Expression of p21^{WAF1/CIP1} in fetal and adult tissues: simultaneous analysis with Ki67 and p53

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Abstract

Aims—To determine the expression of p21^{WAF1/CIP1} in relation to the expression of Ki67 and p53 in various normal adult and fetal tissues, and to investigate its distribution throughout the cell cycle.

Methods—The expression of p21^{WAF1/CIP1} in relation to Ki67 and p53 was analysed in adult and fetal tissues using immunohistochemical techniques. Heat induced epitope retrieval techniques were used to characterise the presence of p21^{WAF1/CIP1} in different tissues, as well as to detect its distribution throughout the cell cycle. In addition, flow cytometry and western blotting were used to test whether the level of p21^{WAF1/CIP1} expression varied at different phases of the cell cycle in phytohaemagglutinin (PHA) stimulated lymphocytes.

Results—p21^{WAF1/CIP1} expression varied from one tissue to another, and it was restricted mainly to the squamous and glandular epithelium, where it appeared in association with p53. Human tissues in which p21^{WAF1/CIP1} was found showed a mutually exclusive topographical sequential expression between p21^{WAF1/CIP1} and Ki67. This was confirmed by double labelling studies, which showed that p21^{WAF1/CIP1} positive cells were in the G₀ phase. Unlike these findings of a decline in p21^{WAF1/CIP1} expression after the G₀ phase, PHA stimulated lymphocytes showed a level of p21^{WAF1/CIP1} expression that rose as the cell progressed through the cell cycle.

Conclusions—The analysis of p21^{WAF1/CIP1} expression in relation to the status of p53 should take into account the existence of variable p21^{WAF1/CIP1} expression in different tissues. This could provide an explanation for the varying frequency of p53 mutations in tumours of different cellular origin. In tissues characterised by regular p21^{WAF1/CIP1} expression, it appears in a pattern that is consistent with the proposed role of this inhibitor of cyclin dependent kinases in cell cycle arrest—that of inducing cell differentiation. The conflicting results of in vivo and in vitro studies could support the hypothesis that microenvironmental conditions may influence the location of p21^{WAF1/CIP1} in different phases of the cell cycle.

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p21^{WAF1/CIP1} is a 21 kDa cell cycle regulatory protein that can inhibit the kinase activities of a variety of cyclin dependent kinases (CDKs) involved in cell cycle control.¹⁻³ This protein was identified originally in normal human fibroblasts as one component of a quaternary complex with cyclin, CDKs, and proliferating cell nuclear antigen (PCNA).⁴ Recent studies have clarified the means by which p21^{WAF1/CIP1} interacts with CDKs, giving rise to observations suggesting that catalytically active cyclin-CDK-p21^{WAF1/CIP1} complexes contain a single p21^{WAF1/CIP1} molecule, while inactive complexes contain multiple p21^{WAF1/CIP1} molecules.⁵

In addition to its kinase inhibitor activity, p21^{WAF1/CIP1} is able to inhibit DNA replication in the S phase of the cell cycle by interaction with PCNA, blocking the ability of PCNA to activate DNA polymerase.⁶ Different and separate domains in the p21^{WAF1/CIP1} molecule have been described for the inhibition of CDKs and PCNA.⁷⁻⁸ Yang *et al*⁹ have confirmed that the growth of malignant cells, both in vitro and in vivo, is inhibited by the expression of p21^{WAF1/CIP1}, while in some cases this blockage of the cell cycle is associated with the induction of terminal differentiation.

p21^{WAF1/CIP1} was described initially as a wild-type p53 inducible molecule, involved as a downstream effector of p53 tumour suppression.¹⁰⁻¹¹ The exposure of cells to agents that cause DNA damage leads to p53 binding to DNA breakpoints, and subsequent p53 dependent induction of p21^{WAF1/CIP1} expression that inhibits CDKs during the G₁/S phase of the cell cycle.¹¹⁻¹² Cells defective in p53 lack active p21^{WAF1/CIP1} after treatment with DNA damaging radiation or chemotherapy.¹¹⁻¹³ Nevertheless, p21^{WAF1/CIP1} has been shown to be regulated independently of p53 in different cell lines, as may be observed also in normal tissue development in p53 deficient mice and following serum stimulation.¹⁴⁻¹⁵ This p53 independent induction of p21^{WAF1/CIP1} has been shown to correlate with the growth arrest associated with differentiation during the G₀ phase of the cell cycle.¹⁶⁻¹⁷

Previous studies on p21^{WAF1/CIP1} expression have been performed mainly in embryonic and adult mouse tissues,¹⁸⁻¹⁹ with the exception of some detailed studies on p21^{WAF1/CIP1} expression in human gastrointestinal tract mucosa,²⁰⁻²¹ and a recent study characterising the staining patterns of different p21^{WAF1/CIP1} antibodies in normal human tissue.²²

An initial search for p21^{WAF1/CIP1} expression in various adult human tissues showed selective

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Table 1 Expression of p21^{WAF1/CIP1}, p53, and Ki67 protein in adult and fetal normal tissues

Tissue	Cell type	Adult			Tissue	Fetal			
		Ki67	p21	p53		Ki67	p21	p53	Rb protein
Skin	Basal cells	–	–	+	Skin basal cells	++	–	–	++
	Suprabasal cells	+++	+	+		–	–	–	
	Spinous layer cells	–	++	–	Cerebellum	++	–	–	+
Cerebellum	Neurons/glia	–	–	–					
Eye		++	–	–	Lymph nodes	+	–	–	++
Reactive lymph nodes	Germinal centre	+++	+	+					
Spleen	Interfollicular area	++	+	+					
Thymus		++	–	–					
Tonsil	Epithelial cells	+	++	+					
	Lymphoid cells	+++	+	+					
	Basal cells	–	–	–					
Salivary gland	Suprabasal cells	+++	+	+					
	Close up suprabasal cells	–	++	–					
	Germinal centre	+++	+	+					
Oesophagus	Interfollicular area	++	+	+					
	Ducts/acines	+	–	–					
	Squamous epithelium:				Primitive epithelium digestive tube	++	–	–	++
Stomach	Basal cells	–	–	–					
	Suprabasal cells	+++	+	+					
	Close up suprabasal cells	–	++	–					
Small and large intestine	Gastric epithelium:								
	Base	–	–	–					
	Neck	–	+	–					
	Isthmus	+++	+	–					
Liver	Pit	+	++	–					
	Intestinal epithelium:								
	Crypt/base	+++	–	–					
Pancreas	Crypt/superficie	–	++	–					
		–	–	–	Liver hepatocytes	–	–	–	++
		–	–	–	Haematopoiesis	+++	–	–	+++
Lung		–	–	–	Pancreas	++	–	–	+++
Heart		–	–	–	Lung	++	–	–	++
Smooth muscle		–	–	–	Heart	++	–	–	++
Skeletal muscle		–	–	–	Muscle	++	–	–	++
Kidney		–	–	–					
Adrenal gland	Glomeruli/tubules	+	–	–	Kidney	+++	–	–	++
	Cortex:				Adrenal gland:				
	Glomerulosa	++	+	–	Definitive cortex	++	+	+	++
Thyroid gland	Fasciculada	+	++	–	Primitive cortex	+	++	–	+++
	Reticular	+	+	–	Medula	++	–	–	+
	Medulla	+	++	–					
Breast	Ducts/follicle	–	–	–					
Cervix		–	–	–					
Placenta	Squamous epithelial:								
	Basal cells	–	–	–					
	Suprabasal cells	+++	+	+					
Testis	Close up suprabasal cells	–	++	–					
	Sincitiotrophoblast	–	++	–					
	Citotrophoblast	+++	–	++					
Hypophysis	Mesenchyme villus	+	–	–					
	Seminiferous tubule	++	–	+					
	Leydig cells	–	+	+					
Pineal	Efferent/epididymis	+	++	–					
Ovary	Mesenchyme/vasos	–	+	–					
Prostate	Nervous/glandular zones	–	–	–					
Uterus		–	–	–					

–, no staining; +, weak staining; ++, medium staining; +++, strong staining. Rb, retinoblastoma.

expression of this protein, restricted to some cell populations in certain tissues. This has led to a more comprehensive characterisation of p21^{WAF1/CIP1} distribution in adult human tissues, in an attempt to establish a relation between p21^{WAF1/CIP1}, p53, and Ki67 expression.

Our results indicate that the p21^{WAF1/CIP1} protein, associated with p53, is expressed selectively by squamous and mucous secreting epithelial cells in adult tissues, in a pattern associated with cell differentiation.

To address the question of whether the expression of p21^{WAF1/CIP1} in different tissues having different growth fractions could be explained on the basis of p21^{WAF1/CIP1} modulation during the cell cycle, we tested whether the level of expression of p21^{WAF1/CIP1} varied throughout the different phases of the cell cycle

in phytohaemagglutinin (PHA) stimulated lymphocytes, using flow cytometry and western blotting.

Materials and methods

TISSUES

Normal adult and fetal human tissues were obtained from the routine files of the pathology department, in the Virgen de la Salud Hospital, Toledo, Spain. Fetal tissue was collected from 12–13 week spontaneous abortion specimens, sent in for study by the pathology department.

Five paraffin blocks of different surgical specimens were analysed in each tissue. They were fixed in buffered formalin for 24 hours shortly after surgery, and embedded in paraffin

Figure 1 Ki67 (A), p21^{WAF1/CIP1} (B), and p53 (C) immunostaining. Reactive lymphoid follicles showing Ki67 labelling of most germinal centre cells, but with scattered p53 and p21^{WAF1/CIP1} positive cells. In contrast to this finding, the squamous epithelium shows Ki67 (D) labelling of suprabasal cells, p21^{WAF1/CIP1} generalised expression by close up suprabasal cells (E), and a less intense p53 staining of basal nuclei (F). Double staining for Ki67 (peroxidase) and p21^{WAF1/CIP1} (alkaline phosphatase) in the tonsillar epithelium (G) showing mutually exclusive Ki67 and p21^{WAF1/CIP1} staining. Immunoperoxidase staining counterstained with haematoxylin. (Original magnification $\times 400$, except G, $\times 1000$.)

following routine procedures. Representative sections of each paraffin tissue block were stained with haematoxylin and eosin and examined microscopically to confirm histological preservation. Parallel sections were then processed for immunohistochemical studies.

PERIPHERAL BLOOD LYMPHOCYTES

Normal peripheral blood was obtained by venepuncture from voluntary healthy donors. Peripheral blood lymphocytes (PBLs) were isolated by Histopaque (Sigma, St Louis, USA) density gradient centrifugation and washed in RPMI-1640 medium. Cells were kept at 37°C in a 5% CO₂ humidified incubator in cell culture flasks, at 2×10^6 cells/ml of RPMI-1640 medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 2% PHA (Gibco, BRL, Glasgow, UK). Aliquots of the activated cells were harvested every 24 hours and prepared for analysis.

IMMUNOHISTOCHEMISTRY

Immunostaining techniques were performed in paraffin embedded tissue. A step of heat induced retrieval antigen, in a solution of sodium citrate buffer, was performed before incubation with antibodies. Xylene dewaxed and alcohol rehydrated paraffin sections were placed in Coplin jars filled with a 0.01 M tri-sodium citrate solution, and heated for three minutes in a conventional pressure

cooker. After heating, slides were rinsed in cool running water for five minutes. They were washed quickly in Tris buffered saline (TBS) pH 7.4, and incubated with primary antibodies specific for: p21^{WAF1} (antibody WAF1; Oncogene Science, Cambridge, Massachusetts, USA); p53 (antibody DO7; Novocastra, Newcastle, UK); Ki67 (antibody MIB1; Novocastra); and Rb (antibody PMG3-245; Pharmin-gen, San Diego, California, USA).

Following incubation with the primary antibody, immunodetection was performed with biotinylated antimouse immunoglobulins, followed by peroxidase labelled streptavidine (Dako) with diaminobenzidine (DAB) chromogen as substrate. All immunostaining was performed using the TechMate 500 (Dako) automatic immunostaining device. Incubation omitting the specific antibody, as well as with unrelated antibodies, was used as a control of the technique.

For double immunostaining, sections were incubated first with the MIB1 monoclonal antibody followed by incubation with biotinylated antimouse immunoglobulin (Dako). Peroxidase labelled streptavidine was then added and developed with DAB. The sections were then incubated with WAF1, followed by biotinylated antimouse immunoglobulin and alkaline phosphatase labelled streptavidine. A mixture of naphthol phosphatase and new fuchsin was used as chromogen.

Figure 2 Thymic immunostaining for Ki67 (A), p21^{WAF1/CIP1} (B), and p53 (C). Although thymic cortical cells exhibit a high growth fraction, p53 and p21^{WAF1/CIP1} expression appears to be restricted mainly to epithelial cells. The mucous secreting epithelium of gastric foveolae staining showing Ki67 labelling of isthmus gastric cells (D), in contrast to the p21^{WAF1/CIP1} staining of gastric pit cells (E). Immunoperoxidase staining counterstained with haematoxylin. (Original magnification $\times 160$, except C $\times 400$.)

The intensity of expression was scored semi-quantitatively by two independent observers as: 0 (no cell staining), 1+ (isolated cell staining), 2+ (intermediate number of cells stained), or 3+ (staining of most cells).

WESTERN BLOTTING

Peripheral blood lymphocytes (PBL) and PHA stimulated PBLs were washed twice with cold phosphate buffered saline (PBS) before the protein extraction procedure. Protein was extracted with a triple detergent lysis buffer (50 mM Tris HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulphate (SDS), 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, phenylmethylsulphonyl fluoride 100 $\mu\text{g}/\text{ml}$, aprotinin 50 $\mu\text{g}/\text{ml}$) for 30 minutes at 4°C. Extracts were cleared by centrifugation.

Extracted protein was resolved by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose-ECL (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. The blots were blocked overnight with 5% bovine serum albumin in PBS at 37°C. p53 and p21^{WAF1/CIP1} proteins were detected by DO7 and WAF1 monoclonal antibodies, respectively, followed by incubation with goat antimouse coupled to

horseradish peroxidase (Amersham). Blots were developed using the chemiluminescence ECL detection kit (Amersham).

Proteins from PBLs and PHA stimulated PBLs were incubated with an actin specific monoclonal antibody (Amersham) to control for the amount and quality of the protein extracted.

FLOW CYTOMETRY

The expression of p21^{WAF1/CIP1}, p53, and Ki67 was also studied using flow cytometry analysis of PHA activated lymphocytes. For detection of the three proteins by immunofluorescence, fixation/permeabilisation for optimal immunodetection was performed with paraformaldehyde/triton X-100. The monoclonal antibodies used included WAF1, DO7-FITC (fluorescein isothiocyanate; Dako), and Ki67-FITC (Dako). An isotype matched negative control was used to estimate the quantity of non-specific binding.

Before labelling, the cells were incubated for 30 minutes with 20% goat serum. They were then incubated for 30 minutes at 4°C with the relevant monoclonal antibodies, followed by two consecutive washes in 0.1% bovine serum albumin diluted in PBS. For p21^{WAF1/CIP1} detection, cells were incubated once again (30 min-

Figure 3 Ki67 (A), p21^{WAF1/CIP1} (B), double Ki67/p21^{WAF1/CIP1} (C), and p53 (D) staining in the placenta: cytotrophoblast cells show Ki67 and p53 labelling, while syncytiotrophoblast cells only have distinct p21^{WAF1/CIP1} labelling. Immunostaining of the testis revealing Ki67 reactivity of germinal cells within the seminiferous tubule (E) and p21^{WAF1/CIP1} staining of Leydig cells (F). Fetal muscle cells showing Ki67 (G) and p21^{WAF1/CIP1} (H) staining. Immunoperoxidase staining counterstained with haematoxylin. (Original magnification $\times 400$, except D $\times 1000$.)

utes at 4°C) with goat antimouse monoclonal antibody (F(ab)'2 fragments) conjugated with FITC (Dako). After two more washes, the cells were analysed.

Parallel samples of PHA activated lymphocytes were prepared for the study of the nuclear DNA content by flow cytometry using the CycleTest DNA reagent kit (BD Immunocytometry System). Sample preparation, data acquisition, and analysis have been described elsewhere.²³

Double staining for p21^{WAF1/CIP1} and DNA was performed in PHA activated lymphocytes

that had been fixed previously, permeabilised, and indirectly labelled for p21^{WAF1/CIP1} using a FITC tagged secondary antibody. The lymphocytes were then incubated (30 minutes at room temperature) with 100 μ l of RNase (2 mg/ml) and 200 μ l of propidium iodide (2 mg/ml), after which the cells were analysed.

Flow cytometry was performed using a FACSORT instrument (Becton Dickinson, San Jose, California, USA), equipped with a double discrimination module. Cell cycle analysis was carried out using the CELLFIT software package for data acquisition and

Figure 4 (A) Flow cytometry analysis of p21^{WAF1/CIP1}, p53, and Ki67 protein expression in phytohaemagglutinin stimulated peripheral blood lymphocytes. Fluorescence histogram of unstimulated PBLs, and PBLs after 24 hours, 48 hours, and 72 hours of PHA stimulation. An increase in the mean fluorescence intensity of p21^{WAF1/CIP1}, p53 and Ki67 is evident. The vertical marker, positioned on negative control samples, separates the non-reactive (left) from the reactive (right) cells. (B) Relation between p21^{WAF1/CIP1} and the cell cycle in PHA stimulated lymphocytes (B). Analysis of p21^{WAF1/CIP1} (ordinate)/DNA (abscissa) double labelled samples; the horizontal windows define the reactivity threshold for p21^{WAF1/CIP1}.

analysis. Protein analysis was undertaken using the LYSIS II software program (Becton Dickinson).

Results

EXPRESSION OF P21^{WAF1/CIP1} IN NORMAL HUMAN TISSUE

A differential pattern of p21^{WAF1/CIP1} expression was observed. Results have been grouped according to the intensity of expression.

Adult tissues with low or null p21^{WAF1/CIP1} expression

In the brain, eye, spleen, salivary gland, liver, pancreas, lung, heart, smooth and skeletal muscle, kidney, breast, hypophysis, pineal gland, and uterus, p21^{WAF1/CIP1} expression was found only in rare isolated cells (table 1). A similar pattern of reactivity was found for p53.

Isolated p21^{WAF1/CIP1} positive cells were identified in nearly all the reactive lymphoid tissue samples analysed (mainly tonsil and lymph node). From one to three lymphoid cells/high power field were detected in the germinal centre and interfollicular areas (fig 1B). p53 positive cells were seen in these lymphoid tissues, paralleling p21^{WAF1/CIP1} distribution and intensity (figs 1B and C) (table 1).

Ki67 expression was present in all these tissues at different levels of intensity. Low Ki67 levels were detected in the majority of tissue types, with the exception of reactive lymphoid tissue, where the majority of germinal centre and large interfollicular cells show strong reactivity (fig 1A) (table 1).

Adult tissues with high p21^{WAF1/CIP1} expression

The squamous epithelium in skin, tonsil, oesophagus, and cervix showed a topographically sequential expression for p21^{WAF1/CIP1} and Ki67. Detailed analysis of reactivity for the different proteins is shown in the table 1. Double staining for Ki67 and p21^{WAF1/CIP1} was performed in tonsillar squamous epithelium; this confirmed the mutually exclusive expression of these two proteins, from Ki67⁺ p21⁻ suprabasal cells to Ki67⁻ p21⁺ spinous stratum cells. (figs 1D–G).

In the thymus, p21^{WAF1/CIP1} staining of epithelial cells was very intense, unlike the weak staining of lymphoid cells. Ki67 reactivity was mainly found in lymphoid cells. Mild p53 expression was observed (figs 2A–C).

The glandular epithelium in the stomach and intestine also showed mutually exclusive and sequential staining for p21^{WAF1/CIP1}, and Ki67 (table 1, figs 2D–E).

The adrenal gland, testis, and placenta also showed p21^{WAF1/CIP1} staining in some cell subsets, although always with an inverse relation between Ki67 and p21^{WAF1/CIP1} staining (table 1, figs 3A–F). Cytotrophoblast cells in the placenta exhibited striking p53 staining, more intense in this cell layer than in any other tissue (fig 3D).

Fetal tissue

p21^{WAF1/CIP1} protein expression was not present in the majority of cell types, even those in which expression was detectable in the adult tissue. In the adrenal gland, p21^{WAF1/CIP1} positive

Figure 5 Flow cytometry data for p21^{WAF1/CIP1}, p53, and DNA index expression in phytohaemagglutinin stimulated peripheral blood lymphocytes. %S, percentage of cells in the S phase of the cell cycle.

cells were observed mainly in the primitive cortex and some definitive cortex cells (simultaneously with p53); the adrenal medulla was negative. Ki67 staining was the reverse (table 1).

In skeletal muscle tissue the p21^{WAF1/CIP1} protein was detected in isolated nuclei (fig 3H; table 1).

On the other hand, Rb protein expression was observed in all tissues at variable levels of intensity. Rb and Ki67 proteins displayed simultaneous expression in most cell types (table 1).

EXPRESSION OF P21^{WAF1/CIP1} IN PHA STIMULATED PBLs

Peripheral blood lymphocytes were stimulated with PHA for 72 hours and analysed for the amount of S phase cells by flow cytometry. Ki67 fluorescence intensity along the cell cycle was investigated in parallel samples. The results are shown in figs 4 and 5.

The amount of p21^{WAF1/CIP1} and p53 protein expression was measured in PHA stimulated PBLs by western blotting and flow cytometry.

Figure 6 Western blot analysis of p53, p21^{WAF1/CIP1}, and actin proteins in phytohaemagglutinin stimulated peripheral blood lymphocytes.

The results of both techniques were similar (figs 6 and 4, respectively). The amount of p21^{WAF1/CIP1} protein increased 24 hours after stimulation with PHA, followed by stabilisation of the p21^{WAF1/CIP1} protein level. On the other hand, the percentage of S phase cells began to increase after 24 hours of PHA stimulation, reaching a peak at 72 hours, which was paralleled by the expression of p53 (fig 5).

Dual colour flow cytometry analysis of PHA stimulated lymphocytes simultaneously labelled with propidium iodide and anti-p21-FITC confirmed the increase in p21^{WAF1/CIP1} after PHA stimulation, as illustrated in fig 4B. This study did not show any significant variation of p21^{WAF1/CIP1} expression between different phases of the cell cycle.

Discussion

This study examined p21^{WAF1/CIP1} expression in relation to the expression of Ki67 and p53, in different normal adult and fetal tissues and in PHA stimulated PBLs.

A selective distribution of p21^{WAF1/CIP1} was observed, with preferential expression by the squamous and glandular epithelium, and weak or undetectable expression in other tissues. One of the striking findings of this study is the relatively low or zero level of p21^{WAF1/CIP1} expression in reactive lymphoid tissue. The germinal centres of lymphoid follicles are the adult topographical compartment where most proliferating cells may be found and it would be expected that p21^{WAF1/CIP1} and other CDK inhibitors would play a role as cell cycle brakes. However, the absence of p21^{WAF1/CIP1} expression suggests that the p21^{WAF1/CIP1} function of negative cell cycle control must be redundant to other negative regulators. The tissues where p21^{WAF1/CIP1} appears in selectively expressed form also show a characteristic expression of p53. This is a rather striking result of this work, and has not been described before. It supports the hypothesis that the expression of p21^{WAF1/CIP1} in these tissues could be secondary to the role that p53 plays in cell differentiation, as has already been suggested.²⁴

This dissimilar expression of p21^{WAF1/CIP1} in the different tissues studied should be taken into account when studies on p21^{WAF1/CIP1} expression in relation to p53 status are performed. It could explain why some of the studies performed to date on this topic reach contradictory conclusions. In non-Hodgkin's lymphoma, p21^{WAF1/CIP1} expression is abolished in cases with p53 mutations,^{25 26} in other tissues it appears to be at least partially independent of p53 status (for example, in pancreatic²⁷ and some colorectal cancers²¹). In addition, the restriction in p21^{WAF1/CIP1} and p53 expression in some normal human tissues could contribute to the variable frequency of p53 mutations in tumours derived from different tissues.

In most of the tissues studied, p21^{WAF1/CIP1} is expressed by Ki67 negative cells, suggesting that p21^{WAF1/CIP1} expression in normal human tissues is restricted to cells in the G₀ phase. A prototype of this situation is found in the squamous epithelium of the tonsil, oesophagus, cervix, and skin where a topographical

alternative pattern of expression between p21^{WAF1/CIP1} and ki67 is observed. Ki67 expression appears to be restricted to proliferative compartments, whereas p21^{WAF1/CIP1} reactivity is found in differentiating cells that have already ceased DNA replication. These observations are consistent with previous descriptions in gastrointestinal tract mucosa, suggesting that there is a topological control of p21^{WAF1/CIP1} expression in normal cells that becomes disordered during neoplasia.²⁰ In those organs in which it is possible to establish a gradient of maturation (squamous and glandular epithelium), p53 appears at an earlier stage than p21^{WAF1/CIP1}.

A surprising finding was the absence of p21^{WAF1/CIP1} expression in the majority of the fetal tissues studied, where it was only weakly detected in the adrenal gland and skeletal muscle. This is consistent with data obtained in mice lacking p21^{WAF1/CIP1}, which appear to develop normally, suggesting that any essential role for p21^{WAF1/CIP1} in terminal differentiation must be redundant to other cell cycle inhibitors.²⁸ Recent studies on p27^{KIP1} deficient mice suggest that this protein could be important in terminal differentiation during fetal development, as its absence leads to a syndrome of multiorgan hyperplasia.^{29,30} Unlike p21^{WAF1/CIP1}, Rb was strongly expressed in all fetal tissues; this is in agreement with studies showing that Rb plays a key role in controlling the cell cycle and differentiation in embryonic development.^{27,31,32}

Lastly, the expression of p21^{WAF1/CIP1} through the cell cycle was investigated, in an attempt to elucidate how modulation of p21^{WAF1/CIP1} expression could explain the differences found in p21^{WAF1/CIP1} localisation in some tissues. Both western blotting and flow cytometry studies showed an increase of p21^{WAF1/CIP1} along the progression of the cell cycle, the intensity found in activated lymphocytes being roughly twice that observed in PBLs. This difference is slightly less than that observed for p53 (1:3), and is significantly less than that observed in the proportion of S phase cells (1:20). Double labelling studies have confirmed that, in this model, activated lymphocytes exhibit p21^{WAF1/CIP1} labelling. Although this cannot explain the differences found in different tissues, it suggests that the cell cycle phases in which p21^{WAF1/CIP1} is expressed may vary in different models. In this respect, it should be emphasised that in PHA stimulated PBLs, p21^{WAF1/CIP1} also appears in parallel with p53.

To conclude, this study shows that in vivo p21^{WAF1/CIP1} expression is restricted to certain cells and tissues (preferentially the squamous and glandular epithelium), and that in these tissues it appears in association with p53. Tissues characterised by p53 and p21^{WAF1/CIP1} expression show a mutually exclusive topographical sequential expression between p21^{WAF1/CIP1} and Ki67, indicating that p21^{WAF1/CIP1} positive cells are in the G₀ phase. Nevertheless, in in vitro PHA stimulated lymphocytes, p21^{WAF1/CIP1} appears to increase as the cell cycle progresses, suggesting that the location of p21^{WAF1/CIP1} in different phases of the

cell cycle may be variable, and dependent on microenvironmental conditions.

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- Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 1993;75:805-16.
- Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nature* 1993;366:701-4.
- Hunter T, Pines J. Cyclins and cancer III: cyclin D and CDK inhibitors come of age. *Cell* 1994;79:573-82.
- Xiong Y, Xiang H, Beach D. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell* 1992;71:505-14.
- Zhang H, Hannon GJ, Beach D. p21-containing cyclin kinases exist in both active and inactive states. *Genes Dev* 1994;8:1750-8.
- Waga S, Hannon GJ, Beach D, Stillman B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* 1994;369:574-8.
- Li R, Waga S, Hannon GJ, Beach B, Stillman B. Differential effects of the p21 CDK inhibitor on PCNA dependent DNA replication and repair. *Nature* 1994;371:534-7.
- Chen J, Jackson PK, Kirschner MW, Dutta A. Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature* 1995;374:386-8.
- Yang ZY, Perkins ND, Ohno T, Nabel EG, Nabel GJ. The p21 cyclin-dependent kinase inhibitor suppresses tumorigenicity in vivo. *Nat Med* 1995;1:1052-6.
- El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, et al. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993;75:817-25.
- El-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, et al. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res* 1994;54:1169-74.
- Bae I, Smith ML, Sheikh MS, Zhan Q, Scudiero DA, Friend SH, et al. An abnormality in the p53 pathway following X-irradiation in many wild-type p53 human melanoma lines. *Cancer Res* 1996;56:840-7.
- Dulic V, Kaufman WK, Wilson SJ, Tlsty TD, Lees E, Harper JW, et al. P53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell* 1994;76:1013-23.
- Michieli P, Chedid M, Lin P, Pierce JH, Mercer WE, Givol D. Induction of WAF1/CIP1 a p53-independent pathway. *Cancer Res* 1994;54:3391-5.
- Steinman RA, Hoffman B, Iro A, Guillof C, Liebermann DA, el-Housseini ME. Induction of p21 (WAF1/CIP1) during differentiation. *Oncogene* 1994;9:3389-96.
- Zhang W, Grasso L, McClain CD, Gambel AM, Cha Y, Travali S, et al. P53-independent induction of WAF1/CIP1 in human leukemia cells is correlated with growth arrest accompanying monocyte/macrophage differentiation. *Cancer Res* 1995;55:668-74.
- Halevy O, Novitsch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, et al. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* 1995;267:1018-21.
- Macleod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, et al. P53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes Dev* 1995;9:935-44.
- Parker SB, Eichele G, Zhang P, Rawls, Sands, Bradley A, et al. p53-independent expression of p21/Cip1 in muscle and other terminally differentiating cells. *Science* 1995;267:1024-7.
- El-Deiry W, Tokino T, Waldman T, Oliner JD, Velculescu VE, Burrell M, et al. Topological control of p21(WAF1/CIP1) expression in normal and neoplastic tissues. *Cancer Res* 1995;55:2910-9.
- Dogliani C, Pelosio P, Laurino L, Macri E, Meggiolaro E, Favretti F, et al. p21/waf1/cip1 expression in normal mucosa and in adenomas and adenocarcinomas of the colon: its relationship with differentiation. *J Pathol* 1996;179:248-53.
- Fredersdorf S, Milne AW, Hall PA, Lu X. Characterization of a panel of novel anti-p21^{Waf1/Cip1} monoclonal antibodies and immunochemical analysis of p21^{Waf1/Cip1} expression in normal human tissues. *Am J Pathol* 1996;148:825-35.
- Vindelov LL, Christensen IJ, Nissen NI. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* 1992;13:502-9.
- Lutzker SG, Levine AJ. A functionally inactive p53 protein in teratocarcinoma cells is activated by either DNA damage or cellular differentiation. *Nat Med* 1996;2:804-10.
- Villuendas R, Pezzella F, Gatter K, Algara P, Sanchez-Beato M, Martinez P, et al. P21^{WAF1/CIP1} and MDM2 expression in NHL. Relation to p53 status. A p53⁺ MDM2/p21-immunophenotype associated with missense p53 mutations. *J Pathol* [In press.]
- Chilosi BM, Dogliani C, Magalini A, Inghirami G, Krampera M, Nadali G, et al. P21/waf1 cyclin-kinase inhibitor expression in non-Hodgkin's lymphomas: a potential marker of p53 tumor-suppressor gene function. *Blood* 1996;88:4012-20.

- 27 DiGiuseppe JA, Redston MS, Yeo CJ, Kern SE, Hruban RH. p53-independent expression of the cyclin-dependent kinase inhibitor p21 in pancreatic carcinoma. *Am J Pathol* 1995;147:884-8.
- 28 Deng C, Zhang P, Harper JW, Elledge SJ, Leder P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* 1995;82:675-84.
- 29 Fero M, Rivkin M, Tasch M, Porter P, Carow CE, Firpo E, *et al.* A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27^{Kip1}-deficient mice. *Cell* 1996;85:733-44.
- 30 Kiyokawa H, Kineman RD, Manova-Todovora KO, Soares VC, Hoffman ES, Ono M, *et al.* Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27^{Kip1}. *Cell* 1996;85:721-32.
- 31 Lee EY-HP, Chang C-Y, Hu N, Wang Y-CJ, Lai C-C, Her-rup K, *et al.* Mice deficient for Rb are nonviable and show effects in neurogenesis and hematopoiesis. *Nature* 1992;359:288-94.
- 32 Szekeley L, Jian W-Q, Bulic-Jakus F, Rosen A, Ringertz N, Klein G, *et al.* Cell type and differentiation dependent heterogeneity in retinoblastoma protein expression in SCID mouse fetuses. *Cell Growth Differ* 1992;3:149-56.