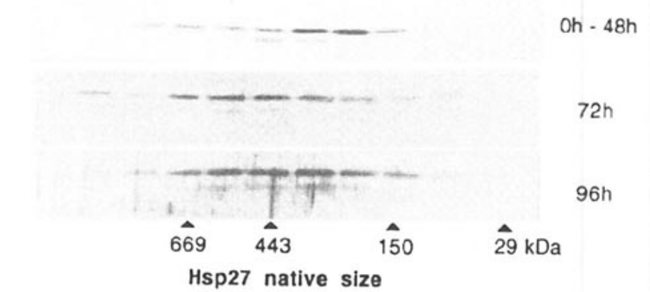

Fig. 5 Redistribution of Hsp27 oligomers in response to tRA. HL-60 cells (5×10^8) were harvested and lysed, as described in Materials and Methods, at various time points prior to and at 72 and 96 h following treatment with tRA. Cell lysates were applied to a Sepharose 6B column and every third fraction was analyzed by electrophoresis on a 12% SDS-polyacrylamide gel. Proteins were then transferred to nitrocellulose and immunoblotted using an anti-Hsp27 serum. The blot was developed with the ECL detection method. The arrowheads 29, 150, 443 and 669 indicate the apparent size (kDa) of gel filtration markers. The autoradiograph representing the 96-h condition was overexposed to allow a better detection of Hsp27 oligomers. Note the transient redistribution of Hsp27 oligomers toward large molecular weights as a result of tRA treatment. The lower molecular weight band detected by the antibody (see also Figs 3B and 6) may reflect a partially degraded form of Hsp27 (see also Arrigo and Welch 1987).

HL-60 cells were harvested and lysed at various time points prior to and following treatment with tRA. Lysates were subjected to sizing chromatography on a Sepharose 6B column. Eluant fractions were then analyzed for Hsp27 levels by immunoblot analysis (Fig. 5). In control untreated cells, Hsp27 was predominantly in the form of medium-sized oligomers that displayed native sizes between 200 and 400 kDa. A similar pattern of Hsp27 oligomerization was observed during the first 48 h of tRA treatment. Thereafter, a broader distribution of Hsp27 oligomers was observed together with a shift of their native sizes toward high molecular masses (up to 800 kDa). By 96 h, the reverse phenomenon was observed and the distribution of Hsp27 oligomers shifted toward lower molecular masses.

The various changes in Hsp27 were plotted together with change in growth rate (Fig. 4). It can be concluded from Figure 4 that the tRA-induced Hsp27 phosphorylation is an early phenomenon that precedes the change in the oligomerization of Hsp27 and occurs before the decrease in [^{3}H]-thymidine incorporation. The increased oligomerization of Hsp27 is a later phenomenon. It occurs concomitantly with the beginning of the period when cells display a reduced growth rate and when the accumulation of Hsp27 is maximal.

We next analyzed the role of Hsp27 during the granulocytic differentiation of HL-60 cells. To this end, an oligonucleotide complementary to the beginning of Hsp27 mRNA coding region was introduced into HL-60 cells by using the streptolysin-O technology described by Barry et al (1993) (see Materials and Methods). As seen in the silver-stained gel analysis presented in Figure 6A, Hsp27 anti-sense oligonucleotide did not alter the overall protein pattern of HL-60 cells. The level of Hsp27 was immuno-detected, since, in growing HL-60 cells, this protein is difficult to detect in a stained gel. The immunoblot presented in Figure 6B shows that Hsp27 cellular content was decreased by about 40% 2 days after introducing Hsp27 anti-sense into HL-60 cells. In contrast, the level of this protein was not altered when the streptolysin-O treatment was performed in the absence of Hsp27 anti-sense oligonucleotide (Fig. 6). Similarly, no alteration of Hsp27 was observed when Hsp27 sense and VSV oligonucleotides were tested (not shown). Another control shows that the level of constitutively expressed Hsp90 was not altered by the presence of Hsp27 anti-sense. Hence, the decreased level of Hsp27 described above appeared specific to Hsp27 anti-sense oligonucleotide.

As seen in Figure 7A and B, HL-60 cells partially depleted of Hsp27 displayed growth rates that were similar to control cells. In this experiment, the growth rate of control HL-60 cells was lower than that described in Figure 1A. This effect was a consequence of the streptolysin-O treatment. It is seen in Figure 7C and D that, following the addition of tRA, the cells partially depleted of Hsp27 were less growth-inhibited than the cells that either did not receive the anti-sense oligonucleotide or had received the control sense or VSV oligonucleotide (not shown). After 6 days in the presence of tRA, this

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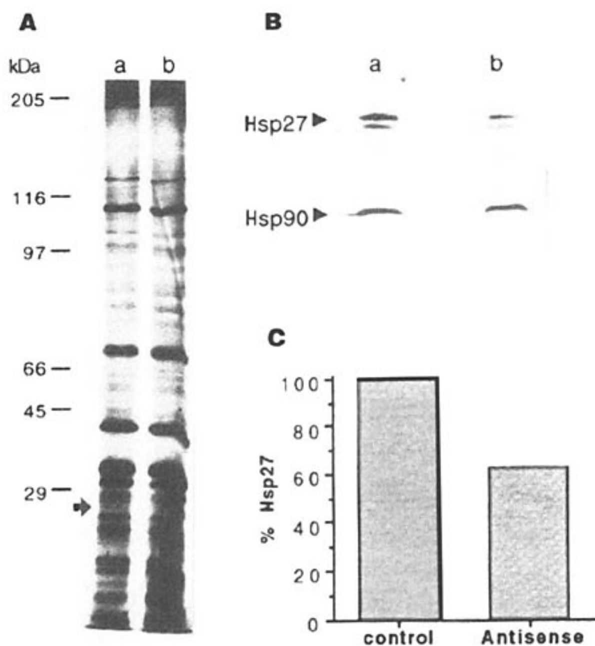

Fig. 6 Reduced Hsp27 level in HL-60 cells containing an anti-sense oligonucleotide specific for Hsp27 mRNA. HL-60 cells were treated by the streptolysin-O method as described in Materials and Methods. Control HL-60 cells were treated with streptolysin-O either in the absence (A) or in the presence (B) of Hsp27 anti-sense oligonucleotide. Cells were then allowed to grow for 2 days in conditioned growth medium in the presence or not of 10 μM of the oligonucleotide. Equal amounts of total cellular proteins were analyzed by SDS-PAGE. Samples (A and B) were loaded twice on the same gel. After electrophoresis the gel was cut in two parts: one part was used for silver staining and the other part was kept for immunoblot analysis. (A) Silver-stained gel. The arrow indicates the position where Hsp27 migrates. Note that Hsp27 is a minor protein that is not detectable in silver-stained gels. (B) Immunoblots. In this case, the nitrocellulose membrane was cut in two pieces that contained the slow and fast migrating proteins, respectively. Immunoblots were probed with either Hsp27 or Hsp90 antisera and were revealed with ECL. Similar results were obtained when blots were revealed with peroxidase staining or were incubated with a mixture of Hsp27 and Hsp90 antisera (in this case the nitrocellulose blots were not cut in two pieces). (C) Quantitative analysis of Hsp27 levels. Internal standards used were Hsp90, determined by immunoblot analysis, and silver-stained proteins from the same samples analyzed in the same gel (see A). Note the reduced level of Hsp27 in cells containing Hsp27 anti-sense oligonucleotide, compared to Hsp90 levels and to the bulk of HL-60 proteins which remain constant. Hsp27 levels were unaltered when control sense or VSV oligonucleotides were analyzed (not shown).

resulted in a 45% increase in the number of cells in anti-sense-treated cultures compared to control cultures. Moreover, after 4 days in the presence of tRA, DNA synthesis in these cells, measured by the incorporation of [^{3}H]-thymidine in the same number of cells was found to be twice that of control cells (Fig. 7E).

To test whether partial depletion of Hsp27 altered the granulocytic differentiation of HL-60 cells, the tRA-mediated ability to produce superoxide anions in the presence of PMA or morphological changes (condensed/indented nucleus and loss of multiple nucleoli) were analyzed. After 4 days in the presence of tRA, the PMA-mediated production of superoxide anions was similar whether or not HL-60 cells had been treated with Hsp27 anti-sense oligonucleotide (Fig. 7F). A similar observation was made when HL-60 cells were treated with Hsp27 sense or VSV oligonucleotides (not shown). Hence, concerning this parameter of differentiation, cells partially depleted of Hsp27 behaved as normal cells. In contrast, the morphological changes induced by tRA were strongly attenuated by the presence of Hsp27 anti-sense oligonucleotide. It can be seen in Figure 2 that, after 10 days in the presence of tRA, control cells or cells treated with VSV or Hsp27 sense oligonucleotide displayed the classical morphology of mature granulocytes characterized by the indentation, convolution and segmentation of nuclei and loss of multiple nucleoli (Fig. 2B and D). In contrast, most tRA-treated HL-60 cells containing Hsp27 anti-sense oligonucleotides showed an increased size and did not display the characteristic changes in nuclear morphology (Fig. 2F). Other morphological criteria, such as the loss of multiple nucleoli and the decrease in the nuclei/cytoplasm ratio, appeared less altered by Hsp27 partial deprivation (Fig. 2F). It is seen in Figure 2A, C and E that the cellular morphology of HL-60 cells not treated with tRA was not altered by the different oligonucleotides. Hence, some parameters of the granulocytic differentiation of HL-60 cells, such as changes in nuclear morphology, appeared altered by a decrease in the level of Hsp27.

DISCUSSION

In the current report, we show that Hsp27 undergoes quantitative as well as qualitative changes in response to retinoic acid-induced granulocyte differentiation of HL-60 promyelocytic leukemic cells. These changes are transient and temporally associated with the beginning of the differentiation process that is characterized by a gradual down-regulation of cellular proliferation. These observations extend our previous report performed with HL-60 cells that differentiated in 72 h in presence of 1 μM of tRA (Spector et al 1994). The production of superoxide anion upon membrane stimulation with PMA was used as a marker of differentiating HL-60 cells. In this report, the PMA-mediated production of superoxide began to be detectable after 4 days of tRA treatment. Morphological changes that are characteristic of granulocytic differentiation, including the loss of multiple nucleoli, indentation, convolution and segmentation of nuclei, were observed in most of the cells after 10 days of treatment with tRA.

Our study shows that the maximal accumulation of Hsp27 occurred at the beginning of the incubation with tRA when DNA synthesis was inhibited by only 20–30%. Thereafter, Hsp27 level rapidly declined. Phosphorylation

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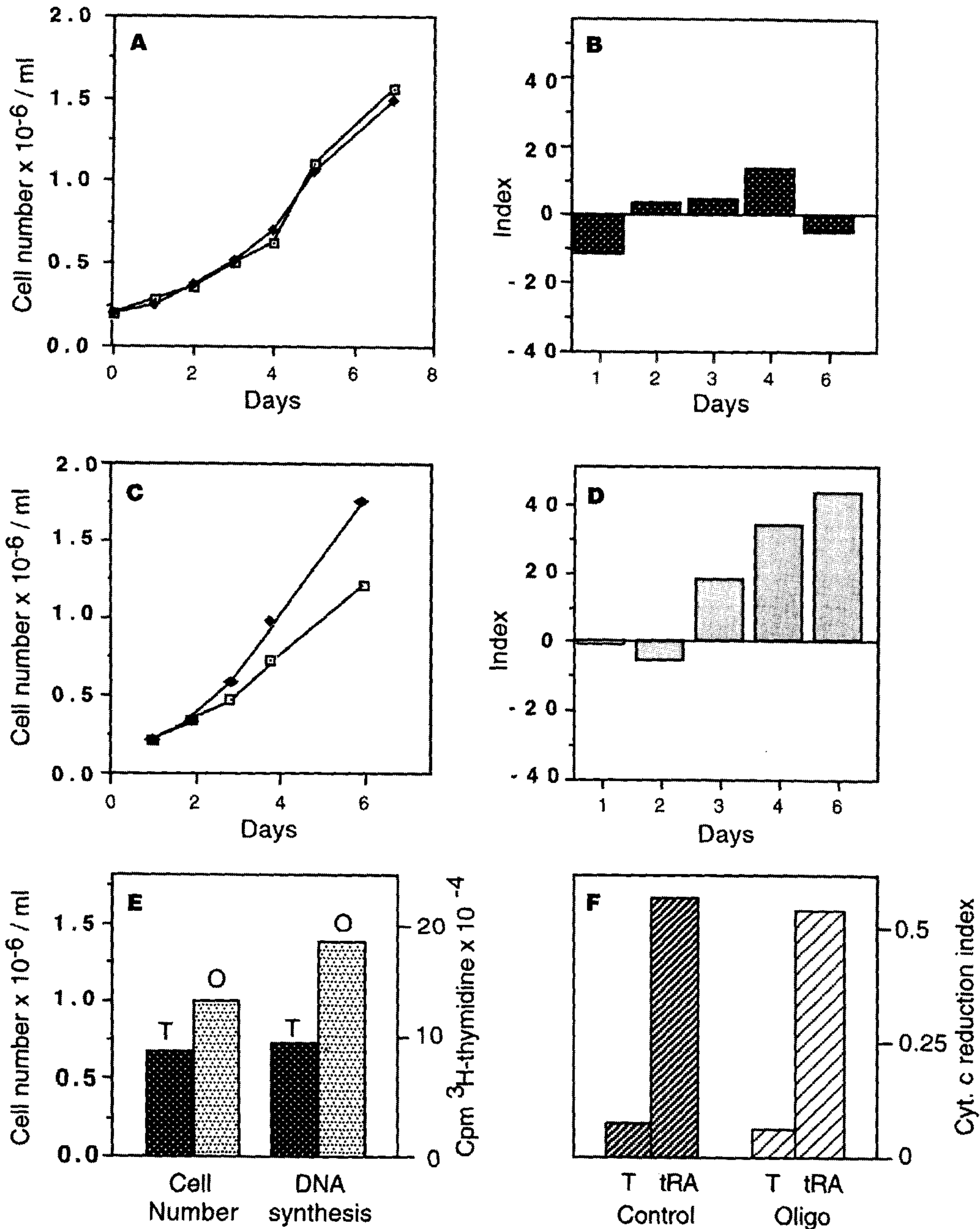

Fig. 7 Decreased Hsp27 levels alter HL-60 decreased cell growth during tRA-induced differentiation but do not interfere with the tRA mediated ability to produce superoxide. Untreated and tRA-treated HL-60 cells containing (closed symbol) or not (open symbol) Hsp27 anti-sense oligonucleotides were analyzed. The control cells that contain no oligonucleotides were nevertheless treated with the streptolysin-O procedure. (A, B) Growth characteristics in the absence of tRA treatment. Cell number as a function of time was measured (A). In (B) the index represents the difference (in cell number) between cells that contain and those that do not contain Hsp27 anti-sense divided by the number of cells that do not contain the anti-sense oligonucleotide × 100. Note that the presence of Hsp27 anti-sense oligonucleotide did not alter the mitotic index. (C, D) as (A, B) but in the presence of 1 μM tRA added at the beginning of the experiment. (E) Comparison of cell number and DNA synthesis in HL-60 cells, exposed for 4 days to 1 μM tRA, that contained (gray bar, O) or not (black bar, T) Hsp27 anti-sense oligonucleotide. Incubation with [^{3}H]-thymidine was for 12 h and for the same amount of cells (2.10^5 cells/ml). (F) Determination of the ability of cells to reduce ferricytochrome c. Light-hatched plot: cells containing Hsp27 anti-sense oligonucleotide; dark-hatched plot: control cells that contain no oligonucleotide; T: untreated cells; tRA: cells incubated 4 days with tRA. Results are indicated as cytochrome c reduction index (as defined in Fig. 1). In these experiments, no effects were observed when Hsp27 sense or VSV oligonucleotides were analyzed (not shown). In these experiments, standard deviations, which were not indicated in order to clarify the figures, were less than 10% (*n*=3).

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of Hsp27 was found to precede the accumulation of this protein. Hence, Hsp27 phosphorylation is a very early phenomenon that occurs before cellular growth is altered. These results may also explain why Minowada and Welch (1995) failed to observe an accumulation of Hsp27 in HL-60 cells. This discrepancy with our observations is probably due to the fact that these authors analyzed Hsp27 properties at the end of the differentiation process when the transient accumulation of Hsp27 is over. In their conditions, which resemble those used in our preceding study (Spector et al 1994), differentiation is complete in 72 h; at that time the level of Hsp27 is back to normal.

Since Hsp27 is oligomeric (Arrigo et al 1988), we have analyzed the native size of this protein following tRA addition to HL-60 cells. Drastic and complex tRA-mediated changes of Hsp27 oligomerization were observed. They ranged from the medium-sized oligomers (200–400 kDa) observed in control untreated cells to the broader distribution (200–800 kDa) of this protein observed after 72 h of treatment with tRA. It should be noted that the particular redistribution of Hsp27 oligomers was concomitant with the maximal accumulation of this protein and occurred, therefore, after the phosphorylation of Hsp27.

Our results clearly indicate that the changes in Hsp27 accumulation and modifications are specific for the early stages of tRA treatment. The mechanism that mediates increased Hsp27 level during HL-60 cellular differentiation was found to vary depending upon the differentiation signal. In the case of the macrophagic differentiation mediated by PMA, increased levels of Hsp27 are preceded by an increase in Hsp27 mRNA (Spector et al 1993). In contrast, tRA raises the cellular content of Hsp27 by a mechanism that appears to increase the half-life of this protein (Spector et al 1994). In this respect, Hsp27 has been described as a substrate of myeloblastin (Spector et al 1995), a serine protease which is rapidly down-regulated by retinoids and linked to cellular growth arrest and myeloid differentiation (Bories et al 1989).

The consistency with which Hsp27 is linked to growth arrest during differentiation of hematopoietic and other mammalian cells suggests that this phenomenon is likely to be functionally relevant. A putative growth-regulatory role for Hsp27 during differentiation was studied in HL-60 cells containing reduced level of this protein. This was assessed by introducing into HL-60 cells an anti-sense oligonucleotide complementary to the beginning of Hsp27 coding sequence. A 40% decrease in the level of Hsp27 was observed that did not alter the growth rate of untreated HL-60 cells. In contrast, Hsp27 partial depletion reduced the inhibition of growth rate and DNA synthesis normally seen in response to tRA. Hsp27 partial depletion was also found to alter some of the morphological changes induced by tRA, but did not interfere with another differentiation marker, i.e the ability to produce superoxide anion in the presence of PMA. This suggests that small variations in the level of Hsp27 can drastically alter the decreased growth rate and some of the morphological changes that occur during granulocytic differentiation of HL-60 cells.

Of interest, during the differentiation of murine embryonic stem cells, Hsp27 also transiently accumulates (Stahl et al 1992) and displays changes in its phosphorylation and oligomerization status (P Mehlen and A-P Arrigo, unpublished data). In this system, we have recently observed that an almost complete inhibition of Hsp27 expression also decreased the differentiation-mediated down-regulation of cell growth and altered the differentiation process (Mehlen P, Mehlen A, Godet J and Arrigo A-P, manuscript in preparation). Hence, our results favor the hypothesis that the transient increase in the level of Hsp27 is of crucial importance for the down-regulation of cell growth and the morphological changes that occur during differentiation.

The molecular function of Hsp27 involved in the modulation of cell differentiation is unknown. The observation that Hsp27 anti-sense oligonucleotide increases the size of most tRA-treated HL-60 cells suggests that Hsp27 may play a role as a chaperone (Jakob et al 1993) modulating actin architecture (Miron et al 1991; Lavoie et al 1995). On the other hand, we have recently reported that Hsp27 expression induces a pro-reducing state that inhibits the activation of the transcription factor NF-κB by oxidative stress (Mehlen et al 1996). Hence, Hsp27 expression may also indirectly regulate transcription factors, such as AP-1 or EGR-1, whose activation is redox-dependent and which are essential during the differentiation process (Abate et al 1990; Esposito et al 1994; Povis et al 1995).

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