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ABSTRACT

A heat shock of 42.5-43.5°C for 1 h applied to HL-60 promyelocytic leukemia cells induced the appearance of between 13 and 34% (n = 6) of cells which showed characteristics of mature metamyelocytes/granulocytes. This is the first time a physical agent has been shown to induce the differentiation of this leukemic cell line. The treatment of HL-60 cells with a variety of agents which have been documented to stress cells and induce a heat shock-like response also induced granulocyte-like differentiation: continuous treatment for 4 days with ethanol (213 μM), sodium arsenite (6 μM), cadmium sulfate (60 μM), lidocaine (3 mM), and procaine (5 mM) induced 73, 54, 14, 54, and 55% of cells, respectively, to reduce the dye nitro blue tetrazolium. They were also capable of the phagocytosis of yeast particles. Examination of differentiated cells showed that those treated with ethanol, arsenite, lidocaine, and procaine also expressed nonspecific esterase activity, typical of monocytes, but did not adhere to plastic and had a cellular and nuclear morphology consistent with differentiation to metamyelocytes. Analysis of protein synthesis of 100 cells treated with 170 mM N-methylformamide, by the pulse labeling of cells for 2 h with 14C]leucine at various times, showed that the constitutive synthesis of both the M, 90,000 and 70,000 heat shock proteins fell substantially after 2 h of exposure to N-methylformamide. When HL-60 cells were incubated with 1 M N-methylformamide, a toxic concentration of this agent, or were heat shocked, the synthesis of both the M, 70,000 and M, 90,000 proteins was induced. We propose that changes in heat shock protein synthesis may be an important element of the induction of differentiation of HL-60 cells, particularly as these proteins have recently been shown to regulate the stability of oncogene proteins, such as myc (Lässcher, B., and Eisenman, R. N., Mol. Cell Biol., 8: 2504-2512, 1988).

INTRODUCTION

A wide variety of drugs and toxins are capable of the promotion of the terminal differentiation of HL-60 human promyelocytic leukemia cells to granulocyte-like cells. These include the polar solvents (2, 3), anthracyclines (4-6), antimetabolites (7-9), an inhibitor of glycoprotein synthesis (10), retinooids (11-12), and benzodiazepines (13). These disparate agents are optimally active at concentrations which are only marginally below those which are cytotoxic, and in the case of the solvents, and related compounds, a quantitative relationship was defined by us which related the concentrations required for toxicity to those required for the induction of differentiation (3). We proposed that the treatment of cells with agents at subtoxic concentrations may serve to “stress” the cells and induce some kind of adaptive or “stress” response (3). It was considered possible that such a response may be involved in those events which result in changes of gene expression leading to terminal cell differentiation, including changes in the expression of certain oncogenes, the activity of which may be responsible for the failure of these cells to mature and for their malignancy.

Recently, there have been suggestions that a family of proteins, which are evolutionarily highly conserved, the hsps, may play an important role in the control of cellular differentiation (14-21). The hsps belong to a class of proteins which are induced in all eukaryotic cells in response to an imposed stress (reviewed in Reference 14), such as a shift to a higher temperature (“heat shock”) or other agents, for example ethanol and other polar solvents (22-24). It is considered that the modification of proteins may be an important trigger in the initiation of the heat shock response (25-27). In addition, certain of the hsps have been shown to modify the degradation of short-lived oncogenes such as p53 (28, 29) and myc (30) and may thus play a role in malignancy.

In order to test the hypothesis that the imposition of a relatively nonspecific stress may bring about HL-60 cell differentiation, we have investigated the effects of a heat shock, and of agents which have been reported to induce the synthesis of hsps, on the growth and differentiated phenotype of HL-60 cells. We also present data on hsp synthesis during the period that HL-60 cells are committed to differentiation by the agent N-methylformamide.

MATERIALS AND METHODS

Cell Culture. HL-60 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (GIBCO, Glasgow) at 37°C, in an atmosphere of 10% CO2 in air. They were maintained in logarithmic phase of cell growth by subculture of between 5 x 104 and 1 x 106 cells/ml at 3-day intervals. A doubling time of between 20 and 24 h was recorded. Initiation of cell growth at 1 x 104 cells/ml allowed a plateau phase of growth to be reached when the cell density was between 1 and 2 x 106 cells/ml. Cell numbers were estimated by the use of a Coulter counter (model ZB) and short-term viability was determined by the exclusion of 0.1% solution of trypan blue.

Induction of Differentiation. (a) By heat shock: 1 x 106 HL-60 cells/ml were exposed to an immediate heat shock (i.e., no preheating) of between 39 and 45°C for various times in a thermostatted water bath, the temperature of which was regulated by a Mercia Scientific (Coventry, UK) thermostat to within ±0.5°C. Measurements of the rate of heating of the culture showed that with adequate agitation the media reached the required temperature within 2 min. At the end of the required period of heat shock, cells were allowed to stand at 21°C for 5 min, by which time they had cooled to ambient temperature, then incubated at 37°C for a further 96 h; cell viability, cell number, incorporation of radiolabeled thymidine and % differentiation (see below) were assessed immediately and at various times. (b) By other agents: 1 x 103 HL-60 cells/ml were incubated continuously for 96 h with various concentrations of the agents, and cell number, % viability, and % differentiation assessed as described below.

Assessment of Differentiation. HL-60 cells which were potentially capable of the production of superoxide anion were assessed by the reduction of the dye nitroblue tetrazolium, as described in detail by us previously (3). Cells which were functionally capable of phagocytosis were assessed by the method of Shaala et al. (31). Cytochemical demonstration of nonspecific esterase activity was performed according to

1The abbreviations used are: hsp(s), heat shock proteins; DMSO, dimethyl sulfoxide; NBT, nitro blue tetrazolium; NMF, N-methylformamide.
the method of Yam et al. (32). Microscopical assessment of the morphology of the cells was made after they had been stained with Wollbach-Giems stain (33). DNA synthesis was measured by incubation of $10^6$ cells with 0.5 μCi of [5-methyl-3H]thymidine (5 Ci/mm; Amersham, UK) for 0.5 h before placing them, in triplicate, on a 2.5-cm Whatman GF/C glass fiber filter, washing with 15 ml each of normal saline, 10% w/v trichloroacetic acid, and a further saline wash. The filters were washed with 15 ml of methanol then air dried, and the radioactivity of each filter was measured in a Packard 2000 CA Tricarb scintillation counter after the addition of Optiphase (Fisons, Loughborough, UK). Preliminary experiments had shown incorporation of the thymidine into acid-insoluble material in the cells was linear for at least 1 h.

Measurement of New Protein Synthesis. Cells were untreated or heat shocked for 1 h at 43.5°C and allowed to recover for 2 h at 37°C, or were treated with various concentrations of NMF or ethanol for different times. The cells were washed twice with leucine-free RPMI 1640, then $5 \times 10^5$ cells were incubated for 2 h at 37°C with 2 ml of leucine-free RPMI plus 10% dialyzed fetal calf serum and 2 μCi of L-[U-14C]-leucine (342 Ci/mmol). The samples were washed twice with leucine-free RPMI, then twice with reticulocyte standard buffer (0.01 M Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂) before resuspending in 1 ml of buffer. The cells were sonicated, then sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (34). Electrophoretic transfer of the proteins onto nitrocellulose (Biorad, Watford, UK) was then performed according to the method of Towbin (35). The dried blot was placed on a (CEA) Singul X-RP nonscreen medical X-ray film (Ceaverken AB, Strangas, Sweden), and exposed in the dark for 3 days before the film was developed. Scanning densitometry was performed using an LKB Ultrascan XL laser densitometer.

RESULTS

A heat shock for 1 h between 42.5 and 43.5°C resulted in the maintenance of a short-term viability of >85% of HL-60 cells as assessed by the exclusion of trypan blue at the end of the heat shock period. Experiments aimed at the assessment of whether heat-shocked cells would subsequently differentiate and divide were conducted at these temperatures. Fig. 1 shows the effect of a heat shock of 43.5°C applied for time periods of up to 80 min, on the viability, cell number, and percentage of cells capable of reducing the dye NBT (termed NBT+ cells), assessed 96 h after return of the cells to 37°C. It shows the averaged results from a series of six experiments; the highest percentage of NBT+ cells after a 1-h heat shock at this temperature was 34% and the lowest 13%. At time intervals before 1 h, less than 5% differentiation was observed, and at longer periods than 1 h cell viability began to decline, with no increase in the numbers of NBT+ cells (Fig. 1). Estimates of cell numbers after 96 h showed that after a 1-h heat shock those cells which had differentiated had gone through just over one cell division, from $1 \times 10^5$ cells to $3.1 \pm 1.0 \times 10^5$ cells/ml, whereas the control cells had completed over three divisions. Heat shock for up to 4 days at temperatures of below 42°C did not result in significant differentiation (data not shown). It was established that the cells did not express nonspecific esterase activity typical of monocytes, but were capable of the phagocytosis of complement-coated yeast particles (see below).

In a further series of heat shock experiments, the rate of development of the differentiated phenotype and changes in DNA synthesis were monitored over a 72-h period immediately following a 1-h heat shock at 42.5°C. Short term viability of the heat-shocked cells, measured by the exclusion of the vital dye trypan blue, remained at >80% over 72 h. Surprisingly, heat-shocked cells rapidly expressed the capability of the phagocytosis of yeast particles and were able to reduce the dye NBT (Fig. 2). We attempted to establish whether there was a significant fall in DNA synthesis, as measured by [3H]thymidine incorporation, as the cells recovered from heat shock and began to express markers representative of a more mature phenotype. No significant depression of thymidine incorporation was observed in the heated cells over a 72-h period following the heat shock. We consider that it is not possible to confirm, using thymidine incorporation, whether the approximately 25% of cells which expressed markers of a more mature phenotype were terminally differentiated because of the superimposition of the growth of the 75% of cells which remained uncommitted, and the possibility that the heat-shocked cells were capable of performing nonscheduled DNA synthesis after receiving the heat shock.
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Table 1 Effects of inducers of the heat-shock response on cell growth and differentiation of HL-60 cells measured after 96 h (±SD)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell number × 10^7 after 96 h</th>
<th>% Viable</th>
<th>% NBT+</th>
<th>% Phagocytosis</th>
<th>% NSE</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12 ± 0.3</td>
<td>96 ± 3</td>
<td>2 ± 1</td>
<td>1 ± 1</td>
<td>&lt;1</td>
<td>6</td>
</tr>
<tr>
<td>170 mM NMF</td>
<td>2.6 ± 0.3</td>
<td>86 ± 9</td>
<td>83 ± 10</td>
<td>76 ± 12</td>
<td>&lt;1</td>
<td>6</td>
</tr>
<tr>
<td>213 mM ethanol</td>
<td>2.3 ± 0.5</td>
<td>82 ± 13</td>
<td>73 ± 11</td>
<td>69 ± 11</td>
<td>22 ± 1</td>
<td>3</td>
</tr>
<tr>
<td>6 μM Na arsenite</td>
<td>2.6 ± 0.4</td>
<td>80 ± 6</td>
<td>54 ± 7</td>
<td>51 ± 10</td>
<td>37 ± 16</td>
<td>3</td>
</tr>
<tr>
<td>3 mM lidocaine HCl</td>
<td>2.8 ± 1.2</td>
<td>83 ± 8</td>
<td>54 ± 2</td>
<td>52 ± 3</td>
<td>45 ± 5</td>
<td>3</td>
</tr>
<tr>
<td>5 mM procaine HCl</td>
<td>2.5 ± 0.9</td>
<td>84 ± 7</td>
<td>55 ± 4</td>
<td>54 ± 3</td>
<td>49 ± 4</td>
<td>3</td>
</tr>
<tr>
<td>60 μM cadmium sulfate</td>
<td>3.5 ± 1.6</td>
<td>75 ± 16</td>
<td>14 ± 11</td>
<td>Not done</td>
<td>Not done</td>
<td>6</td>
</tr>
<tr>
<td>1 h at 43.5°C</td>
<td>3.1 ± 1.5</td>
<td>72 ± 13</td>
<td>20 ± 7</td>
<td>23 ± 2</td>
<td>&lt;1</td>
<td>6</td>
</tr>
</tbody>
</table>

* Initial cell number was 1 × 10^6 cells/ml.
* Short term viability as measured by dye exclusion, see “Materials and Methods.”

Table 1 shows the effects on growth and differentiation of HL-60 cells after treatment for 96 h with a variety of agents which have been documented to induce thermostolerance and/or the expression of hsps (22–24, 36–38). The optimal concentration for the induction of differentiation is as defined previously by us (3). That is, that concentration which reduces cell division and induces the expression of the maximum number of NBT+ cells, while viability was maintained at approximately 80%. Ethanol, procaine, lidocaine, and sodium arsenite are what have been defined as “strong” inducers (39), while cadmium appears to be only a “partial” inducer, and like a heat shock its effects were rather more variable than the other agents (Table 1). We attempted to assay the activity of a variety of lead salts but found all of them to precipitate in the medium, no doubt because of their reaction with sulfhydryl-containing components.

Table 1 also shows that cells which had been treated with some of these agents gave rise to a proportion of cells which expressed significant levels of nonspecific esterase activity more typical of the monocytic pathway of differentiation. The cultures however, did not display any cell attachment to the surface of the falcon flasks. In addition, Giemsa staining and examination of the nuclear morphology of the cells clearly showed reniform and occasionally banded nuclei, typical of metamyelocytes of the neutrophil-granulocyte lineage (40). The plasticity of the leukemic genome has been commented on by Greaves et al. (41); the data presented here support these views, and additionally suggest that it is useful to measure a number of markers of differentiation.

Fig. 3 shows the autoradiograph of the nitrocellulose blot of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the new proteins synthesized in an equal number of cells, after the cells had been pulsed with 2 h with [14C]leucine. Prior to labeling with leucine the cells were either untreated, heat shocked for 1 h at 43.5°C and allowed to recover at 37°C for 2 h, or treated with NMF or ethanol for various times. The blot is presented as the autoradiograph of this is slightly better resolved than that of the original gel. Clearly, those cells which had been subjected to the heat shock (Lane J) ceased the synthesis of many proteins. The most prominent new proteins synthesized in the heat-shocked cells were those of the major hsp families of M, 70,000, 90,000, and 110,000. Treatment of the cells with a toxic concentration (3) of NMF (1 mM) (Lane H) or ethanol (1.28 mM) (Lane I) for 1 h also induced the synthesis of the major hsps; these agents had less effect on normal protein synthesis than a heat shock.

Fig. 3 (Lanes A to G) also shows the effects, with time, on protein synthesis after HL-60 cells had been incubated with a concentration of NMF which was optimal for the induction of differentiation (Table 1 and Reference 3). In the untreated cells (Lane A) there appears to be considerable constitutive synthesis of the M, 90,000 hsp (which appears to be a doublet), and some constitutive synthesis of the M, 70,000 hsp. Incubation with 170 mM NMF rapidly reduced the constitutive synthesis of these proteins and Fig. 4 shows the results of a densitometric scan of the gel comparing the absorbance of the M, 90,000 peak (both bands of the poorly resolved doublet) with that of the M, 45,000 peak, which corresponds to actin. The synthesis of actin had a densitometric average absorbance of 0.36 ± 0.035 units (n = 6).

DISCUSSION

We have tested the hypothesis that a number of disparate agents which have been reported to “stress” cells, possibly by damaging proteins, and which are documented to induce the expression of hsps, might promote the differentiation of HL-60 cells. The finding that a heat shock could induce HL-60 cells to express markers typical of granulocyte-like cells, with the capability of superoxide production under conditions of reduced cell proliferation, is a novel observation (Fig. 1) since it is the first physical agent recorded to promote the differentiation of these cells. Although a significant but rather low degree of differentiation was induced by heat shock, this was variable to a degree not normally seen when chemical agents are used. The reason for this variability is unclear at present. The experiments which measured the induction of hsps in these cells (Fig. 3, Lane J) show that a heat shock had a profound inhibitory effect upon normal protein synthesis while it induced the synthesis of the major hsps: it is possible therefore that the expression of a differentiated phenotype was inhibited by these conditions (cadmium had a similar effect on protein synthesis3). However, ethanol, which as discussed below elicits many of the stress responses induced by heat but apparently, unlike heat, does act as a general inhibitor of protein synthesis (42), was a consistently strong inducer of differentiation (Table 1), and treatment with even a toxic concentration of this agent (Fig. 3, Lane I) had a less profound inhibitory effect on general protein synthesis in HL-60 cells than did heat shock. Similarly, a toxic concentration of NMF (Lane H) had little effect on normal protein synthesis, while it induced the synthesis of the major heat shock proteins. We considered that the imposition of a stress by heat shock was too variable a procedure for detailed analysis of the accompanying cellular and biochemical events and turned to other agents, as discussed below.

A feature of note regarding the promotion of differentiation by a heat shock was that significant differentiation, up to 34% in one experiment, was induced after only a 1-h exposure to this physical agent. Experiments to measure the minimum time of exposure to the polar solvent NMF, which was required to induce significant levels of differentiation, suggest that a minimum of 24 h was necessary,3 while not less than 8 h of

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Fig. 3. Autoradiograph of the nitrocellulose blot of an SDS-PAGE to show protein synthesis in: A, nontreated HL-60 cells; in cells incubated continuously with 170 mM NMF for: B, 2 h; C, 6 h; D, 20 h; E, 30 h; F, 44 h; G, 54 h; or for 1 h, followed by 2-h recovery, with H, 1 M NMF; I, 1.3 M ethanol; J, a 1-h heat shock for 43.5°C.

Fig. 4. Densitometry of the autoradiograph shown in Fig. 3, in which the absorbance of the peak corresponding to the M, 90,000 protein was compared with that of the peak at M, 45,000, corresponding to actin, over the 56-h time period of incubation of HL-60 cells with 170 mM NMF.

incubation with DMSO were required before HL-60 cells were committed to differentiate (43). Heat shock is therefore an extremely effective inducer if defined in terms of time of exposure needed to commit cells to differentiate. An analysis of the rate of appearance of functions typical of cells expressing a more mature phenotype showed that the heat shock rapidly induced changes in gene expression (Fig. 2). We suggest that the variability of the period required for each agent to commit the cells to a program of differentiation may be a reflection of the rate of accumulation of sublethal damage imposed by each substance which then triggers a cellular response which leads to phenotypic change.

The finding that a heat shock induced the differentiation of HL-60 cells led to the investigation of the activity of a variety of agents which had been reported to induce the expression of hsps and/or thermotolerance (22–24, 36–38). We have reported previously that ethanol was an inducer of HL-60 cell differentiation (3) (Table 1) and like other polar solvents, such as dimethylformamide and DMSO, it has been reported to induce the expression of hsps and the thermotolerance of some cells (23, 24, 36). Each of the structurally and pharmacologically disparate agents which were studied induced HL-60 differentiation at concentrations which were marginally below those which were cytotoxic (Table 1). These results lent further support to the hypothesis that the induction of some type of stress response might be involved in the commitment of HL-60 cells to differentiation.

The imposition of a heat shock of 43.5°C induced the expression of the major hsps in these cells (Fig. 3), as did incubation of the cells for 1 h with toxic concentrations of NMF or ethanol (1 M) (Lanes H and I). When we investigated the time course of protein synthesis in HL-60 cells treated under conditions for
the optimal induction of differentiation by 170 mM NMF (3) we were surprised to observe that the constitutive synthesis of the hsps was rapidly and selectively depressed, particularly the synthesis of the Mr 90,000 protein (Fig. 3). As this manuscript was being revised, similar findings were reported for Mr 70,000 hsp synthesis in Friend erythroleukemia cells which were made to differentiate by incubation with dimethylosulfoxide (44). hsp synthesis has been shown to decrease in quiescent cells (45), and it might be considered that in the Friend cell, where DMSO brings about a transient inhibition of DNA synthesis (46), that the changes in the synthesis of hsps which were observed might have simply reflected changes in the replicative status of the cells. However, treatment of HL-60 cells with 170 mM NMF does not inhibit their replication until after 24 h of continuous treatment (47) so that we do not consider that the early (between 2 and 6 h) falls in hsp synthesis simply represent an adverse effect of NMF on cell kinetics. Moreover, preliminary evidence suggests that the constitutive synthesis of the heat shock proteins is reestablished as time progresses, albeit at lower levels. The mechanism whereby low concentrations of NMF (170 mM) reduced constitutive hsp synthesis, whereas a high concentration (1 m) induced synthesis is, extremely puzzling and is currently under investigation by us. Another puzzling feature of our results is the observation that a heat shock induced significant, albeit low and variable, amounts of differentiation (Figs. 1 and 2), but, unlike an effective concentration of NMF, the heat shock induced hsp synthesis (Fig. 3). One possible explanation of this paradox may be that while heat is a strong inducer of certain hsps (hsp 70) there are others which appear to be regulated differently, for example those called hsp 70 and hsc 70 (40), or it is possible that there were temporal changes in heat shock protein synthesis during the period of heat shock, perhaps involving an initial reduction of synthesis. Our present experiments aim to resolve the questions of precisely which of the proteins of this family of proteins is being regulated under different conditions and their temporal control. Preliminary results (1) suggest that there is also temporal regulation of cellular amounts of these proteins, in addition to the rate of their synthesis, as the cells are induced to differentiate.

Recent reports have implicated the hsps in interactions with the products of certain oncogenes, acting to stabilize otherwise transient proteins such as myc and p53, so that the hsps may perhaps play a role in maintaining the malignant phenotype (28–30). The high constitutive synthesis of the hsps in HL-60 cells, particularly hsp 90 (see Fig. 3) may play an important role in maintaining their malignant phenotype. Our current hypothesis is that agents such as NMF may mildly denature other cellular proteins, under conditions where hsp synthesis is not stimulated, so that hsps associated with the stabilization of the products of genes responsible for the malignant phenotype are "competed off" by these modified proteins, allowing proteolysis of the oncogene protein. It is possible that this may then permit the cells to complete their programs of development. We will report on attempts to test this hypothesis in the future.

Finally, the regulation of hsp synthesis has been suggested to occur as a response to the modification of proteins, the presence of nascent unfolded proteins or the accumulation of abnormal amounts of normal cellular proteins (25–27). Inspection of the literature of the promotion of HL-60 cell differentiation suggests that all of the effective agents (see Introduction) could be argued to interact with or modify proteinaceous targets, with the possible effect of changing their normal conformation during the prolonged periods of incubation required to commit cells to terminal differentiation. Direct DNA-damaging agents, such as the alkylating agents, have not been reported to be effective as inducers of HL-60 cell differentiation. In our hands nitrogen mustard is toxic to HL-60 cells but is not an inducer of differentiation. This may give clues as to the mechanism whereby so many disparate agents are able to induce the maturation of HL-60 cells.

REFERENCES

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