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Prostaglandins with antiproliferative activity induce the synthesis of a heat shock protein in human cells

(prostaglandin A/growth regulation/protein synthesis/heat shock)

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ABSTRACT **Prostaglandins (PGs) A_1 and J_2 were found to potently suppress the proliferation of human K562 erythroleukemia cells and to induce the synthesis of a 74-kDa protein (p74) that was identified as a heat shock protein related to the major 70-kDa heat shock protein group. p74 synthesis was stimulated at doses of PGA_1 and PGJ_2 that inhibited cell replication, and its accumulation ceased upon removal of the PG-induced proliferation block. PGs that did not affect K562 cell replication did not induce p74 synthesis. p74 was found to be localized mainly in the cytoplasm of PG-treated cells, but moderate amounts were found also in dense areas of the nucleus after PGJ_2 treatment. p74 synthesis was not necessarily associated with cytotoxicity or with inhibition of cell protein synthesis. The results described support the hypothesis that synthesis of the 70-kDa heat shock proteins is associated with changes in cell proliferation. The observation that PGs can induce the synthesis of heat shock proteins expands our understanding of the mechanism of action of these compounds whose regulatory role is well known in many physiological phenomena, including the control of fever production.**

Heat shock proteins (HSPs) are a set of polypeptides synthesized by prokaryotic and eukaryotic cells in response to a heat shock or to other environmental stresses (1). HSPs are encoded by a subset of cellular genes known collectively as stress genes but their function is still unknown. The structure of the major HSP (the 70-kDa HSP, HSP70) has been widely conserved through evolution from bacteria to man (2), indicating an important role in the survival of the organism. It has been suggested that proteins closely related to the HSP70 (HSP70-like proteins) that are present in unstressed human cells and whose synthesis is tightly regulated during the cell cycle (3) are involved in the control of cell proliferation (3–6).

Prostaglandins (PGs) are synthesized almost universally in eukaryotic cells in response to external stimuli and function as microenvironmental hormones, playing a regulatory role in several physiological responses such as the control of cell proliferation and differentiation (7).

Several PGs have been shown to inhibit the rate of cell proliferation in animal and human tumor systems *in vitro* and *in vivo* (7–9). Types A and J PGs, characterized by the presence of an α,β-unsaturated carbonyl group in the cyclopentane ring, are the most active in controlling cell proliferation, type J PGs being more cytotoxic (9). They have been shown to enter the cells, to be transported to the nuclei (9), and to act independently of cAMP (10) in specific stages of the cell cycle (11). Even though an increasing amount of literature has now described the antiproliferative activity of these compounds in a large number of experimental models, the mechanism by which selected PGs can control cell proliferation is still mainly unknown.

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We have reported (12) that type A PGs totally suppress the proliferation of the human erythroleukemic cell line K562 at doses that do not affect cell viability. This action is reversible depending on the length of treatment and is accompanied by partial (≈30%) inhibition of protein synthesis and glycosylation and by the synthesis of a 74-kDa polypeptide (p74). In the present report we identify p74 as a HSP related to the major 70-kDa group of HSPs and show how its synthesis is associated to growth inhibition.

MATERIALS AND METHODS

Cell Culture and Fractionation. K562 cells were maintained in RPMI 1640 medium, supplemented with 10% (vol/vol) fetal calf serum, 2 mM glutamine, and antibiotics, at 37°C in a 5% CO_2/95% air atmosphere. Cell numbers were counted in a hemocytometer, and cell viability was determined by vital dye exclusion technique (0.1% trypan blue).

Statistical analyses were performed using a nonparametric test for unpaired data. Data are expressed as mean ± SD, and P values of <0.05 were considered significant.

PGs, stored as 100% ethanolic stock solutions (2 mg/ml), were used at the concentration of 4 μg/ml unless otherwise specified. Control medium contained the same concentration of ethanol (0.02%), which did not affect cell viability or metabolism.

Cell fractionation has been described (13) and is a modification of the method of Gazitt and Friend (14).

Protein Synthesis and PAGE Analysis. Cells were labeled with [^{35}S]methionine (10 μCi per 10^6 cells; 1 Ci = 37 GBq) in RPMI medium containing 10% dialyzed fetal calf serum. The radioactivity incorporated was determined as described (12). After cell lysis (12), [^{35}S]methionine-labeled proteins were separated by $NaDodSO_4$/PAGE in a vertical slab gel apparatus (3% stacking gels and 7.5% resolving gels unless otherwise specified), using the buffer system described by Laemmli (15), and processed for autoradiography as described (12). Molecular masses of polypeptides were calculated using the following ^{14}C-labeled methylated protein mixture: myosin (200 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14 kDa). Densitometric analysis of autoradiographed patterns was performed using a laser-beam densitometer (Ultroscan XL; LKB).

Immunoblot Analysis. Equal amounts of proteins for each sample were separated by $NaDodSO_4$/PAGE and blotted to nitrocellulose, as described by Burnette (16). After transfer, the filters were incubated with a monoclonal anti-72/73-kDa HSP antibody (diluted 1:500) from HeLa cells (Amersham)

Abbreviations: PG, prostaglandin; PGA_1, PGB_2, etc., prostaglandins A_1, B_2, etc.; HSP, heat shock protein; HSP70, the 70-kDa HSP.

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shown to be specific only for the 72-kDa HSP, in TEN/Tween 20 buffer (0.05 M Tris·HCl, pH 7.4/5 mM EDTA/0.15 M NaCl/0.05% Tween 20), and the bound antibody was detected by horseradish peroxidase-linked sheep anti-mouse antibody (Amersham). Molecular masses were calculated using Pharmacia "low MW" markers.

Indirect Immunofluorescence. To determine p74 intracellular localization, after cytocentrifugation (500 rpm in a Cytospin-2 rotor for 10 min) K562 cells were fixed, made permeable by exposure to methanol at −20°C for 2 min, and, after treating with normal sheep serum for 20 min at room temperature to block nonspecific binding, stained with the monoclonal anti-72/73-kDa HSP antibody (diluted 1:500) followed by fluorescein-conjugated sheep anti-mouse antibody (Amersham).

RESULTS

PGs That Inhibit Cell Proliferation Induce the Synthesis of a 74-kDa Protein, p74. We have reported (12) that, when K562 cells were treated with identical concentrations of types A, B, E, and F PGs, only PGA_1 and PGA_2 inhibited cell proliferation. This effect appeared not to be due directly to suppression of DNA synthesis, which was not altered for at least 24 hr after treatment (12).

To investigate whether inhibition of cell replication was associated with alteration of protein metabolism, 2×10^5 K562 cells per ml treated with PGA_1, PGB_2, PGE_1, PGE_2, $PGF_{1\alpha}$, or PGJ_2 (5 μg/ml) or with ethanol diluent (at time 0 and after 48 hr) were labeled with [^{35}S]methionine soon after plating (PGJ_2 is 9-deoxy-Δ^9-PGD_2). After 24 hr, radioactivity of samples was determined and samples containing the same amount of radioactivity were analyzed by $NaDodSO_4$/PAGE. A 74-kDa protein (p74) accumulated in PGA_1-treated and more evidently in PGJ_2-treated cells, whose proliferation, as already reported for PGA_1 (12), was almost completely suppressed up to 4 days after plating (at 96 hr after plating: control = $1.86 \pm 0.04 \times 10^6$ cells per ml; $PGA_1 = 0.28 \pm 0.01 \times 10^6$ cells per ml; $PGJ_2 = 0.24 \pm 0.02 \times 10^6$ cells per ml; $P < 0.01$). PGs that did not inhibit cell proliferation, PGB_2 and PGE_1 (as well as PGE_2 and $PGF_{1\alpha}$, data not shown), did not produce any significant change in protein metabolism at this dose and did not induce p74 synthesis (Fig. 1).

To investigate whether the synthesis of the p74 was associated with the nonproliferative stage, PGA_1-treated K562 cells were released from the PGA_1-induced proliferation block and protein synthesis was analyzed after 4 days, when cells had regained their full growth potential. Fig. 2 shows that p74 no longer accumulated in logarithmically growing K562 cells.

Identification and Intracellular Localization of p74. p74, whose synthesis is induced by PGA_1 and PGJ_2, is not glycosylated and has a pI value of ≈5.5. The synthesis of p74, which starts 1–2 hr after PG treatment, is inhibited by actinomycin D (unpublished data). Its molecular mass and

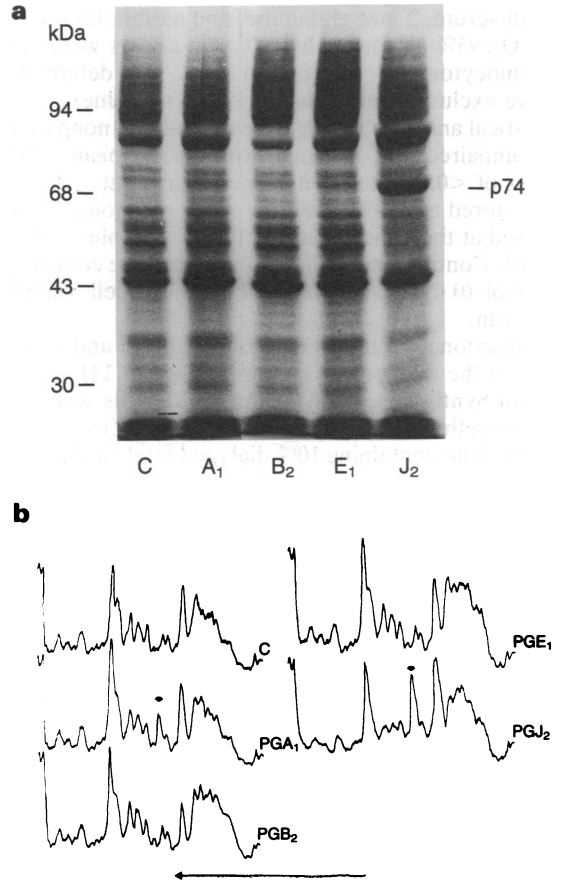

FIG. 1. Effect of various PGs on protein synthesis in K562 cells. $NaDodSO_4$/PAGE analysis (*a*) and densitometric analysis (*b*) of autoradiographic patterns of [^{35}S]methionine-labeled polypeptides synthesized in K562 cells after a 24-hr treatment with PGA_1, PGB_2, PGE_1, PGJ_2, or diluent control solution (C) (lanes A_1, B_2, E_1, J_2, or C, respectively). (*b*) Arrows over scans indicate the position of the 74-kDa protein induced in PGA_1- and PGJ_2-treated cells. Direction of migration is indicated by the arrow at the bottom of the figure.

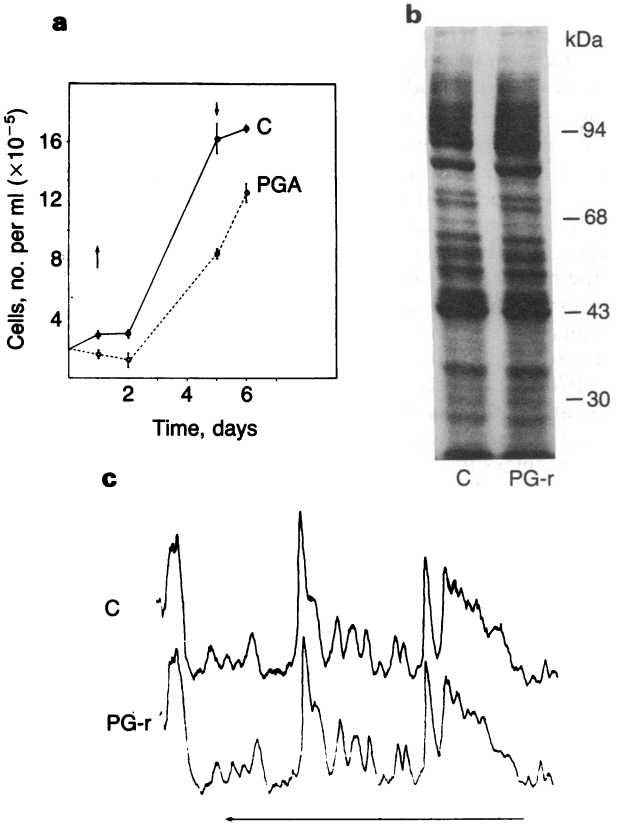

FIG. 2. Analysis of K562 cell proteins after reversal from PGA_1-induced growth inhibition. (*a*) K562 cells treated with PGA_1 for 24 hr were washed three times and replated with fresh medium devoid of PGA_1. (*b* and *c*) After 4 days, PGA_1-pretreated (lane PG-r) and control (lane C) cells were labeled with [^{35}S]methionine (10 μCi per 10^6 cells for 24 hr) and samples were analyzed by $NaDodSO_4$/PAGE (*b*) and densitometry (*c*). p74 synthesis could not be detected at this stage in PGA_1-pretreated cells.

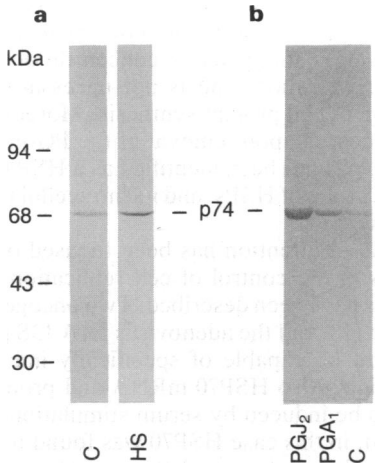

FIG. 3. Identification of p74 as a HSP by immunoblot analysis. (*a*) Proteins from control (37°C) (lane C) or heat shocked (45°C for 10 min) (lane HS) K562 cells were separated by NaDodSO$_4$/PAGE. Immunoblot analysis using anti-72/73-kDa HSP antibodies revealed a 74-kDa polypeptide that was severalfold increased in heat-shocked cells as compared to control cells. (*b*) K562 cells were treated for 24 hr with PGJ_2, PGA_1, or ethanol diluent (lane C) and an increase in HSP synthesis was induced (p74) comparable, or for PGJ_2 superior, to that obtained after heat shock.

other biochemical characteristics were similar to the major mammalian 72/74-kDa HSP (17).

To investigate whether the PGA_1-induced p74 could be identified as a HSP, cell extracts of control, PGA_1-treated, PGJ_2-treated, and heat-shocked K562 cells were separated by NaDodSO$_4$/PAGE and processed for immunoblot analysis, using monoclonal antibodies against the human 72/73-kDa HSP from HeLa cells. K562 cells (5×10^5 cells per ml) were either heat shocked (45°C for 10 min) or kept at 37°C (control). After 10 min control and heat-shocked cells were centrifuged, resuspended in RPMI 1640 medium at 37°C supplemented with 10% fetal calf serum, and kept at 37°C for 6 hr. Then equivalent numbers of control or heat-shocked K562 cells were lysed and equal amounts of proteins for each sample were separated by NaDodSO$_4$/PAGE and processed for immunoblot analysis. Fig. 3*a* shows the 74-kDa polypeptide, recognized by the HSP antibody, whose synthesis appears to be increased severalfold by heat shock as compared to control cells.

In a separate experiment K562 cells (2×10^5 cells per ml) were treated with PGA_1, PGJ_2, or control diluent. After 24 hr cells were counted and equal amounts of proteins for each sample were separated by NaDodSO$_4$/PAGE. Immunoblot analysis, using the monoclonal anti-HSP antibody, revealed the presence of a unique band in PG-treated cells, indicating the accumulation of a 74-kDa protein that was severalfold higher than in untreated uninduced cells (Fig. 3*b*). The increase in p74 synthesis after PG treatment was comparable, and for PGJ_2 superior, to that obtained after heat shock.

To investigate the intracellular localization of p74, K562 cells were plated in medium containing PGA_1, PGJ_2, or ethanol diluent and labeled with [^{35}S]methionine. After 24 hr cells were counted and lysed, and lysates were fractionated. Samples from total cell extracts or from various fractions, containing equal amount of radioactivity, were analyzed by NaDodSO$_4$/PAGE. Fig. 4 shows that in PGA_1-treated cells p74 accumulated in the soluble and the membrane fractions. Moreover, although no other alteration in the pattern of the proteins synthesized in the presence of PGA_1 was found in total cell extracts or in the soluble and nuclear fractions, at least four polypeptides (at 97, 76, 58, and 52 kDa; Fig. 4) appeared to be reduced or missing after PGA_1 treatment. Since at least two of these proteins (58 and 52 kDa) were present in the soluble fraction, it is possible that PGA_1 could interfere with the cell protein transport system. In PGJ_2-treated cells, p74 was found to accumulate in the soluble and membrane fractions but appeared to be present also in the nuclear fraction. In contrast to PGA_1, PGJ_2 treatment partially inhibited the synthesis of several proteins (specially nuclear proteins) in K562 cells.

The intracellular localization of p74 in PGA_1-treated cells was confirmed by immunoblot analysis. K562 cells treated for 24 hr with PGA_1 or ethanol diluent were lysed and fractionated; equal amounts of protein from each fraction were separated by NaDodSO$_4$/PAGE and blotted to nitrocellulose. Immunoblot analysis identified p74 in the soluble and the membrane fraction of PGA_1-treated cells (Fig. 5). By using this technique, a minimal amount of p74 was detected also in the nuclear fraction, and trace amounts of p74 were detected in the soluble fraction of untreated K562 cells, as reported (3) for HSP70 in unstressed human cells during the cell cycle. The intracellular localization of p74 was also determined by indirect immunofluorescence using the monoclonal anti-HSP antibody (Fig. 6). K562 cells grown at 37°C showed light, diffused cytoplasmic staining, as reported for asynchronous populations of HeLa cells (3). After exposure to PGA_1 and PGJ_2, there was an overall increase in the intensity of staining due to the induced synthesis of p74; moreover, this protein appeared to be localized generally in the cytoplasm and partially in small granules in the nucleus of PGA_1-treated cells and in the cytoplasm and dense areas of the nucleus of PGJ_2-treated cells.

Dose-Dependent Synthesis of p74. p74 synthesis increased dose-dependently in response to PGA_1 (Fig. 7). Logarithmically growing K562 cells (2×10^5 cells per ml) treated with PGA_1 for 48 hr, were counted and labeled with [^{35}S]methionine (10 μCi per 10^6 cells for 3 hr). In these conditions PGA_1

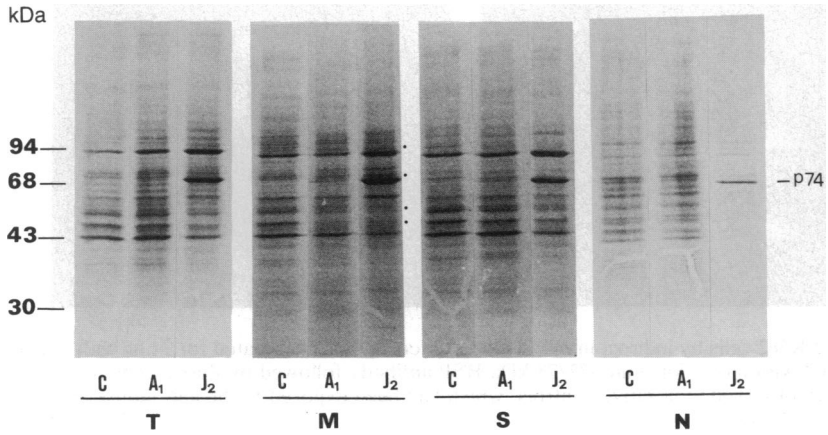

FIG. 4. NaDodSO$_4$/PAGE analysis of proteins from membrane, soluble, and nuclear fractions of PG-treated K562 cells. Cells treated with PGA_1 (lanes A_1), PGJ_2 (lanes J_2), or control diluent (lanes C) for 24 hr were labeled with [^{35}S]methionine, lysed, and fractionated. Proteins from total cell extracts (lanes T) or membrane (lanes M), soluble (lanes S), and nuclear (lanes N) fractions were separated by NaDodSO$_4$/PAGE using a 5–15% gradient gel. Dots indicate polypeptides that are present in reduced amount in the membrane fraction of PG-treated K562 cells.

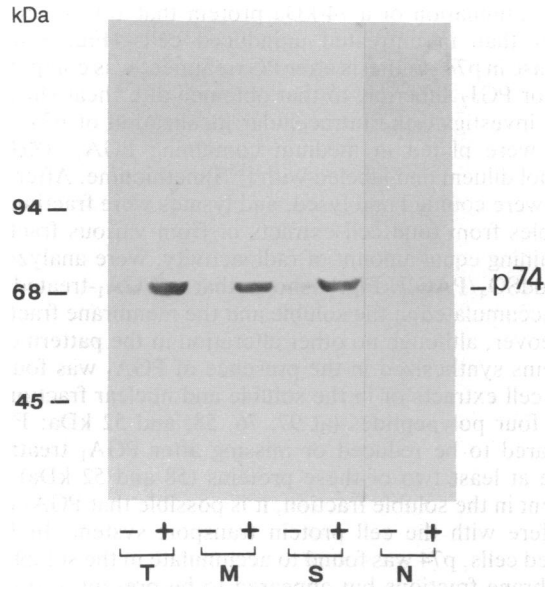

FIG. 5. Intracellular localization of p74 in PGA_1-treated K562 cells by immunoblot analysis. K562 cells were treated with PGA_1 (lanes +) or control diluent (lanes −) for 24 hr. After cell fractionation, equal amounts of proteins from total cell extract (lanes T), membrane (lanes M), soluble (lanes S), and nuclear (lanes N) fractions were processed for immunoblot analysis using the anti-72/73-kDa HSP antibody.

inhibited cell replication dose-dependently beginning at a concentration of 4 μg/ml. Concentrations of PGA_1 as high as 8 μg/ml could totally inhibit cell replication without altering cell viability (cell viability = 98%, equal to control). Concentrations >10 μg/ml were toxic (cell viability ranged from 90% to 95% in K562 cells treated with PGA_1 at 12 μg/ml). Protein synthesis was also inhibited by PGA_1 in a dose-dependent manner (Fig. 7*a*). However, inhibition of protein synthesis was not necessarily associated with inhibition of cell replication (a PGA_1 concentration of 6 μg/ml almost totally suppressed cell replication without significantly inhibiting protein synthesis). p74 synthesis, minimal in control cells, increased dose-dependently in response to PGA_1, up to a concentration of 6 μg/ml. Higher doses of PGA_1 apparently did not further increase p74 synthesis (Fig. 7*b*).

DISCUSSION

The present results show that two PGs with potent antiproliferative activity, PGA_1 and PGJ_2, induce the synthesis of p74 in K562 human erythroleukemia cells, but PGs that do not inhibit cell proliferation do not. Synthesis of p74 is dose-dependent, is stimulated at concentrations of PGs that inhibit cell proliferation, and is not necessarily associated with inhibition of cell protein synthesis. Moreover, accumulation of p74 ceases upon removal of the PG-induced proliferation block. p74 has been identified as a HSP related to the major 70-kDa group of HSPs, and its intracellular localization has been described.

A great deal of attention has been focused on the role of 70-kDa HSPs in the control of cell replication, but contradictory results have been described. Two oncogene products, c-myc protein (18) and the adenovirus E1A 13S gene product (19), appear to be capable of specifically inducing HSP70 gene expression. Also HSP70 mRNA and protein synthesis was shown to be induced by serum stimulation in quiescent HeLa cells (3); in this case HSP70 was found to be diffusely distributed in the nucleus and the cytoplasm during most phases of the cell cycle but to localize mainly in the nucleus during S phase. Recently, HSP70 mRNA and protein synthesis was found to be also specifically induced by interleukin 2 in interleukin 2-dependent human T cells, indicating a potential role for HSP70 in interleukin 2-induced cell cycle progression (20). In contrast, Iida and Yahara (4, 5) have shown that HSP70, as well as other high molecular weight (HMW) HSPs, is synthesized in elevated amounts specifically in quiescent (G_0) yeast cells, chicken fibroblasts, and mouse T lymphocytes and suggested that these proteins might be involved in a cellular machinery that directs cells toward G_0. Similar observations were reported by Kaczmarek *et al.* (6) in human lymphocytes, where HSP70 RNA levels were found to be elevated in quiescent peripheral blood mononuclear cells and to decline to low levels when these cells were stimulated to proliferate upon serum or mitogen induction (6). One of the most reasonable explanations suggested (6) for the discrepancies in these studies has been the possibility that, since the HSP70 gene actually consists of a gene family, the HSP70 gene active in G_0 lymphocytes may be different from the HSP70 gene whose expression in HeLa cells reaches its peak in the G_2 phase.

The present data suggest that in human erythroleukemic cells the PG-induced accumulation of p74 is associated to inhibition of cell proliferation. However, there is no evidence whether p74 synthesis has a direct role in the negative regulation of cell replication or is consequential to the proliferation block in this system. PGJ_2 appears to be a much more potent inducer of p74 synthesis than PGA_1. Moreover, after PGJ_2 treatment, p74 is found to localize into the cell nucleus in much larger amount than after PGA_1 treatment. Since PGA_1 and PGJ_2 have similar effects on the inhibition of

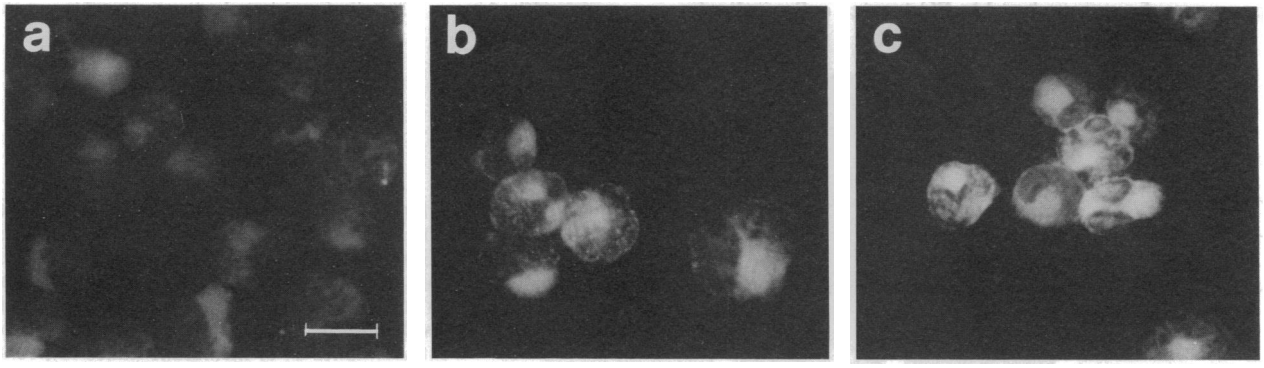

FIG. 6. Intracellular localization of p74 in PG-treated K562 cells by indirect immunofluorescence. K562 cells treated for 24 hr with diluent control (*a*), PGA_1 (*b*), or PGJ_2 (*c*) were fixed and stained with monoclonal anti-72/73-kDa HSP antibody followed by fluorescein-conjugated sheep anti-mouse antibody. No fluorescence staining was observed in negative controls, which had been exposed to the anti-mouse antibody in the absence of the anti-72/73-kDa HSP antibody. (Bar = 10 μm.)

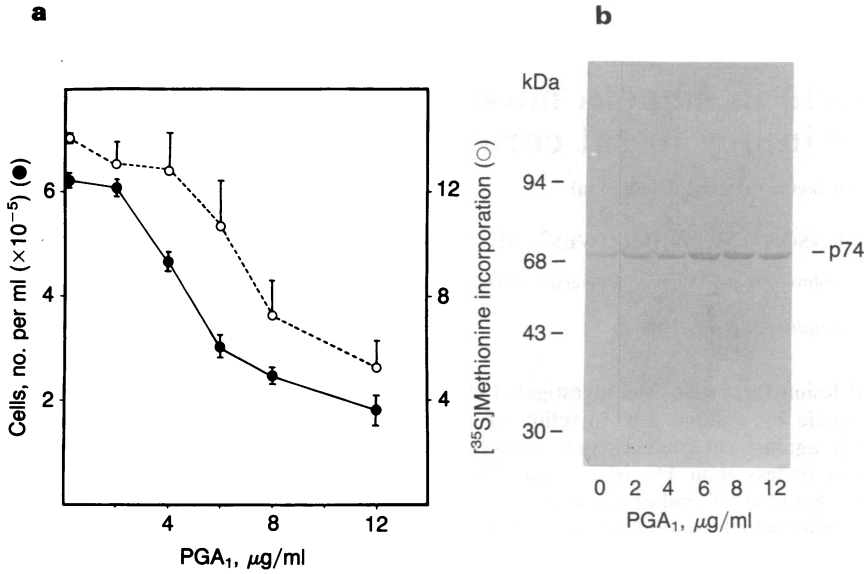

FIG. 7. Dose–response effect of PGA_1 on proliferation, protein synthesis, and p74 accumulation in K562 cells. Logarithmically growing K562 cells (2×10^5 cells per ml) were treated with PGA_1 and, after 48 hr, counted and labeled with [^{35}S]methionine for protein synthesis measurement reported as cpm per 10^6 cells ($\times 10^{-5}$) (*a*). p74 accumulation was measured at the same time (as indicated by lane labels) by immunoblot analysis using the monoclonal anti-72/73-kDa HSP antibodies (*b*).

K562 cell proliferation, these differences could be correlated to cytotoxic properties of PGJ_2 (9). The possibility cannot be excluded that p74 synthesis, even though it can be induced by PGs at concentrations that do not alter cell viability or protein synthesis, could represent the cell response to a stress situation after PG treatment, as HSPs can be transcriptionally and posttranscriptionally activated by a variety of physical and chemical treatments that include, apart from heat, glucose deprivation, sulfhydryl reagents, and heavy metals (21). A hypothetical function of HSP70 is the renaturation or protection of denatured polypeptides, supported by the evidence that overloading of degraded or "abnormal" proteins induces HSP synthesis (22, 23). We have in fact shown that PGs that induce p74 synthesis inhibit protein glycosylation (12) and the incorporation of some polypeptides in K562 cell membranes. Moreover, we have reported PGA_1-induced alterations in the synthesis and glycosylation of specific virus proteins in several virus–host systems, such as the hemagglutinin neuraminidase of Sendai virus and the G glycoprotein of vesicular stomatitis virus, resulting in potent antiviral activity (8, 24). p74 synthesis was induced by PGA_1 in these systems as well and also appeared to be associated with antiviral properties of PGs (unpublished results).

Finally, induction of p74 synthesis by PGs is not unique to K562 cells. We have shown that PGA_1 and PGJ_2 induce the synthesis of a 74-kDa protein with similar biochemical and immunological characteristics in two monkey cell lines, 37RC and MA104, and in a dog kidney cell line, MDCK (8, 24, 25), indicating that synthesis of HSP-like proteins could be a general response of mammalian cells to PGs.

In view of these results and the physiological role of PGs in hyperthermia (26), the study of the relationship between PGs (and their inhibitors) and HSP metabolism could provide insights into the understanding of thermoregulation and a possible link between antitumor PGs and cancer thermotherapy.

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