Virchows Archiv A Pathol Anat (1993) 422.127–132

Virchows Archiv A
Pathological Anatomy and Histopathology

Immunohistochemical detection of the p53 oncoprotein in tumours of melanocytic origin

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Received August 7, 1992 / Received after revision October 16, 1992 / Accepted October 28, 1992

Abstract. We employed the polyclonal anti-p53 antibody NCL-CM1 to cultured cells and pathological tissues in order to investigate the expression of p53 oncoprotein in human malignant melanomas. The results in the cultured cells showed that the antigenic determinant was sensitive to formalin fixation, resulting in a lower reactivity than with fixation by alcohol. In pathological tissues, the expression of p53 oncoprotein increased with progression of the tumour. Among 79 melanomas 37 (47%) showed distinct nuclear labelling and the highest proportion of reactive cells was observed in metastatic melanomas (mean 4.8%). An immunocytochemical study also revealed the presence of mutant-type oncoprotein in human melanoma cell lines, which was recognized by monoclonal antibody P240, and we confirmed that the molecular weight of the antigens recognized by both antibodies was 53 kDa by Western blot analysis. Therefore, although the presence of point mutations in human melanomas is yet to be confirmed our data suggest that the antigen detected by NCL-CM1 is a mutant-type or a complex of mutant and wild-type p53 oncoproteins. This antibody may be useful in retrospective studies of tumours of melanocytic origin.

Key words: Immunohistochemistry – Melanoma – Oncogene – P53 – Tumour suppressor gene

Introduction

Malignant transformation of tumours is believed generally to occur in a series of discrete steps. A large number of possible causes have been proposed for this complex and still poorly understood process. Recent studies using biochemical and molecular biological techniques, however, have revealed that many, though not all, malignant tumours are closely linked to certain types of oncogenes and oncogene products, which may be able to function as growth factors, receptors and transformation inducers (Wynford-Thomas 1991). For example, *ras* mutations and the enhanced expression of c-*myc* and c-*fos* have been reported in malignant melanomas (Shukla et al. 1989; Peris et al. 1991). However, the concept of an anti-oncogene (tumour suppressor gene) involved in maintaining a benign phenotype is also part of modern thinking in this complex area. Extensive studies have been made to clarify the roles and functions of anti-oncogenes (Barbareshi et al. 1992). RB (retinoblastoma) and p53 are the most representative and have been the objects of considerable research. p53 was first reported as a tumour suppressor gene by Lane and Crawford (1979). They identified it from mouse cells transformed with SV40 virus; p53 is located in the short arm of chromosome 17. This gene encodes for a nuclear protein involved in the control of cellular growth and regulation of the cell cycle (Harris 1991). Wild-type p53 protein has a very short half-life and is detected in trace amounts in normal cells; this is the only gene which has been reported to suppress tumours. Mutant-type p53 has a longer half-life and binds to wild-p53 and other oncogenic or viral proteins to form stable complexes, resulting in an accumulation of detectable amounts of the protein (Finlay et al. 1988).

In this study, immunohistochemical studies were carried out in order to investigate the expression of p53 oncoprotein in human melanoma cell lines and pathological tissues from various melanocytic tumours, using polyclonal anti-p53 antibody (NCL-CM1). The significance and something of the role of this oncoprotein in tumours of melanocytic origin were elucidated. This antibody may prove to be useful in tumour pathology.

Materials and methods

The cultured human melanoma cell lines, MM96E and L (Pope et al. 1979), MM418 (Maynard and Parsons 1986), MM-S5 (unpublished), A2058 (Palyi 1989) and SK-MEL-30 (Tai et al. 1983) were used in the present study to elucidate the expression of p53

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Table 1. Immunoreactivity and localization of p53 oncoprotein recognized by NCL-CM1 in human melanoma cell lines treated with three different fixatives

Cell line	Acetone		Ethanol		Formalin	
	Reactivity	Location	Reactivity	Location	Reactivity	Location
MM96E	2+	Nc	2+	Nc	1++	Cyt
MM96L	3+	Nc	3+	Nc	1++	Cyt
MM418	3+	Nc	2++	Nc	1++	Nc/Cyt
A2058	3+	Nc	3+	Nc	1+++	Nc/Cyt
MM-S5	3+++	Nc	3++	Nc	1+++	Nc/Cyt
SK-MEL30	3+++	Nc	3+++	Nc	2+++	Cyt

Reactivity and proportion of positive cells: 1, weak; 2, moderate; 3, strong; +, 1–10%; ++, 11–20%; +++, >21%
Location of immunoproduct: Nc, nuclear staining; Cyt, cytoplasmic staining; Nc/Cyt, nuclear and cytoplasmic staining

Table 2. Expression of p53 oncoprotein recognized by NCL-CM1 in formalin-fixed and paraffin-embedded melanocytic tumours

Tissue	Number of cases		Proportion of positive cells (%)	
	Tested	Positive	Mean	(Range)
Common melanocytic naevi	45	5	0.3	(0–7.2)
Junctional naevi	5	0	0	(0)
Compound naevi	10	1	0.1	(0–1.2)
(e)	10	0		
(d)	10	1		
Intradermal naevi	15	3	0.7	(0–7.2)
Spitz's naevi	5	0	0	(0)
Dysplastic naevi	5	0	0	(0)
Lentigo simplex	5	1	0.1	(0–0.4)
Malignant melanomas	79	37	4.0	(0–46.9)
Primary lesions	42	16	3.4	(0–46.9)
level I	5	0	0	(0)
level II	2	1	0.5	(0–0.9)
level III	2	1	2.3	(0–4.6)
level IV	6	3	2.2	(0–7.6)
level V	14	9	8.7	(0–46.9)
mucosal lesions	13	2	0.1	(0–0.8)
Metastatic lesions	37	21	4.8	(0–38.6)

(e), Epidermal tumour cells; (d), dermal tumour cells

oncoprotein (Table 1). The cell lines were the generous gift of Dr. P.G. Parsons (Queensland Institute of Medical Research, Brisbane, Australia) except for SK-MEL-30, which was kindly donated by Dr. A.N. Houghton (Memorial Sloan-Kettering Cancer Center, New York, USA), and MM-S5, which was established in our laboratory. Cultures were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum (v/v), streptomycin (100 μg/ml), penicillin (100 units/ml), and 3 mM 4-(2-hydroxyethyl)-1-piperazine ethane-sulphonic acid (HEPES) and incubated in a humidified atomosphere containing 5% carbon dioxide/air.

One hundred and twenty-four tumour lesions of melanocytic origin, routinely fixed in 10% neutral-buffered formalin and embedded in paraffin, were selected for this study. Details of these lesions are listed in Table 2.

Polyclonal anti-p53 antibody, NCL-CM1 (Novocastra, UK), was employed for most of the present study. This antibody was developed against recombinant human wild-type p53 protein expressed in *Escherichia coli*, and has been reported to recognize both wild- and mutant-type p53 protein (Bartek et al. 1991). Furthermore, P240, a monoclonal antibody against mutant-type p53 (Oncogene Science, USA) (Gannon et al. 1990) effective only on frozen tissues, was used in cell cultures and Western blot analysis to elucidate the expression of mutant-type oncoprotein in melanoma cells.

For ease of detection of p53 in cultured melanoma cell lines, cells were plated in culture chamber slides (Nunc, Denmark) overnight at 37° C to allow attachment. Plates were then fixed with acetone for 15 min at 4° C, ethanol for 1 min at room temperature (RT), or 10% neutral-buffered formalin for 15 min at RT, followed by extensive washes in phosphate buffered saline (PBS), pH 7.4. The subsequent procedures were followed in immunohistochemical staining of pathological tissues. Sections (4 μm thick) were deparaffinized through xylene and graded alcohols, and endogeneous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol for 15 min. All staining procedures were carried out at RT unless otherwise stated. Normal serum was used to reduce background staining and primary antibody NCL-CM1 (diluted 1:1000) was applied overnight at 4° C. For the staining of cultured cells, NCL-CM1 and P240 (at a dilution of 1:200 in PBS) were reacted for 1 h at RT. After the specimens had been washed in PBS, they were incubated with biotinylated anti-rabbit and anti-mouse immunoglobulins. The specimens were treated with avidin-biotin peroxidase complex reagent (Histofine Kit, Nichirei Labs, Japan) for 5 min following application of diaminobenzidine tetrahydrochloride as a chromogen. After confirming the presence of nuclear labelling, specimens were lightly counterstained with Giemsa.

Counting of nuclear labelling was carried out under high-power magnification in 2–4 representative areas, each containing over 100 tumour cells.

The Western blot and immunostaining procedure have been described in detail elsewhere (Takahashi and Parsons 1990). Briefly, cultured melanoma cells (approximately 5×10^6 cells) were harvested, resuspended in cell lysis buffer [20% glycerol, 1% sodium dodecylsulphate (SDS), 10 mM TRIS, pH 7.4 and 2 mM phenylmethyl sulphonyl fluoride], and sonicated for 3 min. Lysates were immersed in boiling water for 4 min, then ultracentrifuged for 10 min at RT. From each cell lysate, 20 μl was loaded onto 10% polyacrylamide gel and electrophoresed in 25 mM TRIS containing 192 mM glycine and 0.1% SDS. After transfer of proteins onto nitrocellulose membrane, immunostaining was carried out according to the method reported previously (Takahashi et al. 1991b). As a molecular weight marker of immunostaining, monoclonal anti-vimentin (57 kDa, Dakopatts, Denmark) was used, as vimentin is expressed in melanoma cells.

Results

Three different fixatives were used to investigate the presence of p53 oncoprotein in cultured melanoma cells

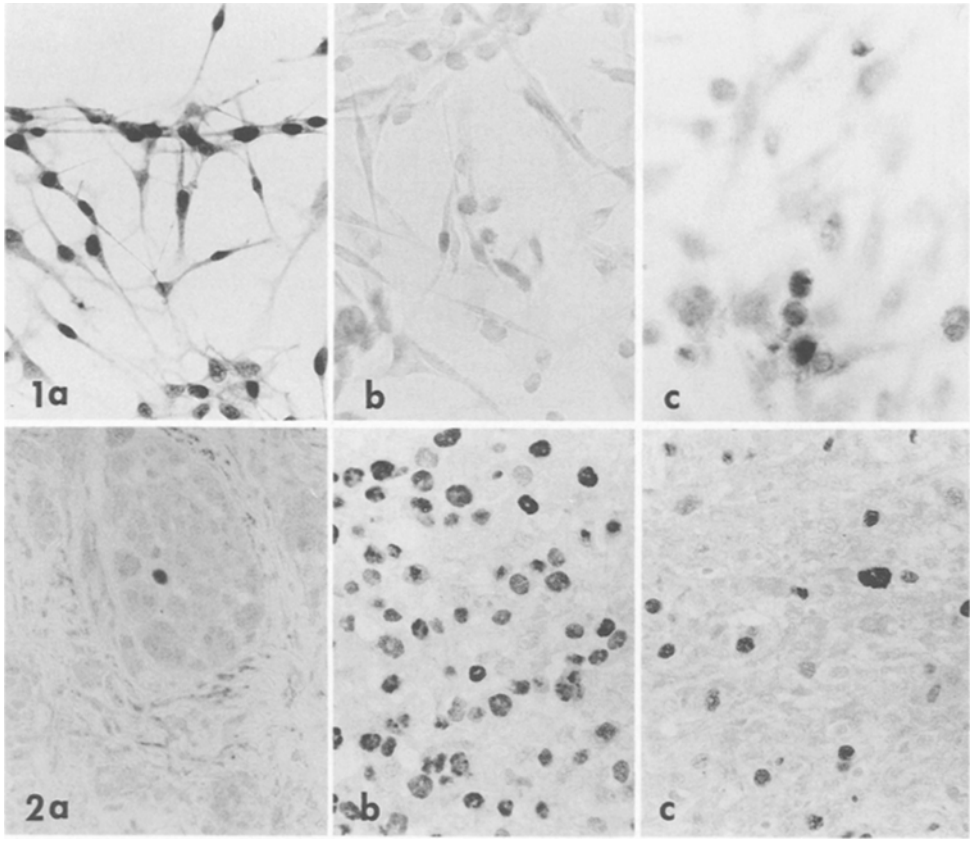

Fig. 1a–c. Immunocytochemistry of cultured human melanoma cells. Distinct nuclear labelling of NCL-CM1 is shown in acetone-fixed A2058 melanoma cells (**a**), whereas immunoproduct is weakly recognized in both the nucleus and the cytoplasm of formalin-fixed cells (**b**). Mutant protein is shown in the nucleus and/or perinuclear region of MM 96E melanoma cells by monoclonal antibody P240 (**c**). ×240

Fig. 2a–c. Immunohistochemistry of pathological tissues by NCL-CM1. Nuclear staining is weakly shown in dermal naevus (**a**), whereas uniform and intense labelling is observed in primary melanoma cells (**b**). Compared to the monotonous labelling in primary lesions, that seen in metastatic lesions is heterogeneously expressed (**c**). ×240

and the effect of fixatives on antigenic expression. In acetone and alcohol fixation (ethanol), distinct nuclear labelling by NCL-CM1 was observed in 5–30% of the cells. Staining of the cytoplasm and of the nucleus was only occasionally observed in formalin-fixed cells, and the reaction intensity in general tended to be weaker than in alcohol fixation (Fig. 1). No significant differences in immunoreactivity were observed among five melanoma cell lines with respect to either the proportion of reactive cells or intensity of reactions in individual cells. The cell lines showed varying levels of tyrosinase activity and melanin content, indicating that p53 expression is not correlated with melanogenesis or type of pigment cells. Regarding the reactivity of monoclonal antibody P240, the proportion of reactive cells and staining intensity were similar to those with NCL-CM1, except for formalin fixation, which abolished nuclear staining. The results of the proportion of positive cells and staining intensity by NCL-CM1 are summarized in Table 1.

Because of the inapplicability of P240 antibody to paraffin-embedded tissues, immunohistochemical studies were carried out using NCL-CM1 alone. In all 124 pathological specimens used in this study, the rate of p53 expression was observed to increase with advance in the stage of malignancy. In normal skin, no nuclear staining was observed in any cutaneous components. In addition, no reactive cells were seen in the skin from exposed areas, suggesting solar exposure (including ultraviolet irradiation) does not activate the expression of p53 oncoprotein at the histological level.

Among 45 lesions of common melanocytic naevi (CMN), 3 lesions of dermal naevi, 1 dermal component of compound naevi, and 1 lesion of lentigo simplex indicated nuclear staining, though at a low intensity (Fig. 2a). No reactive cells were recognized in any lesions of Spitz's naevi and dysplastic naevi. The overall proportion of positive cells in CMN ranged between 0 and 7.2% (mean 0.3%).

Nevertheless 16 (38%) of 42 primary malignant melanoma lesions showed distinct nuclear labelling by the NCL-CM1 antibody (Fig. 2b). Although a uniform intensity of nuclear staining was observed in primary melanomas, the proportion of p53-expressing cells in these lesions was found to rise in parallel with the level of tumour invasion (Fig. 3). However, once melanoma cells had invaded deeper than level III, no statistical significance was obtained between primary and metastatic lesions, suggesting that the accumulation of p53 oncoprotein levels off after a certain stage of tumour progression. Interestingly, in contrast to cutaneous primary melanomas, mucosal lesions showed a lower proportion of reactive cells (mean 0.1%) (Table 2). Among 37 lesions of metastatic melanomas, 21 (57%) were labelled in the nucleus despite being more heterogeneous than primary lesions (Fig. 2c), and the overall mean reactivities in primary and metastatic lesions were 3.4% and 4.8%, respectively.

The expression of p53 oncoprotein was also confirmed by Western blot analysis using P240 antibody against mutant-type oncoprotein and NCL-CM1 anti-

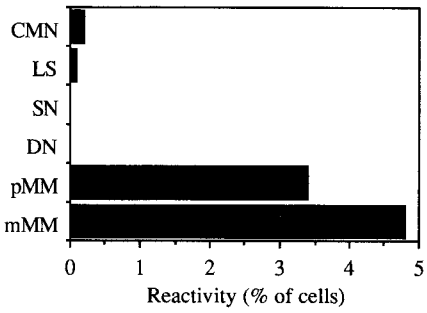

Fig. 3. The proportion of p53-expressing cells detected by NCL-CM1 in various melanocytic tumours. The proportion of nuclear labelling increases according to the stage of the tumour. CMN, Common melanocytic naevi; LS, lentigo simplex; SN, Spitz's naevi; DN, dysplastic naevi; pMM, primary melanoma; mMM, metastatic melanoma

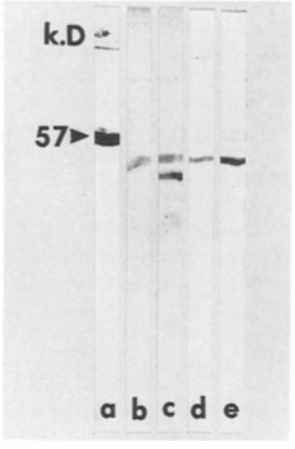

Fig. 4a–e. Western blot analysis of extracts from human melanoma cells. Molecular weight of vimentin is seen at 57 kDa in MM96E cell lysates (**a**). Monoclonal antibody against mutant protein, P240, shows a distinct single band in extracts of MM96E (**b**), whereas two bands are found in MM418 cell extracts, probably because of a degradation of oncoprotein (**c**). NCL-CM1 also reveals a intense band at 53 kDa in extracts from MM96E (**d**) and MM418 (**e**), respectively

body (Fig. 4). As shown in Fig. 4, a distinct band was recognized by P240 monoclonal antibody; its molecular weight was slightly lower than the 57 kDa indicated by the anti-vimentin antibody (Fig. 4a). Interestingly, MM96E showed a single band while MM418 had two bands. The second band was probably induced by a degraded protein (Fig. 4b, c). In addition, polyclonal antibody NCL-CM1 also revealed a major band at the same molecular weight of P240 antigen in melanoma cell extracts (Fig. 4d, e).

Discussion

Although various human malignant tumours, including breast (Ostrowski et al. 1991), lung (Iggo et al. 1990; Caamano et al. 1991; Iwaya et al. 1991; Hiyoshi et al. 1992), colon (Purdie et al. 1991) and others (Barton et al. 1991; Mazars et al. 1992; Villuendes et al. 1992), have been reported to show point mutations of genes and to express mutant-p53 oncoprotein (Porter et al. 1992), few studies have evaluated the expression and role of p53 oncoprotein in malignant melanomas (Strech et al. 1991; Akslen and Morkve 1992) or used polyclonal antibody against p53 (Barton et al. 1991; Slater et al. 1992). In the present study, we found that p53 oncoprotein was expressed in cultured human melanoma cell lines, extracts from melanoma cells, and pathological tissues. Of three different fixatives, the alcohol (ethanol) and acetone seemed to provide the most reliable record of p53 oncoprotein and the expression of p53 oncoprotein was mainly restricted to the nucleus of tumour cells, no matter what fixative was used. The molecular weight of the antigen recognized by two antibodies (NCL-CM1 and P240) was shown to be 53 kDa by Western blot analysis. p53 expression increased in parallel with the degree of malignant transformation; mucosal melanomas showed lower expression, hinting at a better prognosis for this type (Natali et al. 1989; Iida et al. 1990). Although the reactivity was not identical, the rate of p53 expression tended to be similar to that of proliferating nuclear cell antigen recognized by 19A2 monoclonal antibody (Takahashi et al. 1991a). Application of polyclonal antibody enabled a retrospective study of tumours of melanocytic origin to be made.

The occurrence of point mutations of genes in malignant melanoma has not been clearly established and thus the question arose whether the indications of p53 oncoprotein by NCL-CM1, which have been accepted as reliable in tumours other than melanomas (Bartek et al. 1991; Barton et al. 1991; Midgley et al. 1992; Sim et al. 1992; Slater et al. 1992), actually reflected a gene mutation in our specimens. There are two possible explanations for the readings of p53 oncoprotein in this study. First, malignant transformation, whatever the incidence of gene mutations, may cause a retardation of the degradation rate of the protein, resulting in an accumulation of the wild-type oncoprotein. Second, there may be an increase of mutant product with subsequent formation of complex of mutant- and wild-type proteins, resulting in an accumulation of total p53 oncoprotein.

Wynford-Thomas (1992) reported that a spontaneous transformation of rat thyroid cells expressing mutant *ras* oncogene was regularly associated with a strong p53 positivity by immunocytochemistry, but that the stabilized p53 protein was not the mutant type. Yamazaki et al. (1992) have shown that no gene mutation was observed in melanomas by the PCR-SSCP method.

However, point mutation was reported in 1 of 9 human melanoma cell lines (Volkenandt et al. 1991), while Stretch et al. (1991) showed an expression of mutant-p53 oncoprotein in 85% of frozen melanoma sections in an immunohistochemical study using monoclonal antibody P240 against mutant protein. In this latter study, metastatic melanomas showed a significantly higher prevalence of p53 protein over primary lesions. Bartek et al. (1992) also immunostained methacarn-fixed and paraffin-embedded sections of melanoma; 92% of the lesions

expressed p53 in tumour cells. Both of the latter authors suggested p53 mutation as a common factor in human malignant tumours, including melanomas. Furthermore, recent study showed that antigen recognized by NCL-CM1 was reported to be present in extracts from p53-mutant expressing cells (Midgely et al. 1992). In the present study, we have confirmed the expression of a mutant-type protein in cultures both by immunocytochemical method and by electrophoretical analysis using P240 monoclonal antibody. Therefore, it seems certain that melanoma cells express some mutant forms of p53 oncoprotein, although the nature of these is still unknown. The antigen recognized by NCL-CM1 is likely to be mutant type or a complex of mutant- and wild-type proteins.

However, malignant transformation occurs in a series of steps, and the overexpression of p53 oncoprotein may be reflecting only one aspect of the phenotypic changes resulting from random gene alterations and/or be a result of interaction with other oncogenes, including the *ras*- oncogene. Further studies will explore many additional questions, particularly on the role of this gene in melanocytic tumours. Chemical reagents, which directly activate various genes, and ultraviolet irradiation may provide the richest evidence, as this radiation is one of the most serious contributing factors for the progression of malignant melanoma in Caucasians. No evidence has yet come to light of p53 expression in tissues from UV exposed areas of Mongolians.

Acknowledgements. The authors thank Prof. M. Takahashi and Dr. T. Horikoshi for critical advice, N. Kajiura for technical assistance, and H. Ito for photographic work.

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