**QUEEN'S
UNIVERSITY
BELFAST**

An investigation into the role of Bcl-2 in neuroendocrine differentiation

Cadden, I. S., Johnston, B. T., Connolly, R., Gates, D., Tsujimoto, Y., Eguchi, Y., & McGinty, A. (2005). An investigation into the role of Bcl-2 in neuroendocrine differentiation. *Biochemical and Biophysical Research Communications*, 326(2)(2), 442-448. <https://doi.org/10.1016/j.bbrc.2004.11.046>

Published in:
Biochemical and Biophysical Research Communications

Queen's University Belfast - Research Portal:
[Link to publication record in Queen's University Belfast Research Portal](#)

General rights

Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

An investigation into the role of Bcl-2 in neuroendocrine differentiation

I.S. Cadden^a, B.T. Johnston^a, R. Connolly^a, D. Gates^a, Y. Tsujimoto^b,
Y. Eguchi^b, A. McGinty^{a,*}

^a Department of Medicine, Queen's University Belfast, Mulhouse Building, RGH, Belfast BT12 6BJ, N. Ireland, UK

^b Division of Molecular Genetics, Department of Post-Genomics and Diseases, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan

Received 5 November 2004

Available online 19 November 2004

Abstract

Introduction: In addition to its role in apoptosis suppression, Bcl-2 has been reported to be co-expressed with neuroendocrine markers in several tissues, leading to speculation that this oncoprotein may promote neuroendocrine differentiation.

Aim: This study investigated whether Bcl-2 modulated neuroendocrine biopeptide expression.

Methods: Levels of chromogranin A, neurone specific enolase, protein gene peptide 9.5, pancreatic polypeptide, and the chromogranin-derived peptides, intervening peptide and vasostatin-1 were examined by immunocytochemistry in rat pheochromocytoma (PC12) cell lines genetically engineered to over-express Bcl-2 and their mock-transfected controls. Intensity of fluorescence was graded using a semi-quantitative scale from (–) indicating negative expression to (+++) indicating intense positivity.

Results: Mann–Whitney *U* analysis indicated that no significant differences in expression existed between control and Bcl2 over-expressing cell lines for any of the six peptides examined.

Conclusions: The results of this study do not support the hypothesis that Bcl-2 promotes the acquisition of a neuroendocrine phenotype.

© 2004 Elsevier Inc. All rights reserved.

Keywords: Differentiation; Pheochromocytoma; Chromogranin A; PC12; Biopeptide; Immunocytochemistry

Bcl-2 is an anti-apoptotic cellular protein [1]. Studies have shown that elevated expression of this protein is associated with several malignancies [1–4]. Models of over-expression suggest that Bcl-2-dependent inhibition of apoptosis is important in tumour formation [1–4]. In addition to its well-characterised role in the suppression of programmed cell death, evidence exists that Bcl-2 is expressed both in true neuroendocrine cells and in cells that have acquired a neuroendocrine phenotype [5–7], leading to speculation that this protein may not be involved solely in cytoprotection. For example, the anterior pituitary neuroendocrine cells of the adult rhe-

sus monkey represent the only brain cell population consistently exhibiting a non-microglial pattern of Bcl-2 expression [5]. Bcl-2 and neuroendocrine marker co-expression has also been demonstrated in a range of histologically distinct lung tumours [6]. In this study Bcl-2 expression was found to be closely associated with the neuroendocrine differentiation of tumour cells, leading the authors to suggest that this protein may be involved in cell development and differentiation [6]. Similarly, in colon carcinoma cells and neighbouring crypt cells, elevated Bcl-2 expression has been found to correlate with the expression of a number of bioactive neuroendocrine markers. Double immunostaining techniques were used to retrospectively analyse whether advanced colorectal carcinomas and tumour neighbouring mucosa produced both Bcl-2 and a range of gastrointestinal neuropeptides.

* Corresponding author. Fax: +44 28 9063 5900.

E-mail address: a.mcginity@qub.ac.uk (A. McGinty).

These workers provided evidence that Bcl-2 immunoreactivity correlated with the expression of key neurohormonal polypeptides and amines including vasointestinal polypeptide (VIP), pancreatic polypeptide (PP), and somatostatin in both carcinoma tissue and in the neighbouring mucosa, and suggested that in this setting Bcl-2 may promote endodermal stem cell neuroendocrine differentiation [7]. Similarly, Azzoni et al. [8] reported that in the normal oxyntic mucosa Bcl-2 was consistently expressed by a distinct population of neuroendocrine cells located predominantly in the central mucosal layer, suggesting a role for the protein in the migration of maturing endocrine cells. These authors also found that increased probability of gastric carcinoid development was paralleled by greater Bcl-2 expression in hyperplastic oxyntic endocrine cells. Indeed, they found that the ratio of Bcl-2 to chromogranin A (CGA)-positive cells was highest in atrophic fundal gastritis, a condition conferring the highest risk of carcinoid development, and lowest in sporadic Zollinger–Ellison syndrome, a condition characterised by virtually no risk [8].

In addition to studies reporting a correlation between the levels of expression of Bcl-2 and those of neuroendocrine biopeptides, this oncoprotein has also been reported to be physically associated with the neuroendocrine-associated reticulum (RTN) family member RTNxs [9]. Tagami et al. [9] found that the interaction between Bcl-2 and RTNxs sequestered the former in the endoplasmic reticulum, thereby reducing its anti-apoptotic activity. While this study employed cell culture models of Bcl-2 over-expression, the putative modulation of levels of RTNxs, its family members, or indeed any other neuroendocrine markers, by this oncoprotein was not reported. The aim of the current study was, therefore, to examine the relative levels of a panel of neuroendocrine markers in an established cell culture model of Bcl-2 over-expression in order to determine whether Bcl-2 positively modulates levels of these biopeptides.

Materials and methods

The cell lines employed in the current study were rat adrenal phaeochromocytoma (PC12) cells genetically engineered to constitutively over-express Bcl-2 (PC12 HB2-2 and PC12 HB2-3) and their mock-transfected controls (PC12 V1 and PC12 V4) [10]. In addition, a well-characterised PC12 model of Cox-2 over-expression (PCXII) and their mock-transfected controls (PC-MT) were employed in optimisation experiments [11]. In PCXII cells exogenous Cox-2 expression is induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG, 2.5 mM) to the cell culture media [11].

All cell lines were cultured on sterile plasticware obtained from Iwaki, Japan. Cell culture media including Dulbecco's modified Eagle's media (DMEM), heat-inactivated horse serum (HS), and foetal calf serum (FCS) were obtained from Invitrogen Life Technologies, CA, USA. Trypsin, versene, and all supplements used during culture were obtained from Sigma Aldrich, Missouri, USA. Rat-tail collagen type I was from Roche Diagnostics, Germany.

For immunocytochemistry, cells were cultured in collagen-coated BD Falcon slides which were obtained from BD Biosciences, UK.

The primary antibodies for immunocytochemistry included anti-Cox-2 (SC-1745, Santa Cruz Biotechnologies, USA), anti-CGA (20085, Diasorin, USA), and anti-Protein Gene Peptide 9.5 (PGP 9.5) from UltraClone, UK. The remaining primary antibodies used in immunocytochemistry [namely anti-vasostatin (VST-1), anti-intervening peptide (IP), anti-neuron specific enolase (NSE), and anti-pancreatic polypeptide (PP)] were produced in-house [12–14]. Swine anti-rabbit fluorescein isothiocyanate (SWAR-FITC) was obtained from DakoCytomation, Denmark (F0205), the biotinylated donkey anti-rabbit (BAR)-streptavidin-FITC system (RPN 1004 and 1232, respectively) came from Amersham Biosciences, UK, and rabbit anti-goat fluorescein isothiocyanate (RAG-FITC) was also supplied by DakoCytomation (F0233).

For experiments all cells were routinely subcultured in growth media onto collagen-coated slides. The following day growth media were replaced with restriction media (DMEM, 0.5% horse serum/calf serum). In the case of PC-MT and PCXII cells restriction media also routinely included IPTG (2.5 mM). Media were removed from cells 18–24 h later and these were washed three times in PBS (0.15 M sodium chloride, pH 6.0, containing 0.03 M sodium dihydrogen orthophosphate-1-hydrate and 0.09 M anhydrous disodium hydrogen orthophosphate). The cells were then fixed in 4% paraformaldehyde (30 min, 4 °C) after which they were washed three times in PBS. Primary antibodies, namely anti-Cox-2 (1:100) anti-CGA (1:500), anti-NSE (1:50), anti-PGP 9.5 (1:400), anti-IP (1:400), anti-PP (1:200), and anti-VST-1 (1:200), were then applied as appropriate. These primary antibodies were diluted in PBS containing 0.5% bovine serum albumin Fraction V, 0.5% Triton X-100, and 0.015 M sodium azide (antibody diluent). Brain cortex, brain cerebellum, pancreas head, and ileal carcinoid tissue sections were employed as positive controls for the expression of NSE, PGP 9.5, PP, and VST-1, respectively, with adrenal medulla tissue sections serving as positive control for CGA and IP. For immunocytochemistry, negative controls included were a secondary antibody only (antibody diluent) control and the appropriate serum control (1:500). The cells were then placed in an immunostaining chamber and left at 4 °C overnight.

Following this incubation the cells were washed with PBS (3 $\times$ 5 min). The appropriate secondary antibodies were then applied to the cells, namely SWAR-FITC for anti-IP, anti-PP, and anti-VST-1 (1:100, for 1 h at room temperature in darkness), RAG-FITC for anti-Cox-2, and BAR-streptavidin for anti-CGA, anti-NSE, and anti-PGP 9.5. The latter was applied in a two-step process. First cells were incubated with BAR (1:100 for 30 min at room temperature). Following a PBS wash, the cells were then incubated in streptavidin-FITC (1:200 for 30 min in darkness at room temperature).

Following incubation, the cells were washed in PBS in the dark for 20 min. Coverslips were then applied to the slides using 0.1 M PBS/glycerol (1:9; v/v) containing 2.5% 1,4 diazabicyclo(2,2,2)octane (antifade). The slides were viewed under the fluorescence microscope and images were taken. The fluorescent microscope used was fitted with exciter filters that produced blue light (495 nm) for the excitation of fluorescein.

Quantification of the fluorescence intensity emitted by the FITC tag was undertaken by two independent observers. Fluorescence intensity was scored on 4 mark scale ranging—negative (–), weakly positive (+), moderately positive (++) or intensely positive (+++). For each of the six neuroendocrine markers, five independent experiments were carried out on all four cell lines and images were recorded.

For immunoblotting, cell lines were routinely subcultured from confluent T25 flasks at a split ratio of 1:10 in order to achieve 70–80% confluency at time of cell lysis. Growth media were replaced with restriction media, $-/+$ 2.5 mM IPTG, 18–24 h prior to cell lysis. Routinely, cells were placed on ice and restriction media were removed. Cells were then washed three times with ice-cold PBS. Following the complete removal of PBS, cells were lysed in hypotonic lysis

buffer, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes), pH 7.5, containing 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride, and 200 µM orthovanadate (100 µl) as described previously [11]. Samples standardised for protein were separated by SDS–polyacrylamide gel electrophoresis using 5% and 8% acrylamide stacking and resolving gels, respectively, and immunoblotted for Bcl-2, Cox-2 or CGA expression according to the manufacturer's instructions (anti-Bcl-2, Catalogue No. 610538, BD transduction Labs, Oxford, UK; 1:500, anti-Cox-2 Catalogue No. SC-1745, Santa Cruz, Biotechnology, USA, 1:200, and anti-CGA, Catalogue No. SC-1488, Santa Cruz, Biotechnology, USA, 1:200, in Tris buffered saline, pH 7.8, containing 5% [v/v] BSA). Blots were visualised using an ECL Western blotting detection kit, also exactly according to the manufacturer's instructions.

Statistical analysis. For each of the six neuroendocrine markers, five independent experiments were carried using the two control (V1/V4) and two Bcl-2 over-expressing (HB2-2/-3) cell lines. On the advice of a statistician, expression of the neuroendocrine markers by the control (V1/V4) cell lines was compared by means of the Mann–Whitney *U* test. Similarly, neuroendocrine marker expression in the Bcl-2 over-expressing (HB2-2/-3) cell lines was compared by means of this statistical test. Mann–Whitney *U* analysis was then carried out using grouped data from the control and Bcl-2 over-expressing cell lines.

Results and discussion

In view of the reports implicating the anti-apoptotic oncoprotein Bcl-2 in the acquisition/promotion of a neuroendocrine phenotype, the current study sought to determine whether forced expression of this protein was associated with modulated levels of the neuroendocrine markers, CGA, NSE, PGP 9.5, PP, and the CGA-derived peptides VST-1 and IP. A PC12 cell culture model of constitutive Bcl-2 over-expression ([10]; Fig. 1) was used in conjunction with immunocytochemical methods. Since PC12 cells are a neuroendocrine line—derived originally from a rat adrenal pheochromocytoma—which do not basally express Bcl-2 [10,15,16], they represent an ideal model in which to examine the putative effects of exogenously expressed Bcl-2 on neuroendocrine peptide expression. Moreover, the use of immunohistochemical methods in the present study facilitated the semi-quantitative determination of marker expression, some of which would have proven difficult to detect by immunoblotting due to their low molecular weight.

Immunoblot analysis of the four cell lines confirmed that under basal conditions the PC12 HB2-2 and PC12 HB2-3 cell lines expressed Bcl-2, whilst the control cell lines, PC12 V1 and V4, did not (Fig. 1; [10,15,16]). It was next necessary to validate the methodologies chosen in order to confirm that immunohistochemical protocols would be sufficiently sensitive to detect differential neuroendocrine marker expression. In parallel with the current study expression array analysis in our laboratory involving a second, and equally well-characterised, PC12 model of Cox-2 over-expression [11] had indicated that levels of CGA were 2-fold higher in PCXII than their control (PC-MT) counterparts (Connolly et al., unpublished observations). Immunocytochemical and immunoblotting analyses were subsequently carried out to verify this observation. These experiments confirmed that, following the induction of Cox-2 expression in response to IPTG in PCXII cells (Fig. 2A), levels of CGA were elevated compared to those found in their control cell line (Figs. 2B and C). Interestingly, levels of NSE were similar in the two cell lines indicating that the effect of Cox-2 on CGA expression was specific and not due to a global enhancement of neuroendocrine marker expression/differentiation. Critically for the purposes of the current study, the finding that Cox-2-dependent modulation of CGA expression was clearly detected by immunocytochemistry (Fig. 2B) validated the use of this technique for the detection of putative Bcl-2-dependent modulation of neuroendocrine marker expression in PC12 HB2-2/HB2-3 cells and their mock-transfected controls.

Having confirmed that increased expression of neuroendocrine marker expression could be detected immunocytochemically using a PC12 cell model of over-expression, levels of the neuroendocrine markers CGA, NSE, PGP 9.5, PP, IP, and VST-1 were next examined by this method in the HB2-2, HB2-3, V1, and V4 lines. This particular panel of markers was chosen since CGA, NSE, PGP 9.5, and PP represent established neuroendocrine-specific proteins/peptides whose co-expression with Bcl-2 has been demonstrated in a range of tissues [6–8] and whose expression in PC12 cells has also been reported [17]. The expression of the CGA-derived peptides IP and VST-1 was also examined since, as well as representing additional, somewhat less ubiquitous, neuroendocrine biopeptides, it was reasoned that

Fig. 1. Immunoblot analysis of Bcl-2 expression in control (PC12 V1 and V4) and Bcl-2 over-expressing (PC12 HB2-2 and HB2-3) cell lines.

Fig. 2. Immunohistochemical and immunoblot analysis of Cox-2-dependent CGA upregulation in PCXII cells. (A) Immunoblot analysis of IPTG-inducible Cox-2 expression in PC-MT and PCXII cells. (B) Immunohistochemical analysis of Cox-2, CGA, and NSE expression in PC-MT and PCXII cells. (C) Immunoblot analysis of CGA expression in PC-MT and PCXII cells.

this would allow the putative effect of Bcl-2 on CGA-processing to be examined.

For each of the six neuroendocrine markers, five independent experiments were carried out on all four cell lines and images were recorded. Expression of all six neuroendocrine markers was confirmed in both the mock-transfected and Bcl-2 over-expressing cell lines (Figs. 3–8). Not surprisingly, as these represent ubiquitous neuroendocrine markers expressed in the majority of neuroendocrine cells and tissues [6–8,17], high levels (3+) of CGA, PGP 9.5, and NSE were demonstrated in all cell lines (Table 1). PP and the CGA-derived peptides IP and VST-I were also detected in all four cell lines, albeit at lower levels of expression (Table 1; [17]).

On the advice of a statistician, expression of the neuroendocrine markers by the two control cell lines was compared by means of the Mann–Whitney *U* test. Similarly, neuroendocrine marker expression in the two Bcl-2 over-expressing cell lines was compared by means of this statistical test. This analysis confirmed that no significant differences in the expression of any of the six peptides existed between paired control or paired Bcl2 over-expressing cell lines. Having thus ruled out potential clonal influences on neuropeptide expression levels, Mann–Whitney *U* analysis was carried out using grouped data from the control, and also from the Bcl-2 over-expressing, cell lines. Critically, no significant differences in expression were observed between control

Fig. 3. Immunocytochemical analysis of NSE expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

Fig. 6. Immunocytochemical analysis of PP expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

Fig. 4. Immunocytochemical analysis of CGA expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

Fig. 7. Immunocytochemical analysis of IP expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

Fig. 5. Immunocytochemical analysis of PGP 9.5 expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

Fig. 8. Immunocytochemical analysis of VST-1 expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

and Bcl-2 over expressing cells for any of the six neuropeptides examined (Table 2). Given the similarity in levels of neuroendocrine marker expression between the two pairs of cell lines—with the exception of IP (control vs Bcl-2 over expressing lines; 2+ vs 1+; $p = 0.07$)—the

finding that there were no differences between the CGA, NSE, PGP 9.5, PP, and VST-I expression levels was not unexpected (Table 2). Taken together, these results indicate that neither neuroendocrine marker expression nor

Table 1

Immunohistochemical assessment of neuroendocrine marker expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines

Peptide	V1	V4	HB2-2	HB2-3
CGA	+++	+++	+++	+++
NSE	+++	+++	+++	+++
PGP 9.5	+++	+++	+++	+++
PP	+	+	+	+
IP	++	++	+	+
VST-1	++	++	++	++

Shown are the immunopositivity grades from a representative experiment ($n = 5$).

Table 2

Statistical analysis of neuroendocrine marker expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines

Peptide	V1/V4 vs HB2-2/-3
CGA	$p = 1.00$
NSE	$p = 1.00$
PGP 9.5	$p = 1.00$
PP	$p = 1.00$
IP	$p = 0.07$
VST-1	$p = 1.00$

CGA processing appears to be modulated in response to Bcl-2 over-expression.

To our knowledge this is the first study to investigate whether Bcl-2 actually causes, rather than is simply associated with, enhanced neuroendocrine differentiation. It is also the first study to specifically quantify expression levels of neuroendocrine markers relative to those of Bcl-2. Obviously the results from the current study do not support the hypothesis that, in addition to its apoptosis-suppressing properties, Bcl-2 also promotes the acquisition of a neuroendocrine phenotype by positively modulating the expression of key neuroendocrine markers. As such the current results are at variance with previous reports that have suggested a link between Bcl-2 expression and enhanced neuroendocrine differentiation [6–8]. None of these reports have, however, demonstrated an inter-dependence between Bcl-2 expression and the development of a neuroendocrine phenotype, nor have they quantified relative levels of expression. For example, the association between Bcl-2 and neuroendocrine marker expression described by Ohmori et al. [7] was based on double immunostaining for Bcl-2 and several neuroendocrine markers in colon cancer tissue. While this, and similar studies [6,8], have confirmed that both Bcl-2 and various neuroendocrine proteins/peptides are present within the same cell, significantly, in none of these studies has a causal relationship been established.

In summary, the results of the current study do not support the hypothesis that Bcl-2 plays a role in the development of a neuroendocrine phenotype. In view

of these findings it would appear that the reported expression of Bcl-2 in neuroendocrine cells [5–8] relates either to the well-documented anti-apoptotic actions of this oncoprotein or to additional, as yet undiscovered, functions. Previous studies have shown that forced Bcl-2 expression suppresses apoptosis of PC12 cells [15,16] and it has been speculated that the observed expression of Bcl-2 in oxyntic endocrine cells may promote cytosurvival during their maturation and downward migration from isthmal/neck-localised stem cells [8]. Numerous studies have documented Bcl-2 expression in neuroendocrine tumours such as pheochromocytomas [18], thymic neuroendocrine tumours [19], and gastroenteropancreatic neuroendocrine tumours [20]. Investigations into the function of this oncoprotein in the pathology of neuroendocrine tumour development are, however, sparse and whether in this setting its actions differ from the apoptosis-inhibitory role it plays in more common cancers remains to be established.

Acknowledgments

The authors thank Mrs. Kathy Pogue and Mrs. Maureen McDevitt for their excellent technical support and advice. This study was funded by a grant (EAT/1889/01 to A.M. and B.J.) from the NIHPSS.

References

- [1] S. Willis, C.L. Day, M.G. Hinds, D.C. Huang, The Bcl-2-regulated apoptotic pathway, *J. Cell Sci.* 116 (2003) 4053–4056.
- [2] J.L. Townson, G.N. Naumov, A.F. Chambers, The role of apoptosis in tumor progression and metastasis, *Curr. Mol. Med.* 3 (2003) 631–642.
- [3] R.H. Pierce, M.E. Vail, L. Ralph, J.S. Campbell, N. Fausto, Bcl-2 expression inhibits liver carcinogenesis and delays the development of proliferating foci, *Am. J. Pathol.* 160 (2002) 1555–1560.
- [4] N. Shivapurkar, J. Reddy, P.M. Chaudhary, A.F. Gazdar, Apoptosis and lung cancer: a review, *J. Cell. Biochem.* 88 (2003) 885–898.
- [5] D.E. Merry, D.J. Veis, W.F. Hickey, S.J. Korsmeyer, Bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS, *Development* 120 (1994) 301–311.
- [6] S.X. Jiang, T. Kameya, Y. Sato, N. Yanase, H. Yoshimura, T. Kodama, Bcl-2 protein expression in lung cancer and close correlation with neuroendocrine differentiation, *Am. J. Pathol.* 148 (1996) 837–846.
- [7] T. Ohmori, S. Asahi, C. Sato, F. Maki, A. Masumoto, K. Okada, Bcl-2 protein expression and gut neurohormonal polypeptide/amine production in colorectal carcinomas and tumor-neighbouring mucosa, which closely correlate to the occurrence of tumour, *Histol. Histopathol.* 14 (1999) 37–44.
- [8] C. Azzoni, C. Doglioni, G. Viale, G. Delle Fave, M. De Boni, P. Caruana, G. Ferraro, C. Bordini, Involvement of Bcl-2 oncoprotein in the development of enterochromaffin-like cell gastric carcinoids, *Am. J. Surg. Pathol.* 20 (1996) 433–441.
- [9] S. Tagami, Y. Eguchi, M. Kinoshita, M. Takeda, Y. Tsujimoto, A novel protein, RTN-xs, interacts with both Bcl-xL and Bcl-2 on

- endoplasmic reticulum and reduces their anti-apoptotic activity, *Oncogene* 19 (2000) 5736–5746.
- [10] S. Shimizu, Y. Eguchi, W. Kmiike, S. Waguri, Y. Uchiyama, H. Matsuda, Y. Tsujimoto, Retardation of chemical hypoxia-induced necrotic cell death by Bcl-2 and ICE inhibitors: possible involvement of common mediators in apoptotic and necrotic signal transductions, *Oncogene* 12 (1996) 2045–2050.
- [11] A. McGinty, Y.W.E. Chang, A. Sorokin, D. Bokemeyer, M.J. Dunn, Cyclooxygenase-2 expression inhibits trophic withdrawal apoptosis in nerve growth factor differentiated PC12 cells, *J. Biol. Chem.* 275 (2000) 12095–12101.
- [12] C.M. Gleeson, W.J. Curry, C.F. Johnston, K.D. Buchanan, Occurrence of WE-14 and chromogranin A-derived peptides in tissues of the human and bovine gastro-entero-pancreatic system and in human neuroendocrine neoplasia, *J. Endocrinol.* 151 (1996) 409–420.
- [13] R.T. Cunningham, C.F. Johnston, G.B. Irvine, E.M. McIrath, A. McNeill, K.D. Buchanan, Development of a radioimmunoassay for neurone specific enolase (NSE) and its application in the study of patients receiving intrahepatic arterial streptozotocin and floxuridine, *Clin. Chim. Acta* 189 (1990) 275–286.
- [14] R.T. Cunningham, K.M. Pogue, W.J. Curry, C.F. Johnston, J.M. Sloan, K.D. Buchanan, Immunostaining for vasostatin I distinguishes between ileal and lung carcinoids, *J. Pathol.* 187 (1999) 321–325.
- [15] S.P. Mah, L.T. Zhong, Y. Liu, A. Roghani, R.H. Edwards, D.E. Bresden, The protooncogene bcl-2 inhibits apoptosis in PC12 cells, *J. Neurochem.* 60 (1993) 1183–1186.
- [16] L. Lindenboim, R. Haviv, R. Stein, Inhibition of drug-induced apoptosis by survival factors in PC12 cells, *J. Neurochem.* 64 (1995) 1054–1063.
- [17] R.T. Cunningham, K.M. Pogue, W.J. Curry, C.F. Johnston, K.D. Buchanan, PC12 cells show immunoreactivity to a number of proteins and peptides, including vasostatin, *Peptides* 17 (1996) 1297–1301.
- [18] R.R. De Krijger, E. Van Der Hurst, F. Van Der Ham, T. Stijnen, W.N.M. Dinjens, J.W. Koper, H.A. Bruining, S.W.J. Lamberts, F.T. Bosman, Prognostic value of p53, Bcl-2 and c-erbB-2 protein expression in pheochromocytomas, *J. Pathol.* 188 (1999) 51–55.
- [19] A.A. Gal, M.N. Sheppard, J.D. Nolan, C. Cohen, p53, cellular proliferation and apoptosis-related factors in thymic neuroendocrine tumours, *Mod. Pathol.* 17 (2004) 33–39.
- [20] D.G. Wang, C.F. Johnston, K.D. Buchanan, Oncogene expression in gastroenteropancreatic neuroendocrine tumours. Implications for pathogenesis, *Cancer* 80 (1997) 668–675.