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Regulation of the 28 kDa heat shock protein by retinoic acid during differentiation of human leukemic HL-60 cells

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Abstract

Dysregulation of hematopoietic cellular differentiation contributes to leukemogenesis. Unfortunately, relatively little is known about how cell differentiation is regulated. Considering that heat shock proteins (hsp) and specifically the small hsps have been increasingly linked to growth regulation, we sought to determine whether the mammalian small hsp (hsp28) is a growth-regulatory candidate during hematopoietic cell differentiation. Because of its effects on cell growth and differentiation and its increasing clinical use as a differentiating agent, we examined the effect of retinoic acid (RA) on hsp28 during differentiation of the human leukemic HL-60 cell line. Although hsp28 was constitutively expressed at low levels in untreated HL-60 cells, steady state hsp28 protein increased transiently, concomitant with the onset of G1 cell cycle arrest. Furthermore, hsp28 phosphorylation transiently increased within one hour following treatment with RA. Interestingly, in contrast to other differentiating agents the induction of hsp28 by RA was post-transcriptionally mediated with hsp28 protein and mRNA being discordantly regulated. These observations underscore the complex regulation of hsp28 by RA during granulocytic differentiation of human leukemic cells and indicate hsp28 as an intermediary in the pathway through which retinoids exert their growth and differentiative effects.

Key words: Heat shock protein; Phosphorylation; Retinoic acid; HL-60; Differentiation

1. Introduction

Differentiation is a fundamental cellular program which limits the clonal expansion of hematopoietic cell populations since in general, differentiation and cell growth are mutually exclusive. The importance of this process is highlighted by the fact that its dysregulation contributes to leukemogenesis [1]. Unfortunately, relatively little is known about how cell differentiation is regulated. One of the prototypic models used to study this process is the promyelocytic leukemic HL-60 cell line, which represents cells arrested at the promyelocytic stage of myleoid development. The block in development is reversible however, since these cells undergo either granulocytic of monocytic maturation in response to a number of exogenous agents [2]. HL-60 cells have therefore been used to study the role of proto-oncogenes as well as other growth-regulatory molecules during hematopoietic differentiation. Interestingly, the small mammalian heat shock proteins (hsp28) was identified as a novel marker of growth arrest during phorbol ester-induced monocytic differentiation of HL-60 cells. Changes in hsp28 protein expression and phosphorylation state mark the onset of G1 cell cycle arrest during monocytic differentiation [3,4]. Similar changes in hsp28 protein expression have also been observed in differentiating human B lymphocytes and in phorbol ester-induced growth arrest of lymphoma cell lines [5]. These mammalian studies extend those in *Drosophila* where the constitutive expression of the small hsps during development suggests a multifactorial role for these proteins in growth regulation [6–10]. Importantly, a direct link between small hsps and growth regulation was recently demonstrated in a murine tumor model where overexpression of the murine low molecular hsp (hsp25) led to growth inhibition [11].

In the current study we examine the regulation of hsp28 during retinoic acid-induced granulocytic differentiation of HL-60 cells. RA was particularly interesting because of its effects on cell growth and differentiation in vitro as well as its increasing clinical use as a differentiating agent in the treatment of human malignancies [12–14]. We now show that RA has significant effects on

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hsp28 protein expression and phosphorylation. These changes are temporally associated with down-regulation of cell growth and the onset of G1 cell cycle arrest and are reminiscent of those changes observed following PMA-induced monocytic differentiation of HL-60 cells [3,4]. However, in contrast to PMA, RA-mediated induction of hsp28 protein appears to be regulated at the post-transcriptional level.

2. Materials and methods

2.1. Cell Culture

HL-60 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, and 5 μg/ml gentamycin in tissue flasks (Corning Glass Works, Corning, NY).

2.2. Induction of terminal differentiation

HL-60 cells were maintained at logarithmic growth phase at 3×10^5 cells/ml. To induce granulocytic differentiation, cells were treated with all-*trans*-retinoic acid (Sigma, St. Louis, MO) at a final concentration of 1 μM. At various time points prior to and following treatment with RA, Wright-Giemsa stains were obtained on cytosmears.

2.3. Radiolabeling and immunoprecipitations

HL-60 treated or not with RA were incubated for 30 min at 37°C in RPMI 1640 lacking phosphate before being exposed to 0.25 mCi/ml [^{32}P]orthophosphate (carrier free; Amersham, UK) in the same medium. Labeling was for 30 min. The labeling medium was then removed and the cells were quickly washed in ice-cold phosphate-buffered saline (PBS) and harvested. Equal amount of protein from labeled cell lysates were immunoprecipitated with anti-hsp28 antibody as previously described [3,15].

2.4. One and two-dimensional gel electrophoresis and immunoblot analysis

Gel electrophoresis and immunoblotting were performed as previously described [3,16]. Purified hsp28 was from StressGen Corp. (Victoria, Canada). For 2D immunoblots, 1×10^7 cells were used. Immunoblots were probed with anti-hsp28 antibody [16] and developed with the ECL-kit from Amersham (UK).

2.5. Preparation of RNA and Northern blot analysis

Total cellular RNA was purified by the guanidium thiocyanate/CsCl method [17]. Intactness of RNA was verified by ethidium bromide staining (data not shown). Equal amounts (20 μg) of RNA were run on a 1.3% agarose gel and transferred onto a Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, CA). Hybridization was performed using a ^{32}P-labeled cDNA probe from the human hsp28 gene (StressGen Corp., Victoria, Canada) [18]. To control for equal loading of RNA, blots were hybridized with a ^{32}P-labeled β-actin cDNA probe.

2.6. Cellular proliferation and cell cycle analysis

Microcultures were pulsed with 1 μCi [^{3}H]thymidine (Amersham, Arlington Heights, IL) for 4 h. Dried filters were counted on a Packard Tri-carb scintillation counter (Packard, Downers Grove, IL). The stimulation index (S.I.) represents the following: S.I. = sample (cpm)/blank (cpm). 1×10^6 cells were harvested at various time points following treatment with RA. Cells were processed with the Coulter DNA-Prep reagent (Coulter Immunology, Hialeah, FL) and examined by flow cytometric analysis as described by the manufacturer.

3. Results

3.1. Increased hsp28 steady-state protein expression in differentiating HL-60 cells

Hsp28 steady-state protein expression was examined by immunoblot analysis at various time points prior to and following treatment of HL-60 cells with RA (1 μM). In order to control for differentiation-induced changes in cell size and protein content, equal amounts of total cellular protein (50 μg) were loaded at each time point. As shown in Fig. 1a, hsp28 was constitutively expressed in untreated cells. Twenty-four hours following treatment with RA, hsp28 steady-state protein had significantly increased. Similar to both monocytic differentiation of HL-60 cell [3] and differentiating B lymphocytes [5], increased hsp28 protein was transient, returning to baseline levels by 48 and 72 h (Fig. 1a). As a control, purified hsp28 was also immunoblotted (Fig. 1a, lane Ctl).

Historically, changes in cell morphology have been used to follow the progression of myelomonocytic differentiationC. Granulocytic differentiation of HL-60 cells is marked by a decrease in the nuclear:cytoplasmic ratio, condensation of nucleoplasm, and loss of multiple nucleoli. In response to RA, HL-60 cells differentiate to a more mature metamyelocyte or band form. In Fig. 1b, HL-60 cells prior to and at 24 and 72 h following treat-

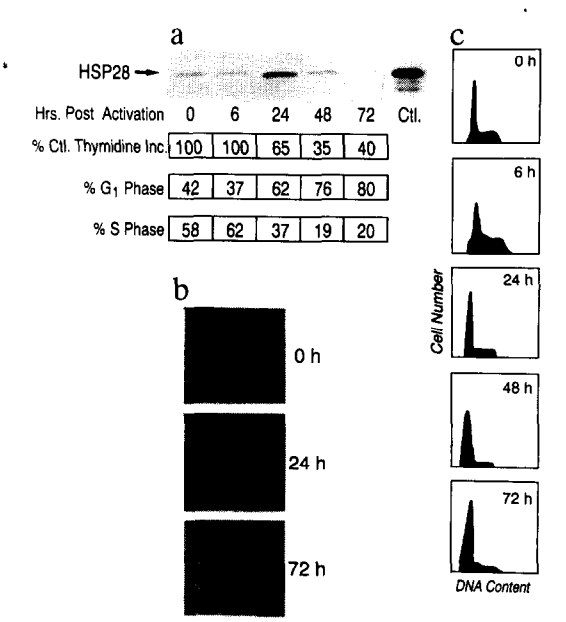

Fig. 1. Transient increase in hsp28 steady-state protein expression accompanies growth arrest of HL-60 cells treated with RA. (a) Hsp28 steady-state protein expression was examined by immunoblot analysis at various time points prior to and following treatment with RA (1 μM). At each time point, equal amounts of total cellular protein (50 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose and immunoblotted using a polyclonal anti-hsp28 primary antibody. Protein signal was detected using a secondary alkaline phosphatase-conjugated goat anti-rabbit antibody. As a control, purified hsp28 was immunoblotted (lane Ctl). The corresponding level of cellular proliferation (% of control thymidine incorporation) is shown below each time point. Cells were incubated with 1 μCi [^{3}H]thymidine for 4 h. (b) Wright–Giemsa stain of HL-60 cytosmear at 0, 24 and 72 h following treatment with RA. (c) Cell cycle analysis at various time points prior to and following treatment with RA. Corresponding peaks for G1 and S phase are indicated.

ment with RA were examined by Wright-Giemsa stain. In our hands, less than 1% of untreated HL-60 cells exhibited morphologic features characteristic of mature metamyelocytes or bands. Although there was gradation of differentiative changes at each time point, significant maturation was not observed until 48 to 72 h, at which time at least 75% of cells exhibited characteristic features of metamyelocytes or bands (condensed/intended nucleus, and loss of multiple nucleoli) (Fig. 1b). In addition at these morphologic changes, the expression of granulocytic differentiative cell surface markers (i.e. CD11b) did not significantly increase until approximately 72 h after RA (data not shown). Growth inhibition and cell cycle growth arrest preceded morphologic differentiation. As shown, [^{3}H]thymidine incorporation decreased 35% twenty-four hours after exposure to RA, with continued growth-inhibition at 48 and 72 h (70% inhibition) (Fig. 1a). Decreased [^{3}H]thymidine incorporation was associated with the onset of G1 cell cycle arrest. As shown in Fig. 1c, the decrease in the percentage of cells in S phase during the first 24 h following treatment with RA was associated with a concomitant increase in the percentage of cells blocked in G1. Therefore, peak hsp28 protein expression occurred concomitant with decreased [^{3}H]thymidine incorporation and the onset of G1 cell cycle arrest.

3.2. *Stimulation of hsp28 phosphorylation in response to RA*

The small hsps are rapidly phosphorylated in response to heat shock and display an accumulation of phosphorylated isoforms as seen in 2D immunoblots analysis ([16,20] and Fig. 2). The most basic isoform ('a') represents the unphosphorylated form of the protein while the more acidic isoforms ('b' and 'c') are phosphorylated. Therefore, one can assess hsp28 phosphorylation state through the relative expression of its isoforms. To this end, 2D immunoblotting was performed on HL-60 cell lysates prepared at various time points prior to and following treatment with RA (Fig. 2A). In control (untreated) cells, isoform 'a' predominated. However, within 1 h following treatment with RA, isoform 'b' increased significantly and isoform 'c' was now detectable. Increased expression of the phosphorylated isoforms was transient, with the steady state levels of the 'b' isoform returning to baseline by 17 h. This isoform was no longer detectable by 45 h. 2D gel analysis of immunoprecipitated hsp28 isoforms from cells labeled for 1 h with [^{32}P]orthophosphate was also performed prior to and after RA addition. As seen in Fig. 2B an increased phosphorylation of the 'b' and 'c' isoforms is observed during the first hour of RA treatment. 17 h after RA addition, the pattern of hsp28 phosphorylation resembled that observed in untreated cells. By 45 h the protein was dephosphorylated (not shown).

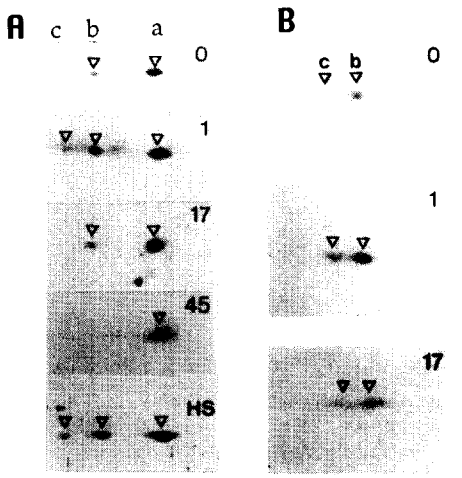

Fig. 2. Retinoic acid induced granulocytic differentiation modulates hsp28 phosphorylation. (A) Analysis of hsp28 isoforms by 2D immunoblotting. The profile of hsp28 isoforms at various time points prior to (0) and at 1 h, 17 h and 45 h (indicated 1, 17 and 45 in the figure) following treatment with RA (1 μM) was determined by 2D immunoblot analysis. At each time point, cells were harvested, lysed in Laemmli sample buffer and then analyzed in 2D immunoblots probed with anti hsp28 antibody as described in Section 2. The blots were developed and visualized using the ECL detection method. The pattern of hsp28 isoforms following exposure to a heat shock treatment for 1 h at 43°C is indicated (HS). (B) 2D gel analysis of immunoprecipitated hsp28 isoforms from cells labeled for one hour with [^{32}P]orthophosphate either prior to (0) and during the first (1) and the seventieth (17) hour following RA treatment. Acidic end is to the left. Only the portion of the autoradiographs showing hsp28 isoforms is presented. The unphosphorylated hsp28 isoform is indicated (a) as well the phospho-isoforms (b and c). Note the transient increased phosphorylation of hsp28 following RA treatment which resembles that observed after heat shock treatment.

3.3. *Increased hsp28 steady-state protein in response to RA is post-transcriptionally mediated*

Total cellular RNA was isolated from HL-60 cells at various time points prior to and following treatment with RA. Equal amounts of RNA were separated by Northern blotting and hybridized with a ^{32}P-labeled hsp28 cDNA probe. In contrast to hsp28 protein, corresponding hsp28 steady-state mRNA levels rapidly decreased in response to RA (Fig. 3, top panel). By 24 h, hsp28 steady-state message was barely detectable. Equal loading and intactness of RNA was verified by re-hybridizing with a ^{32}P-labeled β-actin probe (Fig. 3, lower panel).

4. Discussion

The rationale for using retinoic acid as a treatment for promyelocytic leukemia stems from its ability to induce these developmentally arrested leukemic cells to undergo terminal differentiation [20–22]. Very little is currently known about the molecules involved in mediating the growth and differentiative effects of RA. Considering the increasing role that small hsps play in growth regulation,

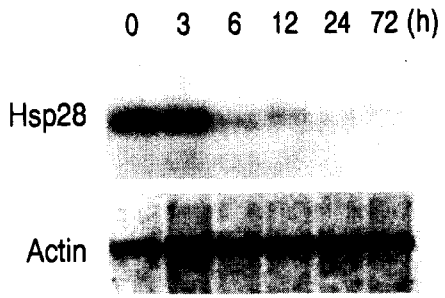

Fig. 3. Down-regulation of hsp28 steady-state mRNA in response to RA. Total cellular RNA was extracted from HL-60 cells at various time points prior to (0 h) and following treatment with RA (3, 6, 12, 24, and 72 h). Northern blot analysis was performed using a ^{32}P-labeled cDNA probe to the human hsp28 gene. To verify that equal amounts of RNA (5 μg) were loaded at each time point, the blot was rehybridized with a β-actin cDNA probe.

we sought to determine how RA affected hsp28 protein expression during granulocytic differentiation of HL-60 cells. In the present study, we demonstrate that hsp28 protein is transiently upregulated by RA, concomitant with the onset of G1 cell cycle arrest. In contrast to other differentiating agents, hsp28 protein and mRNA are discordantly regulated by RA. This suggests that RA-induced changes in hsp28 protein are regulated at the post-transcriptional level. In addition to quantitative changes in hsp28 protein expression, there are also rapid changes in hsp28 phosphorylation state. These observations implicate this hsp as an intermediary in the retinoic acid differentiative pathway.

Phosphorylation of hsp28 is an important modification in response to a number of different stimuli. For example, increased hsp28 phosphorylation is one of the most rapid and pronounced of the cellular responses to heat shock. In addition, non-heat shock stimuli such as tumor necrosis factor, interleukin-1, and phorbol ester also increase hsp28 phosphorylation [23–27]. Interestingly, most of these have significant effects on cell growth suggesting that changes in phosphorylation state are likely to be important in understanding the role of hsp28 in growth-regulation. We now demonstrate that RA also modulates hs28 phosphorylation. A transient increased phosphorylation was observed, followed by a dephosphorylation of the protein 45 h after RA addition. This is particularly intriguing in light of the recent observation that small hsps are protein chaperones [28]. As such, chaperone/hsp physically associate with and often modify the activity of other proteins. Formation of these complexes can have profound effects on cell growth when the associated protein is itself a growth-regulatory molecule [29]. For example, in adenovirus-transformed baby rat kidney (BRK) cells the presence of an hsp27–22 kDa protein complex correlates inversely with the oncogenicity of transformed BRK cells [30]. Since changes in the phosphorylation state of the prokaryotic hsp GroEL regulate its association with other proteins [31], it is likely that phosphorylation may also affect the interaction of hsp28 with associated proteins, such as growth-regulatory molecules.

In addition to being a molecular chaperone, hsp28 has also been shown to inhibit actin polymerization by acting as an actin capping protein [32]. Although it is now known how the interaction between hsp28 and actin is regulated, it is interesting to speculate as to how changes in hsp28 phosphorylation state might affect its interaction with actin. Considering actin polymerization is essential for cell growth, inhibition of this process by hsp28 would have profound effects on growth regulation.

Finally, the discordant regulation of hsp28 protein and mRNA in response to RA is particularly intriguing in light of the involvement of serine proteases in RA-induced differentiation of leukemic cells. Proteinase 3 (PR3) or myeloblastin is a serine protease whose down-regulation by RA is directly involved in initiating growth arrest and terminal differentiation of promyelocytic leukemic cells [33]. Virtually nothing is known about the substrates of PR3, which are likely to be effector molecules in the RA-differentiative pathway. We are currently investigating whether hsp28 is a substrate for PR3 which would suggest that increased hsp28 protein in response to RA might be mediated through changes in protein stabilization.

Recently, the role of the mammalian small hsp in growth regulation was directly demonstrated in a murine tumor model where its overexpression led to reduced growth [11]. The consistency with which hsp28 is linked to growth arrest during differentiation of hematopoietic cells [4,5] suggests that it is likely to be functionally relevant. However, further studies will be required to demonstrate a growth-regulatory role for hsp28 during this process. The identification of molecules such as hsp28 provides novel targets for the development of therapeutic strategies designed to overcome the maturational arrest characteristic of many human malignancies.

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