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Nuclear Accumulation of p53 in Normal Human Fibroblasts Is Induced by Various Cellular Stresses which Evoke the Heat Shock Response, Independently of the Cell Cycle

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Nuclear accumulation of p53 is induced by various DNA damaging agents (the p53 response). Induction of nuclear accumulation of p53 after various cellular stresses, mostly other than DNA damage, including heat shock, was examined in normal human fibroblasts by immunostaining and flow cytometry using a mouse anti-p53 monoclonal antibody. Immunostaining revealed nuclear accumulation of p53 within 6 h after various stresses [heat shock, osmotic shock, heavy metal (Cd), blockers of the cellular respiratory system (NaN_3), amino acid analogues (azetidine and canavanine), an inhibitor of protein synthesis (puromycin), and oxygen free radicals (H_2O_2)]. Heat shock proved to be one of the most effective inducers among these stresses. FACScan analysis revealed that this induction of p53 occurred regardless of the stage in the cell cycle and that accumulation of cells in G2/M occurred. As all of these stresses are known to induce the heat shock response, the mechanism of p53 induction after stresses and that of heat shock response may share, at least partly, some common signaling pathway(s).

Key words: p53 response — Heat shock response — Cell cycle — Cellular stress

The p53 gene is the most frequently mutated gene in human cancers.[1)] Wild-type p53 protein acts as a negative regulator of the cell cycle,[2,3)] and thus the p53 gene is thought to be a tumor-suppressor gene.[4)] Only a small amount of p53 is present in normal cells because of its very short half-life.[5)] p53 is induced and accumulates in the nucleus several hours after treatment of cells with various DNA-damaging agents such as ultraviolet light (UV), ionizing radiation, mitomycin C, cyclophosphamide, cisplatin, restriction enzymes, etc.[6,7)] Recently, nuclear accumulation of p53 after UV-irradiation was shown to be induced through DNA damage of actively transcribed genes.[8)] This induction of p53 was shown to be due to prolongation of its half-life and not to the stimulation of transcription.[9,10)] Accumulated p53 was proposed to block the cell cycle at G1, preventing cells from replicating damaged DNA.[11)] It was suggested that p53 may exert its effect upon cell cycle progression by controlling expression of the gene encoding p21, an inhibitor of cyclin-dependent protein kinase.[12)] The major aim of this study was to examine whether cellular stresses other than DNA damage, including heat shock, can induce nuclear accumulation of p53.

Human primary skin fibroblasts were cultured on cover-slips in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal calf serum in an atmosphere of 5% CO_2. Cells were either exposed to heat shock for 45 min at 43°C followed by incubation for 6 h at 37°C, or treated with various chemical agents (with the exception of azetidine and canavanine) or osmotic shock for 6 h. In the cases of azetidine and canavanine, cells were treated for 16–24 h. After fixation with acetone, indirect immunostaining was performed using a mouse anti-p53 monoclonal antibody (PAb 1801; Ab-2, Oncogene Science, Uniondale, NY) as the first antibody and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG polyclonal antibody (Cappel) as the second antibody as described elsewhere.[8)] Fig. 1 shows that without stress, nuclei of normal human fibroblasts were barely stained with the antibody, but heat shock and other stresses induced nuclear accumulation of p53. The intensities of fluorescence varied depending on the doses and kind of stress applied. The optimal doses which induced maximal nuclear accumulation of p53 for each stress and the relative intensities of p53 induced by the various kinds of stress are summarized in Table I. Heat shock proved to be one of the most effective inducers of p53 among the stresses applied. This result is apparently inconsistent with that of Lu and Lane[7)]; using mouse primary prostate cells, they reported that UV, ionizing radiation, and the restriction enzyme *Pvu*II, all of which damage DNA, induced the nuclear accumulation of p53, but heat shock, which is assumed not to cause DNA damage directly, did not. The reason for this

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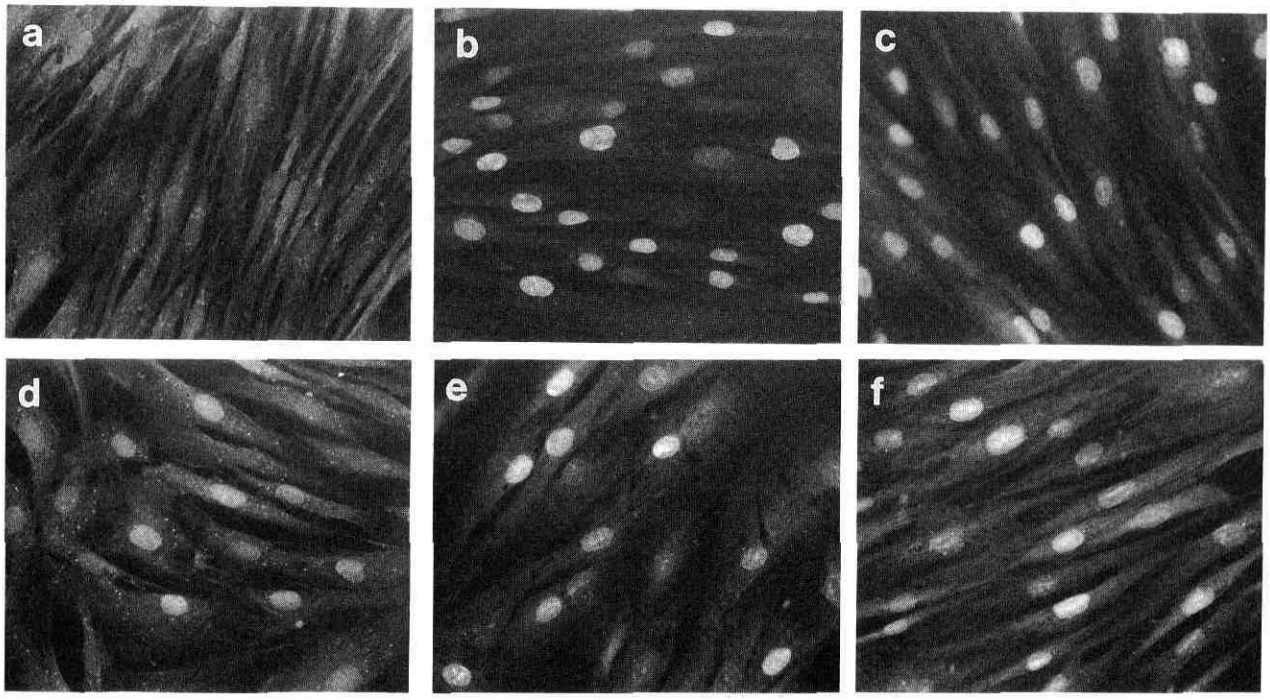

Fig. 1. Induction of p53 by cellular stresses. Normal human fibroblasts were treated for 6 h in the presence of H_2O_2 (0.01 m*M*) (c), or NaN_3 (0.05%) (d), or NaCl (220 m*M*) (e), or for 16 h in the presence of azetidine (10 m*M*) (f). In the heat shock experiment (b), cells were incubated for 45 min at 43°C and then incubated for 6 h at 37°C. After incubation, cells were fixed with acetone and stained for p53 with anti-p53 monoclonal antibody (PAb 1801) and FITC-conjugated anti-mouse IgG. (a) Untreated control cells.

Table I. Optimal Conditions for Induction of p53 in Normal Human Fibroblasts by Various Cellular Stresses

Agent[a)]	Optimal dose[f)]	(Range of doses tested)	Intensity of nuclear fluorescence[g)]	Induction of Hsp70[h)]
Untreated			−	−
Heat shock[b)]	43°C, 45 min	(15–90 min)	+++	+++
$CdCl_2$[c)]	20 μM	(1–40 μM)	++–+++	++
Na_2HAsO_2[c)]	10 μM	(1–40 μM)	+–++	+–++
NaN_3[c)]	0.05%	(0.001–0.09%)	++	±
H_2O_2[c)]	10 μM	(2.5–250 μM)	++	±
Azetidine[d)]	10 m*M*	(0.3–30 m*M*)	+++	+–++
Canavanine[d)]	10 m*M*	(0.4–40 m*M*)	+++	++
Puromycin[c)]	2 μM	(1–100 μM)	+–++	++
Osmotic shock[e,e)]	250 m*M*	(0–300 m*M*)	++	++

a) Normal human fibroblasts were grown on cover-slips and treated for either 6 h (*c*) or 16–22 h (*d*) in the presence of various concentrations of inducers. In the heat shock experiment (*b*), cells were cultured for different incubation times (15–90 min) at various temperatures (38–45°C) before incubation for 6 h. In the osmotic shock experiment (*e*), cells were cultured in DMEM containing different concentrations of NaCl (0–300 m*M*) for 6 h. After the treatment, cells were fixed with acetone and stained for p53 by an indirect immunofluorescence method. Intensity of nuclear fluorescence (*g*) at the optimal doses (*f*) was determined by inspection and evaluated in four grades (−, +, ++, +++). Induction of Hsp70 (*h*) was examined by immunostaining using a mouse anti-Hsp72/73 monoclonal antibody (Ab-1, Oncogene Science). Cells were treated with the inducers under the optimal conditions (doses, periods) shown in (*f*), and fixed with methanol. The intensity of fluorescence was determined by inspection (five grades: −, ±, +, ++, +++).

discrepancy is not clear. It is possible that the response to heat shock might be different between mouse and human cells. As the sensitivity of the p53 response to various stresses is reduced with aging of cells (unpublished observation), it is also possible that the above discrepancy is due to differences in cell culture conditions. In the osmotic shock experiment, cells were cultured for 6 h in DMEM containing different concentrations of NaCl. Nuclear accumulation of p53 was observed between 210 m*M* and 270 m*M* NaCl (concentration of NaCl in normal DMEM is 120 m*M*). Above 290 m*M*, cells showed shrinkage and became detached from the dishes. We observed no sign of p53 induction with lower concentrations of NaCl (80 m*M*, 30 m*M* and 0 m*M*). Hyperosmotic stress also induced the heat shock response.[13] While most stresses induced nuclear accumulation of p53 within 6 h, it took more than 16 h before azetidine and canavanine, analogues of proline and arginine, respectively, manifested their effect. This was probably because a considerable time is required for replacement of the amino acids by the analogues and for accumulation of analogue-containing abnormal proteins in the cells. Puromycin induced p53 only at specific concentrations. Induction of the heat shock response by puromycin has also been observed previously at specific concentrations.[14] Under these conditions, the drug may produce sufficient aberrant proteins to evoke the response. At higher concentrations, it acted as an inhibitor of the p53 induction evoked by UV-irradiation (data not shown) similarly to cycloheximide.[10] Sodium azide, which blocks intracellular respiration through interference with cytochrome *c* oxidase in mitochondria and induces the heat shock response,[14] evoked p53 at the concentration of 0.05%. Cd,[15] H_2O_2[16] and As[17] are strong inducers of the heat shock response, and also induced p53 accumulation. Although we cannot completely exclude the possibility that small amounts of DNA damage were indirectly introduced by these stresses through some as-yet-unknown mechanism(s), the contribution of these mechanisms to the induction of p53 in the present study would be very small. Considerable amounts of DNA damage are required to induce clearly visible nuclear accumulation of p53 within 6 h in normal fibroblasts, and in the case of UV-irradiation, more than 8 J/m^2 is required.[8] Consequently, it is highly likely that nuclear accumulation of p53 can also be induced by cellular stresses other than DNA damage. Since all of the agents listed in Table I are known inducers of the heat shock response and induced Hsp70 at the optimal doses for the p53 response (Table I), we suggest that both cellular responses share, at least partly, some common signaling pathway(s). Another possibility is that some heat shock protein(s) induced by these stresses might have inhibitory activity on the degradation system for p53 or might facilitate the nuclear transportation of p53. Further study will be required to clarify the relationship between the two cellular responses.

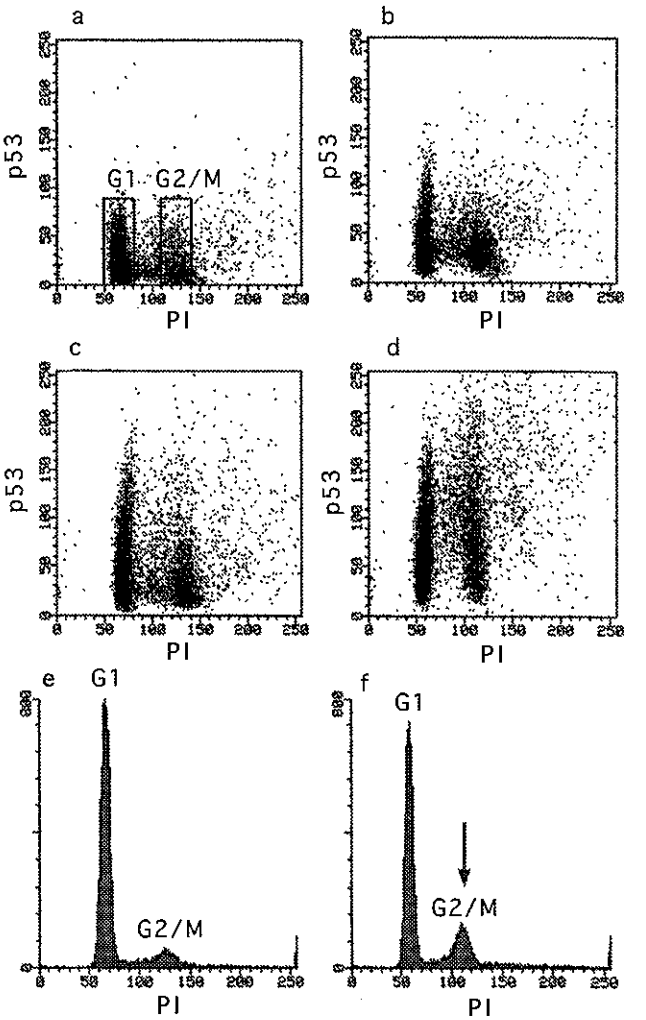

Fig. 2. Nuclear accumulation of p53 evoked by cellular stresses in relation to the cell cycle. Normal human fibroblasts were treated for 6 h either in high-salt medium (220 m*M* NaCl) (b), or in the presence of $CdCl_2$ (10 μM) (c). In (d), cells were treated for 22 h in the presence of azetidine (10 m*M*). The pattern of untreated control cells is shown in (a). The cells were fixed with 88% methanol in suspension and double-stained with FITC-conjugated IgG for p53 and with propidium iodine (PI) for DNA content as described elsewhere.[8] Stained cells (10^4) were analyzed on a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems). The patterns for DNA content of control cells and cells treated with azetidine are shown in (e) and (f), respectively. As shown with an arrow, the population in G2/M of treated cells (f) was increased to about twice that of the control (e).

To examine whether the nuclear accumulation of p53 induced by these cellular stresses occurred independently

of the cell cycle or whether only cells at a specific point in the cell cycle have the ability to respond to these stresses, flow-cytometric analysis was performed. Cells were exposed to the stresses in the same manner as described in Fig. 1 and Table I, and detached with trypsin and EDTA. After fixation with methanol and staining of p53 by the indirect method, cells were analyzed by FACScan (Becton-Dickinson Immunocytometry Systems). As shown in Fig. 2, the intensity of fluorescence of the cells increased after stress regardless of their cell cycle stage. A previous study also showed that DNA damage caused by UV-irradiation induced nuclear accumulation of p53 independently of the cell cycle.[8] p53, when induced by DNA damage, prevents cells from entering S-phase (G1 block).[10] More recently, it was shown that p21 induced by p53 inactivates DNA polymerase δ through interaction with proliferating cell nuclear antigen (PCNA) *in vitro*.[12] Thus, p53 is assumed to function as a negative regulatory factor for progression of the cell cycle in G1 and S phases. FACScan analysis, the results of which are shown in Fig. 2e and f, also showed that after treatment with azetidine the population in G2/M phase increased to almost twice that of control cells. Similar phenomena were observed with all stresses examined (heat shock, osmotic shock, H_2O_2, and Cd) (data not shown). Therefore, it is suggested that p53 induced in cells in G2/M phase functions to arrest the cell cycle at G2/M.

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