p53, Rb and bcl-2 expression during the cell cycle: a study in phytohaemagglutinin stimulated lymphocytes and microwave irradiated lymphoid tissue sections

M S Mateo, M Sanchez-Beato, J C Martinez, A Orfao, J L Orradre, M A Piris

Abstract

Aims—To determine the expression of p53, Rb, and bcl-2 during the cell cycle in stimulated peripheral blood lymphocytes (PBLs) and microwave heated reactive lymphoid tissue sections.

Methods—The expression of p53, Rb and bcl-2 proteins in paraffin wax embedded tonsil tissue sections was detected by immunohistochemistry using an (APAAP) technique following microwave irradiation. Flow cytometric analysis as performed on phytohaemagglutinin (PHA) stimulated PBLs, with simultaneous S fraction determination.

Results—Expression of p53 protein was detected in reactive tonsil germinal centre cells, in some suprabasal cells in the surface and cryptic epithelium, and in some endothelial cells. Analysis of p53 in PHA stimulated PBLs revealed expression of p53 by non-tumoral activated lymphocytes. Rb protein expression was increased in PHA stimulated PBLs and was usually detected in most germinal centre B cells, in isolated paracortical cells, in a fraction of endothelial cells, and in most epithelial suprabasal cells. Expression of bcl-2 in stimulated lymphocytes was inversely correlated with proliferation. This confirms findings in reactive tonsil tissue samples, where proliferating cells located in the germinal centres and paracortical area are mostly bcl-2 negative.

Conclusions—Expression of these three oncogenic and tumour suppressor proteins varies during the cell cycle in non-tumoral cells. Consequently, tumoral growth fraction must be taken into account when analysing dysregulation of these three genes in lymphomas and other tumours. The p53 protein may be detected in benign conditions, as its expression is not synonymous with malignancy or mutation of the p53 gene.

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promotion, as well as inactivation of growth suppressor genes.¹ The final consequence of this genetic dysregulation is the loss of control of normal cell proliferation and differentiation. Oncogenes, or tumour suppressor genes, involved in lymphomagenesis include the p53, bcl-2 and the retinoblastoma (Rb) genes.

The p53 tumour suppressor gene is located on the short arm of chromosome 17, which encodes a nuclear phosphoprotein that plays an important role in the negative control of cell proliferation and regulation of the cell cycle.²⁻⁴ Moreover, p53 plays a fundamental role in the prevention of genomic instability. Following DNA damage, concentrations of the p53 protein increase, blocking progression along the cell cycle in the G1/S checkpoint, permitting DNA repair or inducing apoptosis;⁵ p53 also promotes B cell differentiation.^{6,7} Wild-type p53 has a short half life of about five to 20 minutes,⁸ and is usually undetectable by immunohistochemistry. Nevertheless, mutations or other mechanisms (interaction with viral or other proteins), stabilises the protein and increases its half life to four to eight hours,⁹ permitting detection by immunohistochemistry. Detectable p53 protein expression in non-Hodgkin's lymphomas is associated with an advanced stage of tumoral progression¹⁰ and a poor response to chemotherapy.¹¹

Rb is located on chromosome 13. It encodes a nuclear phosphoprotein which is also involved in regulation of the cell cycle.¹² Further studies have shown that alterations in the Rb gene may have an aetiological role in the genesis of other tumours. Deletion or mutation of the Rb gene is present in bone and soft tissue sarcomas, and in carcinomas of the lung, breast, bladder, and prostate.¹³⁻¹⁹ In a previous study we found that, during the cell cycle, both normal and stimulated lymphocytes showed a gradual increase in Rb protein concentration, with a peak in the M phase. Concurrently, a subset of high grade lymphomas seemed to have abnormally low Rb protein expression.²⁰

The bcl-2 oncogene was isolated at translocation site t(14;18), found in follicular CB-CC lymphomas.²¹ This translocation results in the overexpression of bcl-2 oncoprotein, which rescues cells from programmed cell death, or apoptosis. However, bcl-2 protein is expressed by several non-tumoral cell types and different human tumours in the absence of 14;18 translocation,²² suggesting that mechanisms other than translocation may induce overexpression

Department of Pathology, Hospital "Virgen de la Salud", Toledo, Spain

M S Mateo
M Sanchez-Beato
J C Martinez
J L Orradre
M A Piris

General Service of Cytometry, University of Salamanca, Spain
A Orfao

Correspondence to Dr M S Mateo-Guerrero, Department of Pathology, Hospital "Virgen de la Salud", Av Barber s/n, 45004 Toledo, Spain.

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Sequential study of transformation in a variety of lesions (ranging from reactive to neoplastic) indicates that carcinogenesis is a multistep process involving mutation. This includes the activation of oncogenes inducing growth

of bcl-2. Expression of bcl-2 decreases as the cell cycle progresses in stimulated lymphocytes. Heterogeneous staining of tumoral cells has also been observed in follicular lymphomas.²³

Histologically relevant expression of p53, Rb, and bcl-2 proteins has been implicated in tumorigenesis, progression and resistance to treatment of lymphoproliferative disease.^{10 11 20} The importance of this expression can only be determined by comparing normal expression of these proteins in non-tumoral cells. Antigen retrieval techniques have recently become available,²⁴ enabling more precise characterisation of the expression of these three proteins in resting and activated lymphocytes in non-tumoral conditions.

Methods

Ten paraffin wax embedded reactive tonsil tissue samples from routine tonsillectomy specimens were obtained from the archives of the Pathology Department, "Virgen de la Salud" Hospital, Toledo. Normal peripheral blood was obtained from voluntary healthy donors. Peripheral blood lymphocytes (PBLs) were isolated by HISTOPAQUE (Sigma) 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 cells/ml RPMI-1640 medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 2% phytohaemagglutinin (PHA) (Gibco, BRL). Aliquots of the activated cells were harvested every 24 hours and prepared for flow cytometry analysis.

Paraffin wax embedded reactive tonsil tissue sections were stained using the alkaline phosphatase-antialkaline phosphatase (APAAP) technique, as described previously.²⁵

For detection of p53 protein, the following anti-p53 antibodies were used: Pab 1801 (Serotec), 1801 (Biogenex), CM1 (Novocastra), DO7 (Novocastra), DO7 (Biogenex), BP53 (Biogenex), and BP53-12 (Zymed), all of which specifically detect human wild-type and mutant p53. Anti-bcl-2 monoclonal antibodies used included bcl-2 100 and 124 (IgG1 and IgG2a isotypes, respectively) (kindly provided by Dr D Y Mason²²). The Rb protein was detected using the anti-Rb monoclonal antibody PMG3-245 (Pharmingen),²⁶ which binds both phosphorylated and unphosphorylated forms.

Growth fraction was assessed by MIB-1 monoclonal antibody, which recognises a Ki67 epitope that survives formalin fixation and embedding in paraffin wax.²⁷ All paraffin wax sections were heated in a microwave oven in a sodium citrate solution before incubation with antibody.²⁴ Paraffin wax sections were dewaxed with xylene, rehydrated in alcohol, and placed in Coplin jars filled with a 0.01 M tri-sodium citrate solution, and heated twice in a conventional microwave oven for six minutes at 700 W. While undergoing microwave processing, slides were always covered with buffer. After heating, slides were permitted to cool at room temperature for 15 minutes. They were then quickly washed in TRIS buffered saline (pH 7.4), and incubated with specific antibody.

Quantitative immunohistochemical investigation with a computer analysis system (CAS 200) was used to score individual PBLs for the presence of bcl-2 cytoplasmic protein. The total optical density of the immunostained area against that of the cellular area was calculated by the image analyser. The cellular area and staining in 70 single cells in each case were measured, and the values obtained presented graphically. Linear regression statistical analysis was undertaken to establish whether there was any correlation between cell size (related to cell cycle phase) and bcl-2 protein staining.

FLOW CYTOMETRY

Expression of p53, bcl-2 and Rb was also studied using flow cytometric analysis of PHA activated lymphocytes. 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 CELLFIT software package for data acquisition and analysis. Analysis of protein expression was undertaken using the LYSIS II software program.

For detection of the three proteins by immunofluorescence, fixation/permeabilisation for optimal immunodetection was performed with paraformaldehyde/Triton X-100 (PFT) according to Aiello.²⁸ The monoclonal antibodies used included Pab 1801 (Oncogene Science), Bcl-2 124 (D Mason, Oxford), and PMG3-245 (Pharmingen). An isotype matched negative control was used to estimate the amount of non-specific binding.

Before labelling, the cells were incubated for 30 minutes with 20% goat serum. PFT treated cells were labelled by indirect immunofluorescence with each specific monoclonal antibody. After 30 minutes of incubation at 4°C, the cells were washed twice in 0.1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), and then incubated again (30 minutes at 4°C in the dark) with goat antimouse monoclonal antibody (F(ab)'2 fragments) conjugated with fluorescein isothiocyanate (FITC) (Dako) (F479). After two more washes, the cells were analysed using flow cytometry.

Parallel samples of PHA activated lymphocytes were prepared for the study of nuclear DNA content by flow cytometry using the CycleTEST DNA Reagent Kit (B.D. Immunocytometry Systems). Sample preparation, data acquisition and analysis have been described elsewhere.²⁹

Results

Reactive tonsil tissue displayed p53 reactivity in all cases with DO7, BP53, and BP53-12 and in most cases with CM1 and 1801 in scattered lymphoid cells within the hyperplastic germinal centre (figs 1A and 1B). Less intense p53 reactivity was observed in the interfollicular areas, in some endothelial cells (fig 1C), as well as in some suprabasal cells in the surface and cryptic epithelium (fig 1E). Reactivity was nuclear in all cases, although in some mitotic cells the signal was observed throughout the cyto-

Figure 1 Detection of p53 in reactive tonsil tissue samples: A: lymphoid follicle, B: germinal centre/mantle zone transition, C: endothelial nuclei, D: p53 positive paired nuclei germinal centre cells, E: parabaasal epithelial cells, F: tonsil lymphoid follicle and epithelial cells, G: parabaasal and middle third Rb positive epithelial cells, H: bcl-2 expression in lymphoid follicles, I: basal epithelial cells, J: PHA stimulated PBLs—small cells showing strong bcl-2 expression, large cells showing a weak bcl-2 signal, K: bcl-2 positive scattered germinal centre cells; MIB-1 expression. L: parabaasal epithelial cells.

Figure 1—continued.

Figure 1—continued.

Figure 2 Immunofluorescence analysis of p53, Rb and bcl-2 expression in PHA stimulated PBLs. **A:** p53 reactivity from days 0 to 3. Cells were divided into large and small subsets. Peak 1, small cells; peak 2, large cells. The increase in the number of p53 positive cells in conjunction with cell cycle activation appears to be restricted mainly to the large cell subset. **B:** Increased MFI of Rb expression after 72 hours of stimulation is restricted to the large cell subset. **C:** Fluorescence histograms show a decrease in bcl-2 expression as the cell cycle progressed. The fraction of positive cells present in each sample was calculated after subtracting the negative isotype matched control histogram.

plasm. Occasional p53 positive twin nuclei were also present (fig 1D). In most cases lymphoid positive cells were large with no other distinctive morphological features. MIB-1 staining in parallel tonsil tissue sections revealed that the parabasal layer with p53 positive cells also expressed maximum staining for this marker (fig 1L).

Flow cytometric analysis of the p53 fluorescence of PHA stimulated PBLs revealed an increase from day 0 (19:30) to day 3 (87:90) in the number of labelled cells as well in the mean fluorescence intensity (MFI) (figs 2A and 3). This increase parallels the percentage increase in the number of cells in S phase (fig 3). As a further control, the experiment was repeated with both phycoerythrin and FITC, giving similar results.

Cells were sub-divided into large and small, based on forward scatter (FSC) (which relates

to the size of the cell) versus size scatter (SCC) (which relates to internal granularity) dot plots obtained using flow cytometry. The increase in the number of p53 positive cells as the cell cycle progressed was mainly restricted to the large cells (fig 2A).

Rb protein expression was prominent in reactive tonsil germinal centre cells, isolated paracortical cells, a fraction of endothelial cells, and epithelial parabasal cells (figs 1F and 1G). Rb expression in germinal centre cells presented with variable intensity in most lymphocytes. Reactivity was mainly nuclear, but in mitotic cells Rb expression was distributed within the cytoplasm, giving a strong signal. Although double staining was not performed, the fraction of Rb positive cells at the surface and in the crypt epithelium seems to correspond to the same layer in p53 and MIB-1 positive supra-basal epithelial cells (figs 1E, 1G and 1L).

Figure 3 Flow cytometric analysis of p53, Rb, bcl-2, and DNA index expression in PHA stimulated PBLs. % S = percentage of cells in S phase.

Flow cytometric analysis of PHA stimulated PBLs revealed an increase in Rb expression (MFI=42.21 on day 0 and 207.97 on day 3) (figs 2B and 3).

When the cells were sub-divided according to the FSC/SSC dot plot, the fluorescence histogram of the larger cells displayed the highest number of labelled cells and the highest MFI, while the smaller cells had weaker Rb expression (fig 2B).

Germinal centres from reactive tonsil tissue samples were mainly bcl-2 negative, while mantle cells, most interfollicular lymphocytes, and epithelial basal cells were bcl-2 positive (fig 1H and 1I). Occasional bcl-2 positive cells were observed in germinal centres and were characterised morphologically as centrocytes, with occasional larger cells also showing cytoplasmic reactivity (fig 1K). Consecutive staining of the same germinal centres for MIB-1 showed an inverse correlation between both markers, since the area staining most strongly for MIB-1 corresponded to the dark zone of the germinal centre, where fewer bcl-2 positive cells were observed. Some mitotic figures exhibited weak cytoplasmic positivity. In all cases reactivity was mainly confined to cytoplasm. In the interfollicular area the same staining gradient was found, larger cells being bcl-2 negative. Basal bcl-2 positive epithelial cells were situated below the layer of p53, Rb, and MIB-1 positive cells (figs 1E, 1G, and 1L).

Flow cytometric analysis of bcl-2 positive PHA stimulated PBLs showed a decrease in the MFI from 108 on day 0 to 48.57 on day 3, with a concurrent increase in the rate of the cell cycle (figs 2C and 3).

To confirm the decrease in the level of bcl-

Figure 4 Linear regression analysis of bcl-2 expression and cell size revealed a inverse correlation between these two parameters.

2 staining during the cell cycle, static image analysis was performed, revealing an inverse correlation between cell size and bcl-2 expression (fig 4), roughly similar to that identified in paraffin wax sections of reactive tonsil tissue and in PHA stimulated PBLs (fig 1J).

Discussion

In the present study the expression of bcl-2, p53, Rb proteins during the cell cycle has been characterised in PHA stimulated PBLs and in reactive tonsil tissue samples, representing in vitro and in vivo model systems, respectively (fig 5).

Expression of p53 was detected in isolated large cells in germinal centres, in some supra-basal cells in the squamous epithelium, and in some tonsil endothelial nuclei, confirming previous results obtained in frozen sections.³⁰ Flow cytometric analysis of PHA stimulated PBLs also showed increased p53 expression as the cell cycle progressed. This was mainly associated with the large cell subset.

These results confirm previous data showing that p53 expression may be associated with cell proliferation,³¹ and detected using sensitive immunohistological methods in benign conditions. Previous studies confirm that p53 immunoreactivity in non-Hodgkin's lymphoma is also correlated with the proliferation rate.³²

Detection of p53 in occasional cells therefore, cannot be seen as synonymous with malignancy or genetic mutation. Expression of p53 increases following DNA damage and ultraviolet irradiation in non-tumoral cells.³³ However, cellular distribution and the frequency of p53 detection in benign conditions makes such an explanation improbable for reactive tonsil tissue. Our cases could be explained according to Milner's conformational hypothesis in that expression of p53 may occur following a conformational change, which changes the p53 protein into a proliferation permissive conformation.³⁴ An alternative explanation could be that p53 may play a role in B cell differentiation in these reactive lymphoid specimens.⁶⁷

Although the p53 growth suppressor mechanism is not fully understood, a suggested model for p53 function is that in certain cellular environments (DNA damage, cellular stress, ultraviolet radiation) p53 expression is stimulated, blocking progression of the cell cycle thereby facilitating DNA repair. However, this is not the result of direct action by p53. Recent studies have shown that p53 activates

Figure 5 Diagrammatic representation of p53, Rb and bcl-2 expression during the cell cycle.

transcription of the WAF-1 gene encoding the p21 protein. This protein inhibits cyclin dependent kinase activity, thereby blocking the cell cycle.³⁵⁻³⁷

Previous *in vivo* studies using frozen sections revealed Rb expression in germinal centre B cells in reactive tonsil tissue. Improvements in the technique have enhanced localisation of Rb expression in germinal centre cells, endothelial cells, and epithelial suprabasal cells. These results seem to complement those obtained with *in vitro* experiments using PHA stimulated PBLs, where Rb expression was associated with proliferation. Indeed, in the tonsillar epithelium suprabasal cells represent the most active proliferating compartment.

The results obtained on analysis of Rb protein expression in PHA stimulated lymphocytes showed increased expression of this protein as the cell cycle progressed, confirming previous results^{20,38} and suggesting that, in addition to phosphorylation, Rb expression may play an important role in the control of the cell cycle.

Studies performed in transgenic mice suggest that the Rb protein is a dynamic brake in the control of cell proliferation.³⁹⁻⁴¹ The histological distribution of Rb protein in suprabasal and lower/middle third epithelial cells is consistent with this role. This is because it is mainly expressed in proliferative cell compartments below the upper epithelial third where cell differentiation takes place.

Although the absence of Rb protein has been postulated to be a consequence of deletion or mutation of the Rb gene, our results indicate that normal non-activated cells may have very low or undetectable expression of Rb protein. Therefore, the growth fraction of the tumour must be taken into account when assessing alterations in Rb protein expression.

Unlike Rb, expression of the bcl-2 protein has mainly been detected in small lymphocytes in mantle zone or in interfollicular locations. This is consistent with previous studies, showing that bcl-2 protein may not be useful for the diagnosis of lymphomas carrying the t(14;18) translocation, but may be a normal finding in resting cells.²² However, some bcl-2 positive larger cells were also observed. This seems to indicate that selection of cells to be rescued from apoptosis can occur early for blast germinal centre cells.

Our results in PHA stimulated lymphocytes showed a decrease in bcl-2 protein expression during the cell cycle. As previous studies have shown somewhat different results, we also performed static analysis using CAS 200, correlating cell size and bcl-2 expression, which not only confirmed results obtained on flow cytometry, but showed a more clear inverse correlation between cell size and bcl-2 expression. This correlation is similar to that previously reported in tumoral follicles, where smaller cells express higher amounts of bcl-2 protein compared with larger cells.²³

In conclusion, microwave irradiation of tissue samples before incubation with specific antibody facilitates antigen retrieval and reduces the detection threshold.⁴² *In vivo* and *in vitro* observations suggest that p53, Rb and

bcl-2 protein expression is modulated during the cell cycle and consequently that the tumoral growth fraction must be taken into account when analysing dysregulation of these three proteins in lymphomas and other tumours.

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