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Activation of p53-mediated cell cycle checkpoint in response to micronuclei formation

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SUMMARY

Inactivation of p53 tumor-suppressor leads to genetic instability and, in particular, to accumulation of cells with abnormal numbers of chromosomes. In order to better define the role of p53 function in maintaining genome integrity we investigated the involvement of p53 in the control of proliferation of micronucleated cells resulting from abnormal chromosome segregation. Using cell lines expressing temperature-sensitive (ts) p53 or containing p53 genetic suppressor element (p53-GSE) we showed that inhibition of p53 function increases the frequency of cells with micronuclei. Immunofluorescence study revealed that in REF52 cell cultures with both spontaneous and colcemid-induced micronuclei the proportion of p53-positive cells is considerably higher among micronucleated variants as compared with their mononuclear counterparts. Analysis of 12(1)ConA cells expressing the β-galactosidase reporter gene under the control of a p53-responsive promoter showed activation of p53-regulated transcription in the cells with micronuclei. Importantly, the percentage of cells manifesting specific p53 activity in colcemid-treated cultures increased with an augmentation of the number of micronuclei in the cell. Activation of p53 in micronucleated cells was accompanied by a decrease in their ability to enter S-phase as was determined by comparative analysis of 5-bromodeoxyuridine (5-BrdU) incorporation by the cells with micronuclei and their mononuclear counterparts. Inhibition of p53 function in the cells with tetracycline-regulated p53 gene expression, as well as in the cells expressing ts-p53 or p53-GSE, abolished cell cycle arrest in micronucleated cells. These results along with the data showing no increase in the frequency of chromosome breaks in REF52 cells after colcemid treatment suggest the existence of p53-mediated cell cycle checkpoint(s) preventing proliferation of micronucleated cells derived as a result of abnormal chromosome segregation during mitosis.

Key words: p53, Micronucleus, Cell cycle checkpoint

INTRODUCTION

p53 plays an important role in the restriction of proliferation of abnormal cells. Experimental evidence suggests that it orchestrates a number of cellular responses to cytotoxic stresses (for review see Hartwell and Kastan, 1994; Chernova et al., 1995; Ko and Prives, 1996; Levine, 1997). Functioning as a transcription factor p53 up-regulates expression of genes that contain p53-binding elements including *waf1/cip1* and *gadd45* whose products contribute to cell cycle arrest (El Deiry et al., 1993, 1994; Dulic et al., 1994; Zhan et al., 1994; Harper et al., 1995), and *bax, fas* and a set of *PIG* genes whose products may contribute to induction of apoptosis (Miyashita et al., 1994a; Miyashita and Reed, 1995; Owen-Schaub et al., 1995; Polyak et al., 1997). In addition, p53 can down-regulate a number of genes, probably through direct interaction with basal transcription factors such as TBP and CBP (Seto et al., 1992; Ragimov et al., 1993; Horikoshi et al., 1995). Among these genes is *bcl2*, a suppressor of apoptosis (Miyashita et al., 1994a,b). Thus, p53 activates cell cycle checkpoints and triggers apoptosis preventing growth of damaged cells as well as proliferation of undamaged cells under unfavorable conditions (Hartwell and Kastan, 1994; Chernova et al., 1995; Cox and Lane, 1995; Jacks and Weinberg, 1996; Ko and Prives, 1996; Levine, 1997).

One of the consequences of the loss of p53 safeguard activity is genetic instability. It is noteworthy, that p53 abnormalities induce changes in chromosome number along with chromosome breaks and DNA recombinations. In fact, an increased rate of accumulation of aneuploid cells was found in p53-deficient mouse fibroblasts and in human colorectal carcinoma cells expressing mutant p53 (Tsukada et al., 1993; Carder et al., 1993; Agapova et al., 1996). It was supposed that loss of p53 function can lead both to an increase in probability of chromosome non-disjunction and to abrogation of negative control of proliferation of cells with chromosome segregation errors (Agapova et al., 1996). In order to analyze possible pathways that can underlie the latter mechanism we decided to

study the role of p53 in control of proliferation of cells containing micronuclei resulting from abnormal chromosome segregation during mitosis. In the present paper we show that formation of micronuclei triggers p53 activation followed by cell cycle arrest. p53-mediated activation of the cell cycle checkpoint(s) by micronuclei may represent one of the mechanisms by which normal p53 function prevents accumulation of aneuploid cells.

MATERIALS AND METHODS

Cell cultures

Cell lines with different states of endogenous p53 as well as their derivatives expressing various p53 cDNAs were used. They included: (i) rat embryo fibroblasts (REF) at 2-6 passages in vitro and their subline containing the p53-GSE/22 (genetic suppressor element #22) short C-terminal fragment of rat p53 cDNA that inhibits p53 function via a dominant-negative mechanism (Ossovskaya et al., 1996); (ii) spontaneously immortalized REF52 rat fibroblasts retaining expression of the wild-type (wt)-p53 (Ishizaka et al., 1995) and their subline with transduced p53-GSE/22; (iii) murine immortalized fibroblasts 10(1) which are devoid of endogenous p53 expression (Harvey and Levine, 1991) and their subline expressing murine temperature-sensitive (ts)-Val135-p53, which at 32°C has conformation and activities of wt-p53 but at 37°C behaves like a typical mutant p53 (Mikhalovitz et al., 1990; Martinez et al., 1991). As a control in this case we used 10(1) cells harboring insert-free retroviral vector pPS/neo (Kopnin et al., 1995); (iv) murine wt-p53-positive 12(1) immortalized fibroblasts (Harvey and Levine, 1991) expressing bacterial β-galactosidase under the control of p53-responsive promoter, kindly provided by Dr M. V. Chernov, Cleveland Clinic Foundation, Cleveland (Komarova et al., 1997); and (v) human TR9/7 fibroblasts with tetracycline-regulated expression of exogenous wt-p53, kindly provided by Dr G. R. Stark, Cleveland Clinic Foundation, Cleveland (Agarwal et al., 1995). In these cells the level of p53 expression gradually increases upon decrease of tetracycline concentration in culture medium: at 1.0 μg/ml p53 expression is not detected while the withdrawal of tetracycline induces p53 expression to the levels comparable with those observed after DNA damaging insults (Agarwal et al., 1995).

All cells were cultured in DMEM medium supplemented with 10% fetal calf serum (Gibco Booklet, Scotland) in a humidified atmosphere supplied with 5% CO_2. TR9/7 cells were permanently maintained in the medium with 1.0 μg/ml of tetracycline. For the experiments the majority of cell types were plated at an initial density of $10\text{-}20\times10^3$ cells/cm^2 into LabTek Slide Chambers (Nunc Inc., Naperville, Illinois, USA). TR9/7 cells were seeded at an initial density of 40×10^3 cells/cm^2.

Induction of micronuclei by colcemid treatment

At 14-16 hours after plating colcemid (Sigma) at a final concentration of 0.03 μg/ml was added to the culture medium for 36 hours. Then cells were washed twice with Hanks' solution for 10-15 minutes and placed in fresh growth medium. Further experiments were performed after additional culturing in colcemid-free medium for 24 hours allowing restoration of microtubule cytoskeleton, as was determined by staining with anti-α-tubulin DM1A monoclonal antibody (Sigma) (Fig. 1). For visualization of micronuclei the cells were counterstained with Hoechst #33258 (Sigma).

Immunufluorescence analysis of p53 expression

Cells were washed twice with phosphate buffered saline (PBS) at 35-37°C, fixed with 4% formaldehyde for 15 minutes at room temperature and incubated in a mixture of methanol/acetone (1:1) at –20°C for 10 minutes. Then methanol/acetone was replaced gradually with PBS and cells were stained with PAb421 monoclonal antibodies specific to p53. PAb421/p53 complexes were visualized by indirect staining with Biotin-conjugated anti-mouse IgG and FITC-labeled Streptavidin (both Dianova, Germany).

Analysis of p53-responsive transcription in mouse 12(1)ConA cells by β-galactosidase staining

Cells were washed 5-6 times with PBS at 35-37°C, fixed with 1% glutaraldehyde for 15 minutes at room temperature. For staining cells were incubated overnight in a solution of 0.2% X-gal (Sigma), 3.3 mM K $Fe_4(CN)_6$, 3.3 mM $K_3Fe(CN)_6$ and 1 mM $MgCl_2$ at 35-37°C.

Analysis of 5-bromodeoxyuridine (5-BrdU) incorporation

5-BrdU (Serva) at a final cocentration 10^{-5} M was added to the culture medium for 5 hours. After incubation with 5-BrdU cells were washed 2-3 times with PBS at 35-37°C, fixed with 4% formaldehyde for 15 minutes at room temperature, permeabilized with 1% Triton X-100 in buffer M (50 mM imidazole, 50 mM KCl, 0.5 mM MCl_2, 0.1 mM EDTA, pH 6.8), containing 4% polyethylene glycol (M_r 40,000) for 3 minutes and hydrolysed with 4 M HCl at room temperature for 10 minutes. 5-BrdU incorporation was visualized by indirect immunostaining with BU-33 monoclonal mouse antibody specific to 5-BrdU (Sigma) and TRITC-conjugated anti-mouse IgG (Sigma).

Analysis of chromosome breaks

Chromosomal slides were prepared by routine methods. In each case 50-100 metaphases were analyzed.

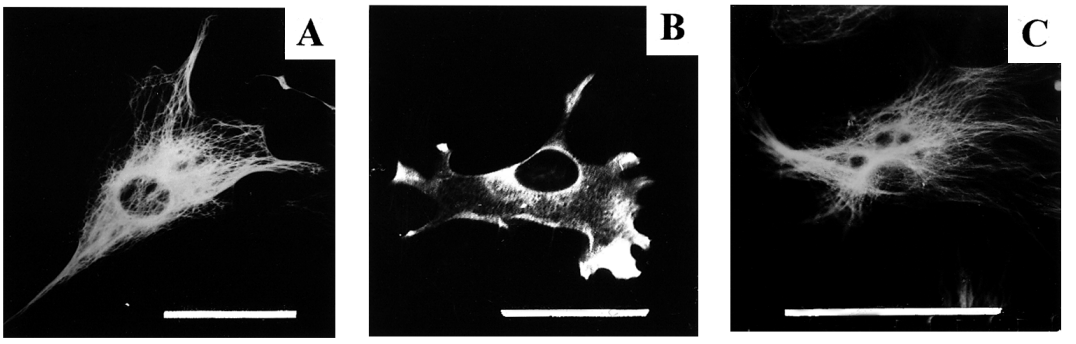

Fig. 1. Restoration of microtubule cytoskeleton in colcemid-induced micronucleated REF52 cell after drug withdrawal.
(A) Immunofluorescence of a control colcemid-untreated REF52 cell after staining with DM1A monoclonal antibody specific to α-tubulin; (B) microtubule disorganization in the colcemid-treated cell; (C) cell with colcemid-induced micronuclei after incubation in drug-free medium for 24 hours. Bars, 20 μm.

RESULTS AND DISCUSSION

The frequency of cells with micronuclei is dependent on the p53 state

To assess the possible role of p53 in restriction of micronucleated cells we first compared the frequency of these cells in cultures with functional or inactivated p53. For this purpose two model systems were used: (i) spontaneously immortalized murine fibroblasts which are either p53-positive [12(1)ConA cell line], or p53-negative [10(1)neo cell line], or express exogenous temperature-sensitive p53 [10(1)Val135 cell line]; and (ii) primary (REF) and immortalized (REF52) rat embryo fibroblasts that express functional p53, or their derivatives expressing dominant-negative p53 fragment (REF/GSE22 and REF52/GSE22 cell lines).

We have found that p53-deficient murine fibroblasts 10(1) show a higher percentage of micronucleated cells as compared with p53-positive 12(1)ConA cells (Table 1). The data obtained with 10(1) cells expressing exogenous ts-p53 provide additional evidence for participation of p53 in control of proliferation of cells with micronuclei. Similar percentages of micronucleated cells were found in 10(1)Val135 cultures permanently incubated at the non-permissive temperature (37°C) and in the control 10(1)neo cell line (Table 1). However, after incubation at the permissive temperature (32°C) for 96 hours the percentage of micronucleated cells was significantly decreased (Table 1). Evidently, the restoration of wt-p53 activity causes elimination of micronucleated variants from a population of mouse 10(1) cells. In contrast, inactivation of p53 function in REF and REF52 cells by dominant negative p53 fragment resulted in substantial increase in the fraction of cells with micronuclei (Table 2). Taken together these data strongly suggest the existence of p53-dependent mechanisms that prevent accumulation of abnormal micronucleated cells.

Table 1. Frequency* of micronucleated cells in mouse cell lines with various p53 states

Cell line	Temperature of incubation	p53 state	Cells with micronuclei (%)*
10(1)neo	37°C	–	24.5±1.7
	32°C†	–	22.0±1.6
10 (1)Val135	37°C	Mutant	22.0±1.6
	32°C†	wt	14.0±1.0
12(1)ConA	37°C	wt	3.2±0.2

*In each case 500-1,000 cells were analyzed.
†Cells permanently cultured at 37°C, were incubated at 32°C for 96 hours.

Table 2. Influence of p53 inactivation by p53-GSE on frequency of cells with micronuclei in rat embryo fibroblasts (REF) and REF52 cell line

Cells	p53-GSE	Cells with micronuclei (%)*
REF	–	<0.1
	+	10.3±0.3
REF52	–	6.5±0.2
	+	12.9±0.4

*In each case 1,000 cells were analyzed.

Activation of p53 in cells with micronuclei

Next we decided to test whether formation of micronuclei can activate p53 function. Since it is known that micronuclei can arise as a result of either abnormal chromosome segregation, or chromosome breaks (Nusse et al., 1987; Hennig et al., 1988) we analyzed both spontaneously derived, and colcemid-induced micronucleated cells. It has been shown that colcemid does not cause a clastogenic effect (Brown and Bick, 1986) and induces formation of micronuclei through disruption of the spindle. In fact, while 50-60% of spontaneously derived micronuclei consist of acentric DNA fragments (probably arising as the result of chromosome breaks), almost all colcemid-induced micronuclei contain one or several kinetochores (i.e. whole chromosomes) (Nusse et al., 1987; Hennig et al., 1988; Stopper, 1994). Under the colcemid treatment conditions that were used in this study (0.03 μg/ml for 36 hours) the majority of REF52 cells became micronucleated while the number of chromosome breaks was not increased (Table 3). So, this approach allowed us to minimize the contribution of chromosome breaks to activation of p53 in micronucleated cells.

DNA damage as well as many other insults leads to p53 stabilization and an increase in its intracellular content (for review see Ko and Prives, 1996; Levine, 1997). To test whether a similar mechanism of p53 activation can be observed in micronucleated cells we performed an immunofluorescence study of REF52 cell cultures with spontaneously derived or colcemid-induced micronuclei. In both cases we have found that a higher proportion of micronucleated cells stains with

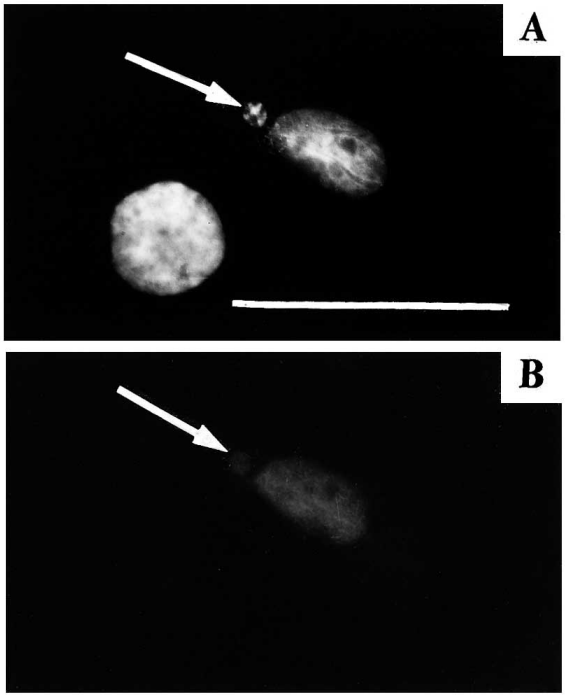

Fig. 2. Comparison of p53 expression in micronucleated and mononuclear cells. Staining with Hoechst #33258 DNA-binding dye revealed in the field the cell containing micronucleus (arrow) as well as mononuclear cell (A), while immunostaining with monoclonal antibody specific to p53 displayed only the cell with micronucleus (arrow) but not mononuclear counterpart (B). Bar, 20 μm.

Table 3. Influence of colcemid treatment used for induction of micronuclei on frequency of chromosome breaks in REF52 cells

	Cells with micronuclei (%)	Metaphases with chromosome breaks (%)
Control	6.5±0.2	8.0±1.5
Colcemid*	55.1±3.1	10.0±1.8

*Colcemid at a final concentration 0.03 μg/ml was added for 36 hours.

anti-p53 antibodies as compared with normal mononuclear cells (Figs 2, 3A). Without colcemid treatment the percentage of p53-positive cells among micronucleated cells was quite low (1.6±0.1%), yet nearly 20-fold higher than that among mononuclear cells (0.1±0.05%). Specific p53 staining in such a small proportion of micronucleated cells might be explained either by low sensitivity of the used method that does not allow detection of p53 in all the cells where it was induced, or by accumulation of p53 only in a proportion of the micronucleated cells. After colcemid treatment the number of p53-positive cells significantly increased. This can be explained by residual activation of p53 in response to disruption of microtubules (Tishler et al., 1995) that persists even after 24 hours of incubation in colcemid-free medium. However, in colcemid-treated cultures the clear difference between the ability of micronucleated and mononuclear cells to stain for p53 was retained (45.8±3.2% vs 6.7±0.3%), see Fig. 3A. Apparently, micronucleated cells are characterized by a further increase in p53 expression over an already elevated basal level that allows visualization of p53 expression in a considerably larger proportion of micronucleated cells. In any case the revealed difference between the frequency of p53-positive mononuclear and micronucleated cells in both colcemid-treated and untreated cultures is in agreement with the suggestion that p53 is activated in cells with micronuclei.

It was shown, however, that p53 accumulation does not obviously lead to enhancement of its activity (Chernov and Stark, 1997). Moreover, in some cell systems an increase in p53 expression is accompanied by inactivation rather than by activation of p53 function. For example, expression of some viral oncoproteins which bind to p53 (SV40 T-antigen and adenoviral E1B) as well as introduction into the cell of dominant-negative p53-GSEs lead to stabilization of transcriptionally inactive p53 (Tiemann et al., 1995; Lowe and Ruley, 1993; Ossovskaya et al., 1996). Also, a high level of functionally inactive wild-type p53 was observed in teratocarcinoma cells (Lutziker and Levine, 1996). In order to detect p53 activation in micronucleated cells we used 12(1)ConA mouse fibroblasts expressing a bacterial β-galactosidase reporter gene under the control of a p53-responsive promoter (Komarova et al., 1997). We have found that micronucleated cells showed a higher proportion of β-galactosidase staining (i.e. p53 activity) as compared with their mononuclear counterparts in both colcemid-treated and untreated cultures (Figs 3B, 4). It is noteworthy that in colcemid-treated cultures the percentage of β-galactosidase-positive cells increased in parallel with the number of micronuclei. Indeed, p53-dependent β-galactosidase expression was found in about 30% of mononuclear cells, in 50-60% of cells with one or two micronuclei, and in 85-90% of cells containing more than four micronuclei (Table 4). This observation along with the data showing no increase in the

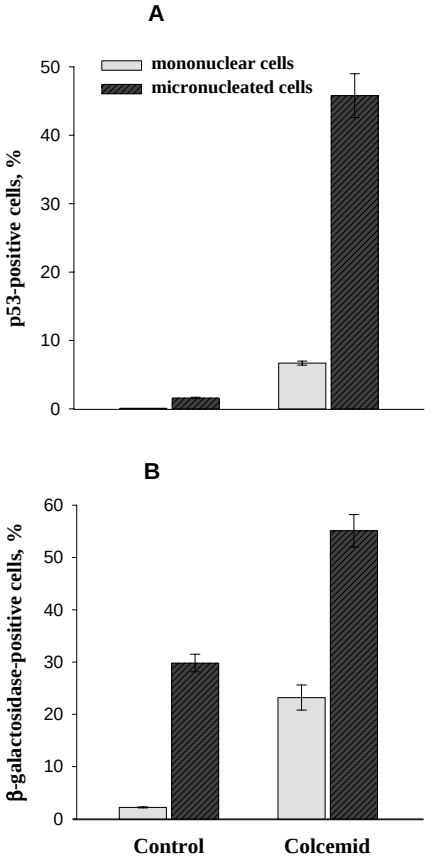

Fig. 3. Frequency of micronucleated and mononuclear cells stained with anti-p53 antibody in REF52 cultures (A) or showed p53-regulated β-galactosidase gene expression in 12(1)ConA cell cultures (B). Both spontaneously derived and colcemid-induced micronucleated cells were characterized by higher proportion of cells showing p53 expression as compared with mononuclear counterparts. (In each case 500-1,000 of mononuclear and 200-500 micronucleated cells were analyzed.)

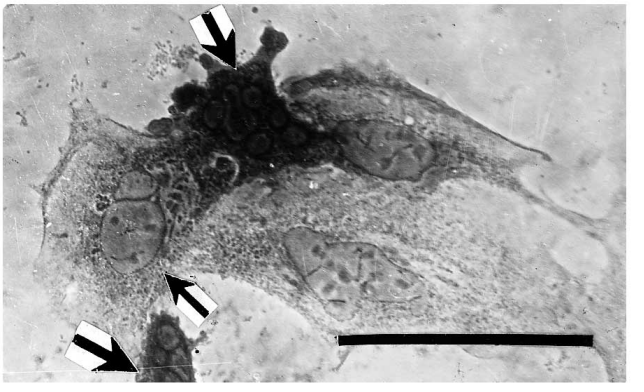

Fig. 4. β-Galactosidase staining of micronucleated (arrows) and mononuclear cells in colcemid-treated 12(1)ConA cultures. Two cells with multiple micronuclei show positive staining (large arrows) while the cell with a single large-sized micronucleus (small arrow) as well as two mononuclear cells remained unstained. Bar, 20 μm.

Table 4. Dependence of β-galactosidase staining on the number of micronuclei in colcemid-treated 12(1)ConA cells

Number of micronuclei	Proportion of β-galactosidase-positive cells (%)*
0	29.8±1.7 (210)
1	55.8±7.5 (103)
3	73.1±9.8 (54)
4	80.1±10.5 (45)
5	84.6±13.7 (16)
>6	90.0±9.3 (39)

*The ratio of the of number β-galactosidase-positive cells to the number of analyzed cells with given number of micronuclei. The number of analyzed cells is indicated in parenthesis.

frequency of chromosomal breaks in colcemid-treated cells (Table 3) strongly points to the emergence in micronucleated cells of some p53-activating signals which are distinct from DNA damage or disruption of microtubules. There is a formal possibility of a reverse consequence of events: p53 activation somehow promotes formation of micronuclei. However, this possibility seems to be unlikely since in mouse 10(1)Val135 cells the 'switching-on' of the wild-type p53 function caused a decrease rather than an increase in the frequency of micronucleated cells (Table 1).

Limited ability of cells with micronuclei to enter S-phase

One of the possible outcomes of p53 activation in micronucleated cells might be the cell cycle arrest that prevents accumulation of cells with abnormally segregated chromosomes. To check this supposition we tested the ability of micronucleated cells to enter S phase of the cell cycle. Three model systems were used: (i) immortalized REF52 rat embryo fibroblasts which express or do not express dominant-negative p53-GSE/22; (ii) murine immortalized fibroblasts which are either p53-negative [10(1)neo cell line], or express exogenous ts-p53 [10(1)Val135 cell line]; and (iii) immortalized TR9/7 human fibroblasts with tetracycline-regulated expression of exogenous p53. The ability of the cells to enter S phase was assessed by analysis of 5-BrdU incorporation in unsynchronized cultures after labeling for 5 hours.

First we have found that in REF52 cells cultures expressing endogenous wild-type p53 a considerably lower portion of micronucleated cells was able to incorporate 5-BrdU as compared with mononuclear cells. The expression of p53-GSE22 in REF52 cells that inhibits at least some of the p53 activity (Ossovskaya et al., 1996) eliminated the difference between mononuclear and micronucleated cells in their ability to enter S phase (Table 5). The dependence of the frequency of 5-BrdU-positive micronucleated cells upon the p53 state was further confirmed by analysis of murine 10(1) fibroblasts expressing ts-p53. At the non-permissive temperature (37°C) allowing expression of inactive p53 the number of 5-BrdU-positive cells with micronuclei practically did not differ from that observed in control p53-negative 10(1)neo subline. However, shifting to the permissive temperature (32°C) that results in restoration of p53 function caused a significant decrease in the ability of micronucleated cells to enter S phase (Table 6). Similar results were obtained in experiments with TR9/7 cells allowing us to compare the ability of various levels of p53 expression to arrest the cell cycle in mononuclear and micronucleated cells. Under conditions permitting minimal expression of p53 (1.0 μg/ml of tetracycline) mononuclear and micronucleated cells show similar percentages of 5-BrdU positiveness (Fig. 5). A stepwise decrease in tetracycline dosages that leads to progressive increase in p53 expression levels resulted in gradual diminishing of 5-BrdU-incorporation that was most prominent in micronucleated cells indicative of their decreased ability to enter the S-phase of the cell cycle. Such effect was observed in the cells with both spontaneously derived (Fig. 5A) and colcemid-induced micronuclei (Fig. 5B).

It is noteworthy that in colcemid-treated TR9/7 cultures repression of p53 function (1.0 μg/ml of tetracycline) eliminated the difference between micronucleated and mononuclear cells, but did not prevent the decrease in the number of 5-BrdU-positive mononuclear cells (from 54.9±3.2% to 36.6±1.5%; see Fig. 5) which evidently resulted from disruption of microtubules. Similarly, in REF52 cells expression of dominant-negative p53-GSE22 abolished the difference in the number of cycling mononuclear and micronucleated cells, however, it did not influence partial cell cycle arrest observed in mononuclear cells after colcemid treatment (Table 5). It is possible that along with p53-dependent mechanisms, p53-independent mechanisms are responsible for the cell cycle arrest in colcemid-treated cells and the latter might dominate after removal of the drug and restoration of microtubule cytoskeleton. Also, the possibility cannot be excluded that disruption of microtubules generates much stronger signals for p53 induction than micronuclei formation does, and the minimal basal quantities of p53 which might be present in untreated TR9/7 and REF52/GSE22 cells (as a result of leakage of the promoter governing p53

Table 5. Influence of inhibition of p53 function by GSE22 on the ability of mononuclear and micronucleated REF52 cells to enter S phase

Cell line	Colcemid treatment	Cells with micronuclei (%)*	5-BrdU-labeled mononuclear cells (%)†	5-BrdU-labeled micronucleated cells (%)†
REF52	–	6.5±0.2	42.0±2.4	12.5±0.8
	+	41.7±2.0	23.0±1.5	4.5±0.3
REF52/GSE22	–	12.9±0.4	57.2±2.9	56.5±4.6
	+	53.1±2.6	21.2±1.5	21.1±0.9

*Related to the number of all analyzed cells in given culture (1,000).
†Related to the number of all analyzed mononuclear cells in given culture (1,000).
‡Related to the number of all analyzed cells with micronuclei in given culture (300-500).

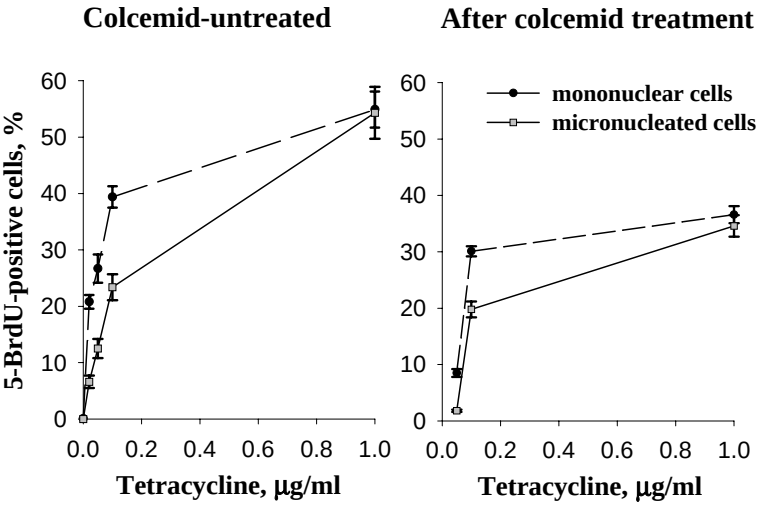

Fig. 5. Influence of various levels of p53 expression on the ability of monuclear and micronucleated TR9/7 cells to incorporate 5-bromodeoxyuridine. In cultures with significant levels of p53 expression (0.01-0.05 μg/ml of tetracycline) both spontaneusly derived and colcemid-induced micronucleated cells show decreased ability to enter S-phase. Repression of p53 function (1.0 μg/ml of tetracycline) eliminates the cell cycle arrest in cells with micronuclei. (In each case 500-1,000 of mononuclear and 200-500 micronucleated cells were analyzed).

expression and incomplete inhibition of the p53 function by GSE22, respectively) are sufficient to be amplified to the levels/activities promoting the cell cycle arrest in response to colcemid treatment, but insufficient to induce p53-mediated arrest in micronucleated cells.

Putative cell cycle checkpoint(s) activated in micronucleated cells

Taken together our results suggest that abnormal chromosome segregation leading to formation of micronuclei can trigger p53 activation and cell cycle arrest. These data allow us to suppose the existence of cell cycle checkpoint(s) that restrict(s) proliferation of cells which either contain additional nuclear structures or have alterations in DNA content (or chromosome number). The former seems to be more likely. In fact, we have observed a correlation between the number of micronuclei and the extent of p53 activation. In the 12(1)ConA cell line the proportion of cells showing p53-driven reporter gene expression increased with an augmentation of the number of micronuclei (see Table 4). In addition we found no difference in p53 activity in the cells with single small-sized and large-sized micronuclei (data not shown). Probably, quantitative changes in DNA content or chromosome number did not affect p53 response.

Activation of p53 can cause cell cycle arrest in different phases. In the majority of cases delay of the G_1/S transition is observed (for review see Ko and Prives, 1996; Levine, 1997), however arrest in G_2 (Agarwal et al., 1995; Stewart et al., 1995) and in S (Mikhalovitz et al., 1990; Ishizaka et al., 1995) was also described. The key role in p53-induced cell cycle arrest is played by *p21^{waf1}*, which binds to and inhibits a number of cyclin and Cdk (cyclin-dependent kinase) complexes: cyclin D1-Cdk4, cyclin E-Cdk2, cyclin A-Cdk2, and cyclin B-Cdc2 (El-Deiry et al., 1994; Dulic et al., 1994; Polyak et al., 1996; Levine, 1997). In addition *p21^{waf1}* binds directly to PCNA and inhibits its function as a DNA polymerase processivity factor in DNA replication (for review see Kelman, 1997). The products of other p53-responsive genes such as *gadd45* can also contribute to cell cycle arrest (Zhan et al., 1994). Variations in the points of the cycle in which the cells arrest in response to p53 activation may reflect differences in cell context, depending on the cell cycle phases of target cells, tissue-specificity, relative activity of various p53-downstream genes, etc. For example, activation of p53 in early G_1 inhibits the G_1/S transition, while the cells which passed the critical point in late G_1 do not show G_1 arrest in response to p53 activation and can be blocked further in G_2 (Linke et al., 1997). It seems quite probable that micronucleated cells can be blocked in G_1 following mitosis in which abnormal chromosome segregation leading to formation of micronuclei had occurred. However, the arrest in other phases cannot formally be excluded. It should be noted that cell cycle arrest evidently is not the sole mechanism by which p53 activation can prevent accumulation of micronucleated cells: in addition it can induce apoptosis. The decision of whether p53 will induce cell cycle arrest or apoptosis depends on many factors such as tissue specificity, growth factor availability, activity of the p53-Rb-E2F pathway, etc. (reviewed by Ko and Prives, 1996; Levine, 1997; Oren and Prives, 1996).

Table 6. Ability of micronucleated cells to enter S-phase in mouse cell lines with various p53 states

Cell line	Temperature of incubation	p53 state	% of 5-BrdU-labeled cells with micronuclei*
10(1)neo	37°C	–	14.0±1.0
	32°C	–	12.0±0.9
10 (1)Val 135	37°C	Mutant	11.0±0.9
	37°C	wt	2.0±0.1

*Related to the number of all 5-BrdU-positive cells. In each case 300-500 cells were analyzed.

While the downstream pathways responsible for p53-induced cell cycle arrest are established, the upstream mechanisms of p53 activation in micronucleated cells remain unclear. Two possibilities exist: (i) micronuclei themselves trigger p53 activation; and (ii) p53 is induced not in response to formation of micronuclei, but as a result of some other cellular changes that regularly accompany appearance of micronuclei. For instance, one intriguing possibility could be that an increase in the number of centrosomes and/or microtubule-organizing centers (MTOC) serves as a signal activating p53 in micronucleated cells. This supposition is

based on the observation of Fukasawa et al. (1996) who showed amplification of MTOC in p53-deficient cells and hypothesized the role of p53 in the regulation of centrosome replication. However, some data indicate that the number of centrosomes is dependent on the ploidy level, but not on the number of nuclei (Onishchenko, 1978; Radley and Scurfield, 1980). Further ideas and studies will be needed to understand the mechanisms of p53 activation in micronucleated cells.

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