Wild-type p53 is required for apoptosis induced by growth factor deprivation in factor-dependent leukaemic cells

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Summary The p53 gene is a growth control gene, abnormalities of which have been implicated in a variety of cancers. Recently wild-type p53 has been shown to exist in two interchangeable conformational variants, which can be distinguished by specific p53 monoclonal antibodies. One conformation acts as a suppressor (PAb240+/PAb1620−) and one acts as a promoter (PAb240−/PAb1620+) of cell proliferation; the latter conformation is also that of mutant p53. We have previously shown that acute myeloblastic leukaemia (AML) blasts which proliferate autonomously in vitro express only p53 in the promoter conformation. In contrast, expression of PAb1620 was found only in blasts with non-autocrine growth in vitro and was diminished following stimulation by exogenous growth factors when there was a switch to p53 in the promoter (PAb240+) conformation. As AML blasts with non-autocrine growth undergo apoptosis when deprived of exogenous growth factors, we studied whether this was mediated by wild-type p53. Antisense oligonucleotides to p53 were used to suppress p53 protein expression in blasts with non-autocrine growth and also the factor-dependent human erythroleukaemia cell line TF-1. Following growth factor deprivation for 48 h, 20.6–53.6% of control blasts were apoptotic and demonstrated a typical ‘ladder’ on DNA electrophoresis characteristic of internucleosomal degradation of DNA. In the presence of p53 antisense, apoptosis was suppressed despite the absence of growth factor, however cell proliferation was not stimulated. We conclude that apoptosis occurring in factor-dependent AML blasts following growth factor deprivation is mediated by wild-type p53 (PAb1620+), and that conformational change of p53 to the PAb240+ conformation occurring either by mutation or by the action of autocrine growth factors would permit leukaemic cell survival by suppressing apoptosis.

A number of regulatory genes which influence cellular susceptibility to enter the physiological process of cell death known as apoptosis have been identified, including c-myc (Evan et al., 1992), bcl-2 (Hockenbery et al., 1990; Bissonette et al., 1992) and p53. Wild-type p53 is classified as a tumour-suppressor gene (Elyahu et al., 1989; Finlay et al., 1989). Introduction of wild-type p53 into cell lines that have lost endogenous p53 function results in growth arrest (Baker et al., 1990; Diller et al., 1990; Mercer et al., 1990) and apoptosis (Yonish-Rouach et al., 1991; Shaw et al., 1992). Recently, further evidence has shown that wild-type p53 (but not mutant p53) is required for radiation-induced apoptosis in thymocytes (Lowe et al., 1993), and that p53 exerts a significant and dose-dependent effect on apoptosis induced by radiation and agents that cause DNA strand breakage, such as chemotherapeutic drugs (Clarke et al., 1993). However, other mechanisms which induce apoptosis have been described, including the withdrawal of trophic factors (Arends & Wyllie, 1991).

The viability and proliferation of leukaemia cells from patients with acute myeloblastic leukaemia (AML) is dependent upon the presence of haemopoietic growth factors (Löwenberg & Toug, 1993). Unlike normal haemopoietic progenitors, some myeloid leukaemia cells produce autocrine growth factors (Young & Griffin, 1986; Reilly et al., 1989; Russell, 1992). We have found that both autocrine and exogenous granulocyte–macrophage colony-stimulating factor (GM-CSF) act to maintain viability and to suppress apoptosis in blast cells from patients with AML. AML blasts which do not produce autocrine GM-CSF rapidly lose viability due to apoptosis when cultured without added growth factors. Similar changes were observed in the factor-dependent human erythroleukaemia cell line TF-1 following growth factor deprivation (Bradbury et al., 1993). In contrast, leukaemic cells which produced autocrine GM-CSF were found to be protected against apoptotic cell death following in vitro culture. Thus, normal haemopoietic progenitor cells and growth factor-dependent myeloid leukaemia cells undergo apoptosis following deprivation of haemo-

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Materials and methods

AML cells

Blood samples were obtained at diagnosis from four patients with AML and peripheral blood blast count of >2 x 10⁹ l⁻¹. The diagnosis of AML was made using FAB criteria following conventional cytochemical stains and surface marker analysis. Mononuclear cells were separated by Ficoll-Hypaque sedimentation and samples were depleted of T cells by Dynabeads M-450 Pan-T (CD2) (Dynal, Oslo, Norway). Samples were cryopreserved in 10% dimethylsulphoxide (DMSO) and 20% fetal calf serum (FCS) in liquid nitrogen. Viability of thawed cells was greater than 90%. TF-1 is a human erythroleukaemia cell line (Kitamura et al., 1989), which was kindly donated by T. Kitamura (DNAX Research Institute of Molecular and Biology, Palo Alto, CA, USA).

Antibodies and oligonucleotides for p53

Three purified mouse monoclonal antibodies for p53 were used (Oncogene Science, NY, USA). PAb1801 recognises an epitope between amino acids 32 and 79 (Banks et al., 1986). PAb240 recognises an epitope between amino acids 156 and 335 (Gannon et al., 1990). PAb1620 was developed by Ball et al. (1984) and has been shown to recognise a conformational epitope specific for wild-type p53 (Ball et al., 1984; Milner & Medcalf, 1991).

Eighteen-mers of p53 oligonucleotides were obtained from British Bio-technology (Oxford, UK). They correspond to the sense or antisense sequences flanking the translation initiation regions of the messenger RNA for p53 (Zakut-Houri et
The sequence of the phosphorothiate oligonucleotides with the ATG initiation codon or its complement CAT in the sense and antisense sequence was as follows: sense, 5′-ACTGCCATGGAGAAGCCG-3′, antisense, 5′-CGGCTCCTCATGCGAGT-3′.

**Western blotting**

Cells (10^6) were washed with PBS (pH 7.2) twice, then lysed for 15 min at 4°C with 300 μl of lysis buffer [50 mM Tris–HCl pH 8.0, 0.25 M sodium chloride, 0.1% Nonidet P-40, 50 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 50 μg ml⁻¹ aprotinin]. The lysates were collected by microcentrifugation for 10 min. Protein concentrations of the lysates were determined by the method of Lowry et al. (1951). Aliquots of 100 μg of each of lysate were analysed by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with the Laemmli buffer system (Laemmli, 1970). The gels were run at 150 V for 45 min in a mini protein II slab cell (Bio-Rad, Richmond, CA, USA). Proteins in the gel were transferred onto nylon membrane by electrotransfer process (0.8 mA cm⁻² membrane, about 60 min) in a continuous buffer system [49 mM glycine, 45 mM Tris, 0.0375% (v/v) SDS, 20% (v/v) methanol]. Non-specific binding was blocked by incubation of the membrane with 0.5% bovine serum albumin (BSA) in TBST (0.01 M Tris–HCl pH 8.0, 0.15 M sodium chloride, 0.05% Tween-20) overnight at 4°C. The membrane was probed for 60 min with mouse monoclonal antibody for p53 (PAb1801), followed by alkaline phosphatase-conjugated goat anti-mouse antibody for a further 30 min. The bands were visualised by alkaline phosphatase substrate solution [100 mM Tris–HCl pH 9.5, 100 mM sodium chloride, 5 mM magnesium chloride] in 10 ml including 66 μl of nitroblue tetrazolium (NBT, 50 mg ml⁻¹) and 33 μl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 50 mg ml⁻¹). The DNA was visualised under ultraviolet light after staining with 5 μg ml⁻¹ ethidium bromide. λDNA (EcoRI and HindIII digest) (Sigma, Dorset, UK) was used as a DNA marker to estimate the size of DNA fragments.

**Statistical methods**

Data were analysed by a two-sided unpaired Student t-test.

**Results and Discussion**

To study the role of wild-type p53 in the induction of apoptosis in factor-dependent leukaemia cells, we studied peripheral blood blast cells from four patients with non-autocrine growth in a clonogenic assay, as well as the factor-dependent TF-1 cells. The growth characteristics of these cells are shown in Table I. Positive expression of p53 was detected in all of these cells by Western blot with PAb1801, which recognises both wild-type and mutant p53 (Banks et al., 1986) (Figure 1). p53 has been demonstrated to exist in two conformational states: one which exhibits a suppressor effect and one with a promoter effect on cell proliferation (Milner, 1991; Ullrich et al., 1992). These two conformations of wild-type p53 can be recognised by different antibodies: PAb1620 and PAb240 recognise the suppressor and promoter conformations respectively (Ball et al., 1984; Gannon et al., 1990; Milner & Medcalf, 1991), and PAb240 also recognises mutant p53 (Gannon et al., 1990). We have previously shown that the conformation of p53 in AML blasts is related to growth factor stimulation and is regulated by exogenous or autocrine haemopoietic growth factors (Zhu et al., 1993). Thus, cells with non-autocrine growth when deprived of growth factor express p53 in both the suppressor and the promoter conformation. However, following growth factor stimulation, p53 was found to be only present in the promoter conformation, which is also the conformation found in blasts with autocrine growth factor production (Zhu et al., 1993). These findings were confirmed in this study. Flow cytometric analysis showed that between 20% and 33% of blasts with non-autocrine growth expressed p53 in the suppressor (PAb1620) conformation when cultured in the absence of exogenous GM-CSF (Figure 2). However, after these cells were induced to grow by exogenous GM-CSF, expression of PAb1620 was found in less than 5% of cells, and the expression of PAb240, which recognises the promoter conformation of p53, was increased (Figure 2). These results suggested to us the possibility that wild-type p53 in the suppressor conformation (PAb1620) may be involved in

| Table 1 | In vitro growth characteristics of AML blasts studied |
|---------|-----------------------------------------------------|
| Patient | FAB type | No. of colonies/2 × 10⁵ cells | NCM | 5637-CM |
| AML-1   | M2       | 0                             | 40  |        |
| AML-2   | M1       | 0                             | 112 |        |
| AML-3   | M1       | 0                             | 61  |        |
| AML-4   | M2       | 0                             | 134 |        |

No. of colonies represents the mean of triplicate cultures. 5637-CM contains GM-CSF, G-CSF and IL-1 (Hoang & McCulloch, 1985). NCM, No conditioned medium, i.e. cells cultured in the absence of exogenous growth factors.
Figure 1 Expression of p53 in AML blasts of totally CSF-dependent and TF-1 cells detected by Western blot with PAb1801.

Figure 2 Conformational change of p53 protein in AML cells following growth factor stimulation. p53 expression was analysed by flow cytometry using PAb240 and PAb1620. AML cells were cultured without added growth factor (NCM, □) and in the presence of 5637-conditioned medium (5637-CM) containing GM-CSF, G-CSF and IL-1 (Hoang & McCulloch, 1985) (□). TF-1 cells were cultured both in NCM (□) and in the presence of recombinant GM-CSF (□). Only AML cells cultured under conditions of growth factor deprivation express p53 in the suppressor (PAb1620⁺) confirmation.

Figure 3 Demonstration of dose-dependent inhibition of p53 expression in TF-1 cells by antisense p53 oligonucleotides. TF-1 cells were treated by varying concentrations of antisense (—○—) or sense (—□—) p53 oligonucleotides for 24 h. Expression of p53 protein was investigated by flow cytometry with the monoclonal anti-p53 antibody PAb1801, which recognised both wild-type and mutant p53. Each point represents the mean ± s.d. of triplicate cultures.

Figure 4 DNA fragmentation induced by growth factor deprivation in AML blasts and its suppression by antisense (a) p53 oligonucleotides. Sense (s) p53 oligonucleotides were used as a control.

The induction of apoptosis in factor-deprived leukaemic cells.

To study this further, antisense oligonucleotides were used to analyse the effect of suppression of wild-type p53 in blasts undergoing apoptosis following growth factor deprivation. Using antisense p53 oligonucleotides which correspond to the translation initiation region of the p53 mRNA, a dose-dependent inhibition of p53 expression in TF-1 cells by antisense p53 oligonucleotides was observed (Figure 3). Using PAb1801 to detect p53, we found that a concentration of 5 μM antisense oligonucleotides reduced expression from 75% to 16%, and this concentration of oligonucleotides was then used for further experiments designed to study the effect of suppression of p53 expression on apoptosis. As also shown in Figure 3, control sense oligonucleotides had no effect on p53 expression.

Following growth factor deprivation for 24 h, the percentage of leukaemic cells expressing morphological features of

TF-1 AML-1 AML-2 AML-3 AML-4

TF-1 AML-1 AML-2 AML-3 AML-4

TF-1 AML-1 AML-2 AML-3 AML-4

TF-1 AML-1 AML-2 AML-3 AML-4

TF-1 AML-1 AML-2 AML-3 AML-4

TF-1 AML-1 AML-2 AML-3 AML-4

TF-1 AML-1 AML-2 AML-3 AML-4

TF-1 AML-1 AML-2 AML-3 AML-4

TF-1 AML-1 AML-2 AML-3 AML-4

TF-1 AML-1 AML-2 AML-3 AML-4
Apoptosis was between 9.3% and 16.0%, increasing to between 20.6% and 53.6% at 48 h (Table II). DNA extracted from these cells showed a characteristic 'DNA ladder' which is a feature of internucleosomal degradation of DNA (Figure 4). In the presence of 5 µM antisense oligonucleotides, apoptosis in all samples was inhibited, with <1.5% of cells showing morphological features of apoptosis at 24 h and a similar degree of suppression at 48 h. These results were confirmed by the absence of a DNA 'ladder' in DNA extracted from cells incubated with antisense oligonucleotides (Figure 4). These results suggested that apoptosis in growth factor-dependent leukaemic cells is mediated via wild-type p53; however, suppression of apoptotic cell death in these cells did not induce DNA synthesis. As shown in Figure 5, the addition of antisense oligonucleotides to cultures of TF-1 cells did not increase DNA synthesis when the cells were cultured without added GM-CSF.

The data presented here suggest a role for wild-type p53 in inducing apoptosis in leukaemic cells deprived of growth factors; indeed, a similar mechanism may operate in normal haemopoietic progenitors deprived of growth factors as these cells have been shown to express p53. Recently, p53 has been implicated in the induction of apoptosis by irradiation and chemotherapeutic drugs (Clarke et al., 1993; Lowe et al., 1993). Thus, p53-deficient cells were markedly resistant to the effects of these agents in inducing apoptosis. Our data would suggest that wild-type p53 is also involved in apoptosis occurring in leukaemic cells as the result of growth factor deprivation, as suppression of p53 protein expression prevented the onset of apoptosis in these cells. Moreover our data suggest that this effect is specifically associated with expression of p53 in the suppressor (PAb1620+) conformation. These findings also explain why AML cells with autocrine GM-CSF production, which only express p53 in the promoter (PAb240+) conformation (Zhu et al., 1993), do not undergo apoptosis following in vitro culture. From these observations we suggest that the loss of the suppressor form (PAb1620+) of wild-type p53 during leukaemogenesis occurring either as the result of mutation or more frequently by the action of autocrine growth factors, would promote the survival of cells deprived of exogenous growth factors. Such a mechanism may be important in permitting the survival and regrowth of the leukaemic cells following chemotherapy.

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Table II Apoptosis of factor-dependent AML cells is inhibited by antisense p53 oligonucleotides

| Source of cells | Antisense p53 | Sense p53 | Antisense p53 | Sense p53 | p-value (48 h) |
|----------------|--------------|----------|--------------|----------|---------------|
| TF-1           | 0.6 ± 0.6    | 14.3 ± 2.5 | 10.6 ± 1.0    | 53.6 ± 3.5 | <0.005        |
| AML-1          | 1.3 ± 1.1    | 11.3 ± 1.1 | 10.3 ± 2.3    | 38.0 ± 2.0 | <0.001        |
| AML-2          | 0.6 ± 0.6    | 9.3 ± 1.5  | 5.6 ± 2.0     | 26.7 ± 3.5 | <0.005        |
| AML-3          | 1.0 ± 1.0    | 16.0 ± 3.6 | 3.3 ± 0.5     | 20.6 ± 2.5 | <0.001        |
| AML-4          | 1.0 ± 1.0    | 11.0 ± 2.0 | 5.6 ± 0.5     | 24.6 ± 3.8 | <0.002        |

DNA synthesis

| GM-CSF | Sense p53 | Antisense p53 | NCM |
|--------|-----------|---------------|-----|
|        | c.p.m.    |               |     |
|        | 0         | 200           | 800 |
|        | 400       | 600           | 1000|

Figure 5 Effect of antisense oligonucleotides to p53 on DNA synthesis in TF-1 cells under different culture conditions: (1) no added growth factors (NCM), (2) 5 µM antisense p53 oligonucleotides, (3) 5 µM sense p53 oligonucleotides and (4) rGM-CSF (200 units ml⁻¹). All experiments were performed in triplicate. Antisense oligonucleotides to p53 suppressed apoptosis following association with growth factor deprivation but did not induce cell proliferation.
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