Virchows Archiv (1994) 424:357–360

ORIGINAL ARTICLE

T. Wiethege · B. Voss · K.M. Müller

p53 accumulation in polynuclear-giant-cells

Received: 2 November 1993/Accepted: 15 February 1994

Abstract Accumulation of *p53* has been reported in nearly all malignant human tumours. Macrophage derived giant cells of sarcoid granulomas in human lung tissue also show intense staining for *p53* while normal alveolar macrophages remain unstained. Since sarcoid giant cells are not considered to be either pre-neoplastic nor to exhibit *p53* gene mutations, two different physiological functions of *p53* may be illustrated. Alveolar macrophages were isolated from rat lungs and cultured in vitro. Accumulation of *p53* was observed by indirect immunohistochemistry after application of polyclonal rabbit serum directed against murine *p53* (CM5). Antiproliferating cell nuclear antigen (PCNA) antibodies were used to study DNA synthesis. Most of the multinucleated giant cells derived from macrophages accumulated *p53* in the cytoplasm, while only few nuclei were stained. PCNA was found in most giant cells nuclei. However, PCNA positivity was visible in few mononucleated macrophages. Isolated alveolar macrophages in vitro clearly divide and since nuclear division is a late event in the cell cycle, *p53* may be involved in G1/S-control and in other cell-cycle-checkpoints between mitosis and cytokinesis.

Key words Tumor suppressor gene · *p53* · Macrophage Giant cell · Cell cycle

Introduction

p53, a 53 kDa nuclear phosphoprotein, is encoded by the *p53* tumour suppressor gene which is localized on the short arm (p13.1) of chromsome 17. It was first described in 1979 as a cellular protein which co-precipitated with the large T-antigen of simian virus 40 (SV 40) from infected murine cells and synthesis was enhanced in chemically transformed tumours (DeLeo et al. 1979; Kress et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979). *p53* is a tumour suppressor gene and wild-type *p53* is able to inhibit the transformation of *ras*-activated rat embryonic fibroblasts (Eliyahu et al. 1989; Finlay et al. 1989) while mutations of the *p53* gene lead to the transformation of affected cells (Laviguer et al. 1989; Nigro et al. 1989).

Mutations of the *p53* gene are the most frequent gene alterations reported in human cancer. *p53* alterations have been detected in nearly 45% of cancer patients (Hollstein et al. 1991) including those with breast and lung tumours, leukaemias, osteosarcomas, ovarian, stomach and cerebral neoplasms. Most of the mutations exhibit the exchange of a single nucleotide only occurring within evolutionary conserved domains of the gene (Caron de Fromentel and Soussi 1992). A mutation in one allele is often accompanied by the loss of the second allele, and leads to a failure of *p53* function in cell cycle control. Reports describe a control function for *p53* in the G1/S phase switch controlling the transition into mitosis (Bischoff 1990; Milner 1991). Cell cycle checkpoints can enhance cell survival and limit mutagenic events following DNA damage, and precise characterization of cell cycle regulation will help to understand the mechanisms of carcinogenesis and apoptosis. However, the specific functions of the *p53* protein and its molecular interaction in cell cycle controlling of normal and transformed cells remain to be clarified.

The functional properties of *p53* have been suggested to be involved in controlling the integrity of the cell and/or the genome (Lane 1992). Wild type *p53* protein was not thought to accumulate in the cytoplasm or in the nucleus of normal and undamaged cells and is not detectable in frozen or formalin-fixed paraffin sections by standard immunohistochemical techniques (Levine 1991). The positive detection of *p53* protein in sections was thus always combined with a gene mutation or sta-

T. Wiethege (✉) · K.M. Müller
Institute of Pathology, University Clinic Bergmannsheil,
Gilsingstrasse 14,
D-44789 Bochum, Germany

B. Voss
Professional Associations'
Research Institute for Occupational Medicine,
Bochum, Germany

bilization of wild type phosphoprotein by viral proteins like SV 40 large T-antigen (Tan et al. 1986) or cellular proteins like mdm2 (Barak et al. 1993). Those changes were strongly correlated to cell transformation and cancer. However, from results on sarcoid granulomas of human sarcoid tissue (Wiethege et al. 1992) *p53* positivity may also occur in benign tissues or cells (see also Koutsleini et al. 1991; Soini et al. 1992).

We have studied the role of *p53* in cultured polynuclear macrophage giant cells of rats.

Materials and methods

Female Wistar rats (180–200 g) were sacrificed under anaesthesia with diethylether. After tracheotomy, a plastic tube (1.4 mm) was fixed in the trachea, and the lungs were washed six times, each with 5 ml phosphate buffered saline (PBS; 137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM sodiumhydrogen phosphate × H_2O, 1.5 mM potassium dihydrogen phosphate, pH 7.4, 37°C). Lavage fluids were collected and centrifuged 5 min at 200 *g*. After centrifugation, the supernatant was removed and the cell pellet was resuspended with standard culture medium (Dulbeco's modified Eagle's medium + 10% fetal calf serum + antibiotics (Boehringer, Mannheim)). Cell quantity was determined using a modified Sysmex Microcellcounter F-300 (Digitana, Hamburg). 0.5×10^6 cells were distributed on a sterile glass slide in a Heraeus Quadriperm culture dish. Cells were cultured at 37°C, 5% carbon dioxide and 90% humidity.

Cell characterization was achieved by standard techniques (Wiethege et al. 1991) and by a propidium iodide staining (500 ng/ml PBS) to demonstrate polynucleated cells. Induction of giant cell formation was obtained by modifying culture conditions, either by an increase of cell density or by adding 10 U/ml interleukin 3 (IL-3; Boehringer, Mannheim) to the culture medium.

A standard immunoperoxidase avidin-biotin complex protocol (Vectastain) was used for the detection of *p53*. Briefly, cells were fixed in 50% (v/v) acetone in methanol for 10 min at –20°C, washed with 70% ethanol and rehydrated in TRIS buffered saline (5 mM TRIS, 0.76% (w/v) sodium chloride, pH 7.6). Endogenous peroxidase acitivity was quenched by incubation in 3% hydrogen peroxide for 10 min. Cells were further pre-incubated with 20% normal goat serum and consequently incubated for 1 h at 25°C with the antiserum CM5, diluted 1: 1000. The polyclonal rabbit anti murine *p53* antiserum CM5 was produced and kindly provided by C. Midgley (CRC Lab. University Dundee, UK) and directed against full length recombinant murine *p53* expressed in *E. coli* (Jerry et al. 1993). In addition three commercialy available monoclonal antibodies, PAb 421 (Harlow et al. 1981), PAb 240 (Gannon et al. 1990) and PAb 1801 (Banks et al. 1986) (Oncogene Sci.), diluted 1: 100 were used for immunohistochemical studies. All monoclonal antibodies have been checked for reaction with rat *p53*: PAb 240 and 421 react well, PAb 1801 shows a weaker cross reaction (Lane DP, personal communication). An antibody direct-

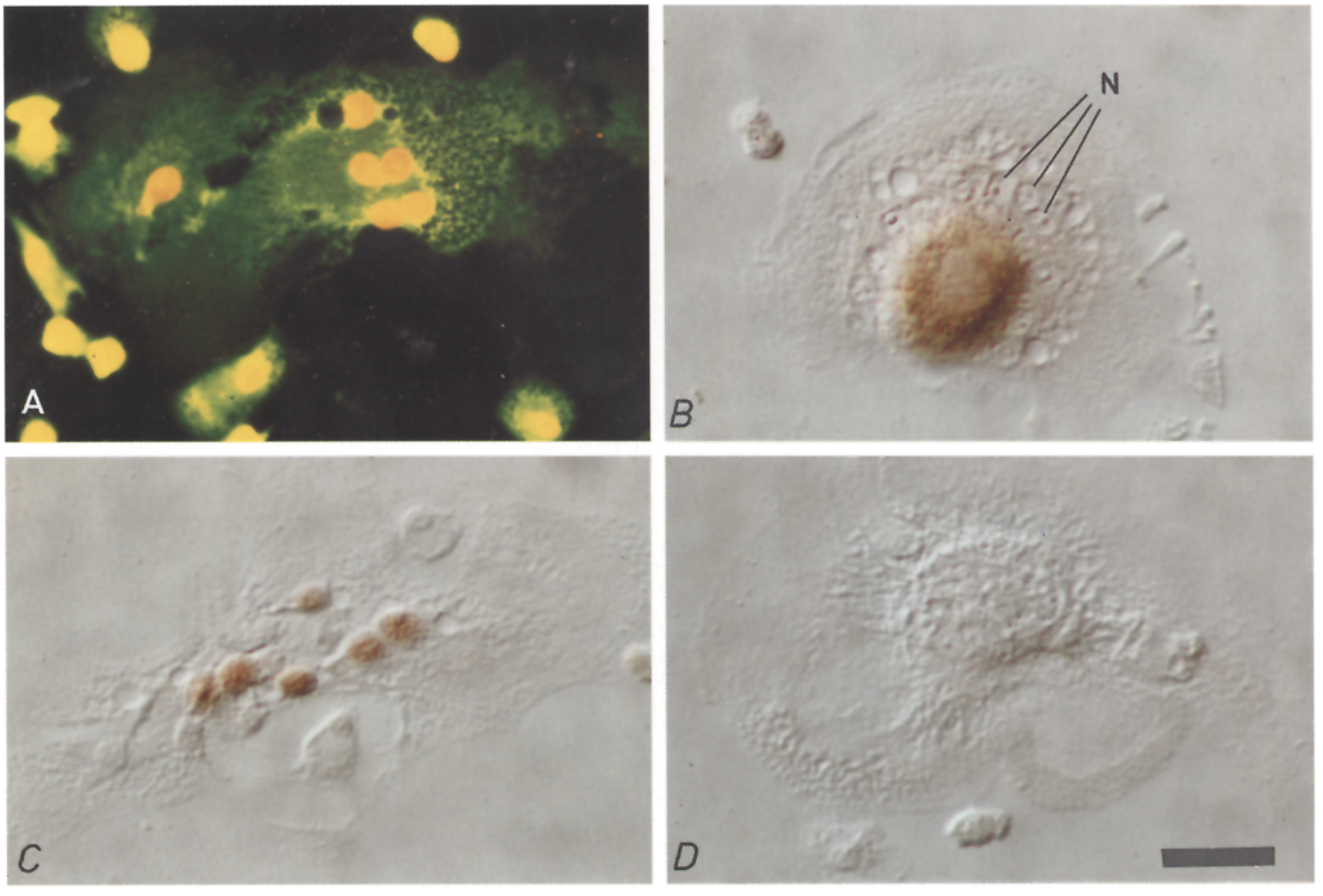

Fig. 1 Rat alveolar macrophage derived giant cells cultured in vitro. By using the CM5 antiserum for immunohistochemistry, polynuclear giant cells, verified by propidium iodide staining (**A**) show a staining in the dense central cytoplasm (**B** *N* nuclei). The antibody directed against PCNA reveals a strong nuclear staining (**C**) where as a reaction in cytoplasm or nuclei is not visible by incubating the cells with rabbit or mouse IgG or pre-immune-serum for control (**D**). *Bar* 40 μm

ed against PCNA (Waseem and Lane 1990; Oncogene Sci., 1: 100) was used as a marker for cell proliferation. For control, cells were incubated with rabbit IgG or pre-immune serum instead of anti *p53* or anti PCNA antibody. Immunoperoxidase staining method was followed as indicated by the manufacturer (Vector, Burlingame, Calif., USA).

Genomic DNA from several different cell cultures was screened by non-radioactive polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis (Wiethege et al. 1993) for mutations in exons 5, 6 and 7 of the rat *p53* gene using the primers P5-1/2, P6-1/2 and P7-1/2 described by Makino et al. (1992). According to Hulla and Schneider (1993), these regions represent the comparable exons 5 to 8 of the human *p53* gene.

Results

The isolation of rat alveolar macrophages by lavage revealed approximately 5×10^6 viable cells per lung. On an average, 95% of these cells were characterized as alveolar macrophages (Wiethege et al. 1991). Multinucleated giant cells, verified by propidium iodide staining (Fig. 1A), developed in primary macrophage cultures within 14 days. The induction of giant cell formation could be achieved either by increasing the culture density to 1×10^6 cells per slide or by adding 10 U/ml IL-3 to the culture medium. About 15–20% of the cells transformed to giant cells. By using antibodies directed against the *p53* tumour suppressor gene product detectable amounts of the phosphoprotein could be demonstrated in multinucleated giant cells. After immunohistochemical demonstration of *p53* gene product using the CM5 antiserum followed by immunoperoxidase staining, nearly 50% of the multinucleated giant cells accumulated *p53* in the cytoplasm. Only 2–3% of the cells showed staining of one or two of their nuclei. Only few mononucleated alveolar macrophages also exhibited positivity for *p53*. Absent or weak staining was observed using the antibodies PAb 421 or PAb 240 and PAb 1801 (Table 1). There was no difference between multinucleated giant cells induced by high cell density or by IL-3.

At the same time, PCNA was found in most giant cells but rarely in mononucleated cells. Although PCNA staining was predominantly intranuclear, a diffuse staining signal was also visible in the cytoplasm. Alteration of the fixation procedure resulted in the same staining pattern.

PCR-SSCP analysis was performed for investigation of the *p53* state. PCR-SSCP analysis of multinucleated giant cell enriched cell cultures did not indicate a mutation within exons 5 to 7 of the *p53* gene.

Table 1 *p53* staining pattern (+++ strong; (+) weak; – no immunohistochemical staining)

	Macrophage giant cells	Mononucleated macrophages
CM 5	+++	(+)
PAb 421	(+)	–
PAb 240	–	–
PAb 1801	–	–

Discussion

Giant cell formation from primary macrophage culture was induced by culture conditions or by cell-cell-fusion. Almost 50% of the multinucleated giant cells accumulated *p53* in the cytoplasm. Although the monoclonal antibodies used were directed against human *p53* predominantly, there is a cross reaction with rat *p53*. While PAb 240 and 421 may react well, PAb 1801 shows a weaker cross reactivity with rat *p53* (Harlow et al. 1981; Gannon et al. 1990; Lane DP personal communication). The CM5 polyclonal antiserum was produced and checked for cross-reactivity with rat *p53* essentially as described by Midgley et al. (1992) except that the antigen was murine *p53* expressed in bacteria (Jerry et al. 1993; Lane DP personal communication). Although *p53* was described to be a nuclear phosphoprotein, cytoplasmic detection was not unusual. Different subcellular locations have been reported for *p53* protein in various cellular systems, ranging from strictly cytoplasmic to strictly nuclear (Gannon and Lane, 1991; Rotter et al. 1983). Takahashi et al. (1993) described the cytoplasmic localization as a result of a translocation of pre-existing nuclear *p53* protein in the cytoplasm after serum stimulation in culture. The positive staining of only half of the giant cells may be due to differing states of cellular activity. The phenomenon of partial staining of transformed cells has already been reported on in investigations of tumour cells, where *p53* accumulation in a randomly distributed staining of nuclei was observed (Vojtesek et al. 1993). Two to three percent of the cells showed staining of only one or two of their nuclei.

PCR-SSCP analysis of the giant cell cultures did not indicate a mutation within exons 5 to 7 of the rat *p53* gene. Since the SSCP technique has been reported to be able to detect point mutations even in mixed cell populations (Wu et al. 1993) it seemed unlikely that there was a failure to detect mutations among 15 to 20 polynuclear giant cells out of 100 cells in culture. Despite this possibility or the chance that a mutation may have taken place in another exon, in a corresponding intron or in a regulating element in *cis*- or *trans*-position, we assume that wild-type *p53* has accumulated in macrophage giant cells. This thesis is supported by the fact that macrophages were of a non-clonal origin and that not all cells exhibited a uniform staining pattern.

Observations with antibodies directed against PCNA indicate that isolated alveolar macrophages synthesize DNA in vitro. Although PCNA may also be induced by DNA damage and can act in repair as well as in replication it seemed here to be an indicator for duplication (Hall et al. 1993). Development or transformation of macrophages into giant cells was accompanied by nuclear division, since this is a late event in the cell cycle, the possibility that *p53* is active in the G1-S and a later stage exists.

There are various explainations of these results. First, detection of *p53* in the cytoplasm may derive from accumulation due to subsequent expression in different nu-

clei. Second, the accumulation of *p53* in PCNA positive mononuclear cells may indicate dysfunction in the cell cycle. With different stages of the cell cycle in different nuclei in the same cell, *p53* may accumulate, recognizing the DNA as damaged and this accumulation may block the polynucleated giant cell cycle just before cytokinesis. The pre-cytokinesis-blockage may prevent cell division prior to DNA repair or may lead to apoptosis. This was observed after a 4 weeks culture period in the present investigations.

Acknowledgements We thank Drs. D. Lane and C. Midgley for providing the CM5 antiserum and Drs. V. Rotter and D. Lane for discussions and comments on the manuscript. The authors wish to thank Mrs. S. Böhm, Mrs. I. Eisenburger, Mrs. H. Sippel and Mrs. S. Freundt for technical assistance. This work was supported by the Europaen Centre for Air Pollution Control Measures (PEF), Kernforschungszentrum Karlsruhe and is part of the Ph.D. thesis of TW.

References

Banks L, Matlashewski G, Crawford L (1986) Isolation of human-*p53*-specific monoclonal antibodies and their use in the studies of human *p53* expression. Eur J Biochem 159: 529–534

Barak Y, Juven T, Haffner R, Oren M (1993) mdm2 expression is induced by wild type *p53* activity. EMBO J 12: 461–468

Bischoff J, Friedman P, Marshak D, Prives C, Beach D (1990) Human *p53* is phosphorylated by p60-cdc2 and cyclin B-cdc2. Proc Natl Acad Sci (USA) 87: 4766–4770

Caron de Fromentel C, Soussi T (1992) TP53 tumour suppressor gene: a model for investigating human mutagenesis. Genes Chromsom Cancer 4: 1–15

DeLeo A, Jay G, Appella F, DuBois G, Law, L, Old L (1979) Detection of a transformation related antigen in chemically induced sarcomas and other transformed cells of the mouse. Proc Natl Acad Sci (USA) 76: 2420–2424

Eliyahu D, Michalowitz D, Eliyahu S, Pinhasi-Kimhi O, Oren M (1989) Wild type *p53* can inhibit oncogene-mediated focus formation. Proc Natl Acad Sci (USA) 86: 8763–8767

Finlay C, Hinds P, Levine A (1989) The *p53* proto oncogene can act as a suppressor of transformation. Cell 57: 1083–1093

Gannon JV, Greaves R, Iggo R, Lane DP (1990) Activating mutations in *p53* produce a common effect. A monoclonal antibody specific for the mutant form. EMBO J 9: 1595–1602

Gannon JV, Lane DP (1991) Protein synthesis required to anchor a mutant *p53* which is temperature sensitive. Nature 349: 802–806

Hall PA, McKee PH, Menage HdP, Dover R, Lane DP (1993) High levels of *p53* protein in UV-irritated normal human skin. Oncogene 8: 203–207

Harlow E, Crawford LV, Pim DC, Williamson NM (1981) Monoclonal antibodies specific for simian virus 40 tumour antigens. J Virol 39: 861–869

Hollstein M, Sidransky D, Vogelstein B, Harris C (1991) *p53* muations in human cancers. Science 253: 49–53

Hulla J, Schneider R (1993) Structure of the rat *p53* tumour suppressor gene. Nucleic Acid Res 21: 713–717

Jerry JD, Ozbun MA, Kittrell FS, Lane DP, Medina D, Butel JS (1993) Mutations in *p53* are frequent in the preneoplastic stage of mouse mammary tumour development. Cancer Res 53: 3374–3381

Koutsleini H, Malliri A, Field J, Spandidos D (1991) *p53* expression in cytological specimens from benign and malignant breast lesions. Anticancer Res 11: 1415–1420

Kress M, May E, Cassingena R, May P (1979) Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus tumour serum. J Virol 31: 742–783

Lane D (1992) *p53*, guardian of the genome. Nature 358: 15–16

Lane D, Crawford L (1979) T antigen is bound to a host protein in SV40 transformed cells. Nature 278: 261–263

Laviguer A, Maltby V, Mock D, Rossant J, Pawson T, Bernstein A (1989) High incidence of lung, bone and lymphoid tumours in transgenic mice overexpressing mutant alleles of the *p53* oncogene. Mol Cell Biol 9: 3989–3991

Levine A, Momand J, Finlay C (1991) The *p53* tumour suppressor gene. Nature 351: 453–456

Linzer I, Levine A (1979) Characterization of a 54 K dalton cellular SV40 tumour antigen present in SV40 transformed cells and uninfected embryonal carcinoma cells. Cell 17: 43–52

Makino H, Ishizaka Y, Tsujimoto A, Nakamura T, Onda M, Sugimura T, Nagao M (1992) Rat *p53* gene mutations in primary zymbal gland tumours induced by 2-amino-3-metyhlimidazo[45-f]quinoline a food mutagen. Proc Natl Acad Sci (USA) 89: 4850–4854

Midgley CA, Fisher CJ, Bártek J, Vojtesek B, Lane DP (1992) Analysis of *p53* expression in human tumours: an antibody raised against human *p53* expressed in *Escherichia coli*. J Cell Sci 101: 183–189

Milner J (1991) The role of *p53* in normal control of cell proliferation. Curr Opin Cell Biol 3: 282–286

Nigro JM, Baker JS, Preisinger AC, Jessup J, Hostetter R, Cleary K, Bigner S, Davidson N, Baylin S, Devilee P, Glover T, Collins F, Weston A, Modali R, Harris C, Vogelstein B (1989) Mutations in the *p53* gene occur in diverse human tumour types. Nature 342: 705–708

Rotter V, Abutbul H, Ben-Ze'ev A (1983) *p53* transformation-related protein accumulates in the nucleus of transformed fibroblasts in association with the chromatin and is found in the cytoplasm of non-transformed fibroblasts. EMBO J 2: 1041–1047

Soini Y, Vähäkangas K, Nuorva K, Kamel D, Lane D, Pääkkö P (1992) *p53* immunohistochemistry in malignant fibrous histocytomas and other mesenchymal tumours. J Pathol 168: 29–33

Takahashi K, Sumimoto H, Suzuki K, Ono T (1993) Protein synthesis-dependent cytoplasmatic translocation of *p53* protein after serum stimulation of growth-arrested MCF-7 cells. Mol Carcinogenesis 8: 58–66

Tan T, Wallis J,Levine A (1986) Identification of the *p53* protein domain involved in the formation of the simian virus 40 large T-antigen protein complex. J Virol 59: 574–583

Vojtesek B, Fisher CJ, Barnes DM, Lane DP (1993) Comparison between *p53* staining in tissue sections and *p53* protein levels measured by an ELISA technique. Br J Cancer 67: 1254–1258

Waseem NH, Lane DP (1990) Monoclonal antibody analysis of the proliferation cell nuclear antigen (PCNA). J Cell Sci 96: 121–129

Wiethege T, Voss B, Pohle T, Fisseler-Eckhoff A, Müller KM (1991) Localization of elastase and tumour necrosis factor a mRNA by non-radioactive in situ hybridization in cultures of alveolar macrophages. Pathol Res Pract 187: 912–915

Wiethege T, Voss B, Müller K (1993) A fast method for nonisotopic single strand conformation polymorphism. BioTechniques 15: 45–48

Wiethege T, Voss B, Müller K (1992) Überexpression des Tumor-Suppressor-Gens p53 in Sarkoidosen. Pneumologie 46: 414–415

Wu JK, Ye Z, Darras BT (1993) Sensitivity of SSCP analysis in detecting *p53* point mutations in tumours with mixed cell populations. Am J Hum Genet 52: 1273–1275