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Bcl-2/Bax ratios in chronic lymphocytic leukaemia and their correlation with in vitro apoptosis and clinical resistance

Sir,

We were interested to read the recent article by Pepper et al (*Br J Cancer* **76**: 935–938, 1997). This paper discussed flow cytometric quantitation of apoptosis as a measure of responsiveness to chemotherapy in B-cell chronic lymphocytic leukaemia (B-CLL), with particular reference to Bcl-2/Bax protein ratios. Our group, using the same antibodies, is pursuing very similar research in superficial bladder cancer. We have compared percentages of positively staining Bcl-2 and Bax tumour cells before and after in vitro exposure to mitomycin C in bladder tumours. We have obtained results (unpublished) which would further support the theory that Bax dysregulation plays an important role in chemoresistant tumours (Boersma et al, 1997; Chresta et al, 1996).

As the next stage to our own experiments, we would be very keen to investigate co-expression of Bcl-2 and Bax in the bladder tumour cells using flow cytometry. Co-expression would indicate the extent of heterodimerization of Bcl-2 with Bax that is likely to affect the inhibition of apoptosis after chemotherapy (Yang et al, 1995). We were therefore very interested to read the authors' description of a triple-colour flow cytometry methodology in B-CLL cells. They described how they sequentially incubated the cells with anti-CD19 Cy5 PE-conjugated antibody, Bcl-2 FITC and Bax followed by PE-labelled secondary antibody. Unfortunately, the authors did not report their findings on the co-expression of Bcl-2 and Bax in the B-CLL cells.

Secondly, the paper did not report the Bcl-2 and Bax protein levels in their clinically untreated patient samples after in vitro exposure to chlorambucil. Considering our own findings, which showed a correlation between apoptotic index >10% and increased Bax protein, these data would have been very relevant.

Thirdly, we were confused by the fact that the authors confirmed that the Annexin V-positive lymphocytes cells were apoptotic by morphological assessment. Annexin V binds to cells in the early stages of apoptosis, are not all likely to exhibit the classical features of apoptosis (Martin et al, 1995). It would be interesting to know what criteria the authors used to confirm that the Annexin V-positive lymphocytes were or were not in early apoptosis.

In summary, the authors designed an important study, but unfortunately they did not exploit or report all of their data.

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Bcl-2/Bax ratios in chronic lymphocytic leukaemia and their correlation with in vitro apoptosis and clinical responsiveness – reply

Sir,

The quantification of Bcl-2 and Bax proteins was achieved by triple-colour analysis as described in our paper, and the co-expression of Bcl-2 and Bax was measured in these experiments. However, our data relate to protein expression in the total gated B-lymphocyte population of cells rather than to individual cell

analysis; all gated cells expressed both Bcl-2 and Bax proteins. The index we used to describe this co-expression was a ratio of the two proteins (Bcl-2/Bax) as there is evidence that the ratio of death promoters to death inhibitors within the cell may determine susceptibility to death signals (Korsmeyer et al, 1993; Thomas et al, 1996; Pepper et al, 1996). These ratios were calculated as

follows. The mean fluorescent intensity (MFI) was calculated for each protein using WinMDI software (J Trotter, Scripps Research Institute, USA) and these values were then converted to molecules of equivalent soluble fluorochrome (MESF) using a calibration curve to standardize the data. The calibration curve was constructed by operating the flow cytometer under identical conditions and monitoring a mixture of beads labelled with known amounts of fluorochrome. In this way, day-to-day variation in fluorescence detection are controlled for, and, in addition, differences in fluorescent intensity between FITC and PE can be normalized. Obviously we would be willing for Williamson et al to have a copy of the raw data derived from these experiments and would also refer them to our publication in the *British Journal of Haematology* (1996).

The Bcl-2 and Bax protein expression presented in this paper relate to the total population of cells, regardless of their status in terms of response to apoptotic signals, i.e. no attempt was made to gate viable and non-viable cells. However, we agree that it is of interest to measure Bcl-2 and Bax protein levels both before and after exposure to drug. This indeed is the subject of our latest publication in *Leukemia & Lymphoma* (1998). Briefly, our results indicate that Bcl-2 and Bax protein levels are significantly different in those cells that resist apoptosis when compared with those that do not, and this difference is particularly pronounced in terms of Bax protein expression.

In the Annexin V positivity experiments we used Annexin V as a measure of apoptosis. It is true that Annexin V is capable of

labelling very early-stage apoptotic cells that have not yet undergone morphological changes. However, we would point out that in these experiments we labelled our cells with Annexin V after incubation with drug for 48 h and most of the cells that were undergoing apoptosis were in fact late-stage apoptotic cells that were easily identified morphologically by membrane blebbing, chromatin condensation and general cellular shrinkage. Recent data from our laboratory have confirmed that morphological evidence of apoptosis can be seen as early as 16 h after drug exposure but is much more marked after 24 h (unpublished).

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Insulin-like growth factor 1 in relation to prostate cancer and benign prostatic hyperplasia

Sir,

We were interested to read the paper by Mantzoros et al (1997) claiming that serum insulin-like growth factor 1 (IGF-1) levels were elevated in prostate cancer patients and suggesting that serum IGF-1 may represent an independent risk factor for this disease. This is in marked contrast to our data demonstrating that serum IGF-1 and IGF-2 levels were not different in patients with prostate cancer relative to age-matched controls (Cohen et al, 1993). Similarly, Ho and Baxter (1997) found normal IGF-1 levels in the sera of patients with prostate cancer. The IGFs circulate complexed to a family of proteins known as the IGF-binding proteins (IGFBPs), as reviewed by Daughaday et al (1986). Apparently, unknown to these authors, IGFBPs have been shown to interfere with radioimmunoassays (RIAs) of the IGFs as a result of their ability to bind to the labelled IGF trace and to artifactually elevate the readings of IGF levels, as reviewed by Rosenfeld and Gargosky (1996). While various methods have been used to attempt to remove the IGFBPs from the IGFs before assay, only techniques involving acidification followed by chromatography have been demonstrated by Bang et al (1991) to successfully perform this separation. RIAs, such as the Nichols kit used by Mantzoros et al (1997), use an ethanol extraction method which, while able to remove most of the IGFBP-3 found as the 150-kDa complex of the IGFs, are completely ineffectual in removing other IGFBPs in serum including IGFBP-2 and IGFBP-4, as shown by

Frey et al (1994). In our report (Cohen et al, 1993), we have shown a two- to threefold elevation in IGFBP-2 levels in the sera of patients with prostate cancer as assayed by both RIA and Western ligand blotting. We have since verified that observation on a second cohort of patients and, in addition, we have demonstrated a more moderate elevation of IGFBP-2 levels in the sera of patients with benign prostatic hyperplasia. We have also recently found a twofold elevation in the serum levels of IGFBP-4 in the sera of prostate cancer patients. Our findings of elevated IGFBP-2 in prostate cancer patients have also been replicated by Kanety et al (1993) and Ho and Baxter (1997). As IGFBP-2 and IGFBP-4 are not removed by the ethanol extraction method used by Mantzoros et al (1997), we believe that their patients actually had normal serum IGF-1 levels, but elevated serum IGFBP-2 and IGFBP-4 levels, leading to an artifactual elevation in the IGF-1 RIA reading. This situation is similar to a report in the *New England Journal of Medicine* by Pintor et al (1989), allegedly discovering a child with phenotypic Laron dwarfism but with normal IGF-1 levels (which are dramatically reduced in that condition). Those authors used the exact same Nichols IGF-1 RIA kit to measure IGF-1 levels in the serum of their subject as Mantzoros et al (1997) did in their paper. Alerted to the possibility of an artifact by a letter to the editor from Laron and Silbergeld (1989), the authors re-assayed their child's serum IGF-1 with an acid-chromatography technique and discovered it to be undetectable, leading them to issue a retraction of their