

Response:

Response to surface IgM engagement in CLL

We appreciate the letter of Dr Zupo and colleagues who like our group have studied the responsiveness of chronic lymphocytic leukemia (CLL) cells to signals through the antigen receptor.^{1,2} Those authors comment on several aspects of our work including the reagents used, the possible existence of CLL subsets in terms of responsiveness to signals through the B-cell receptor (BCR) for antigen, and the biological significance of our observations including apoptosis inhibition and induction of antiapoptotic bcl-2 family members in stimulated CLL cells. Our data are important because they provide the first demonstration of a molecular pathway by which antigen extrinsic to malignant lymphocytes can promote tumor cell survival.

We agree with these investigators that the nature of the immunoglobulin (Ig) reagent used to stimulate human B cells in culture can affect the outcome of survival or death. We utilized an F(ab')₂ preparation of polyclonal goat antibody to human IgM heavy chains (American Qualex, San Clemente, CA) that was selected to avoid any potential nonspecific effects of Fc-receptor engagement in human B cells. As CLL cells express Fc receptors, including the inhibitory receptor FcγRIIb (E.S., unpublished observations by RT-PCR, September 2001; Fridman et al³; and Gamberale et al⁴), the discrepancy between our observations and those of others might arise from negative signals transmitted to CLL cells upon exposure to intact antibody preparations. Our findings, that apoptosis is inhibited upon surface IgM engagement in CLL, are entirely compatible with a tumor that expresses IgM with affinity for autologous structures.

The literature supports 2 patterns of CLL responses to stimulation through the BCR,^{5,6} but we did not observe 2 distinct patterns in our apoptosis studies. Rather, the differences in apoptosis inhibition among cases were a matter of degree. Although the effects of IgM engagement on Annexin V binding were subtle in some experiments, there was a statistically meaningful pattern of apoptosis inhibition among all cases evaluated.² Moreover, in later studies in which we used an alternative method of apoptosis measurement, propidium iodide to mark the subdiploid DNA content of cells, the differences between unstimulated and IgM-stimulated CLL cells were greater and more evident. Other methods we used to measure apoptosis, including evaluation of polyADP-ribose polymerase cleavage by immunoblotting and of cell morphology by fluorescence microscopy, yielded a constant picture. The clinically

relevant question is not whether apoptosis inhibition occurs in CLL upon surface IgM engagement, but what is the degree of apoptosis inhibition in cells stimulated by real, complex antigens *in vivo*? The results of our *in vitro* studies may underrepresent the biological effects of antigens to which tumor cells bind in patients with disease.

Finally, we stand by our findings regarding induction of antiapoptotic genes in CLL cells stimulated by the BCR. In particular, increases of mcl-1 at the protein level have been clear and reproducible in every case we have examined thus far. Consistent with our published model for the role of CD40 ligand and antigen in CLL pathogenesis,⁷ induction of bcl-2, mcl-1, and bfl-1 transcripts has been greatest when both CD40 and surface IgM are engaged. What remains to be elucidated are the precise mechanisms by which bcl-2-type proteins including bcl-2 and mcl-1, as well as other inhibitors of apoptosis that are overexpressed in CLL, interact, are metabolized, and function to prevent cell death.

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To the editor:

Immunohistochemical localization of phosphorylated AKT in multiple myeloma

We read with interest the article by Hsu et al¹ analyzing the activation status of AKT in plasma cells from patients with multiple myeloma (MM) or monoclonal gammopathy of undetermined significance (MGUS). Recent studies based on MM cell lines indicate that the phosphatidylinositol 3-kinase (PI3K) signaling pathway plays a positive role in the survival of myeloma cells.² Although various cellular intermediary proteins are activated by PI3K, recent studies suggest that AKT/PKB activity alone is sufficient to block apoptosis. Possible activators of the AKT/PKB signaling pathways in MM include several growth factors such as insulinlike growth factor-1, epidermal growth factor, basic fibroblast growth factor, interleukin-3 (IL-3), IL-6, and macrophage

colony-stimulating factor.³ Alternatively, loss of the tumor suppressor gene, PTEN, may also promote AKT signal activation. Regardless of the initiating stimulus, full activation of AKT requires phosphorylation at Thr 308 and Ser 473 by the protein kinases, PDK1 and PDK2.

Hsu et al analyzed the phosphorylation status of AKT by using an antibody that recognizes the phosphorylation site of AKT at Ser473 (pSer473-AKT) and demonstrated primarily a cytoplasmic membrane-specific staining pattern in MM cells. We have also analyzed the expression pattern of pSer473-AKT in 18 MM patients. By immunohistochemical staining with an anti-pSer473-AKT antibody (Cell Signaling Technology, Beverly, MA), we

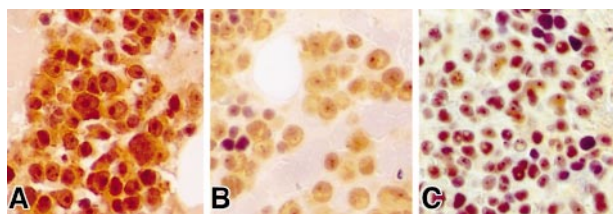


Figure 1. Immunohistochemical analysis of pS⁴⁷³-AKT in MM patients. (A-C) Bone marrow biopsies obtained from MM patients demonstrate immunopositivity for pSer473-AKT in plasma cells. Biopsies were fixed in 10% zinc formalin for 2 hours, subsequently decalcified in Decalcifier II (Surgipath Medical Industries, Richmond, IL) for 2 hours and processed by a standard automated processor. Four μ m sections mounted on glass slides underwent microwave antigen retrieval process in citrate buffer, subsequently incubated with anti-pSer473-AKT antibody at 1/100 dilution overnight at 4°C similar to described previously.⁹ Slides were then stained with a streptavidin/peroxidase detection system.⁹ Note that panels A and C illustrate prominent nuclear and nucleolar staining in the neoplastic plasma cells. Magnification \times 500.

found expression of phosphorylated-AKT in 16 of 18 patients, indicative of constitutively phosphorylated-AKT in primary MM cells. However, unlike the findings of Hsu et al, the majority of our samples showed marked nuclear expression and weaker cytoplasmic reactivity in the plasma cells (Figure 1). Interestingly, plasma cells from 8 patients demonstrated a marked nucleolar staining pattern. Overall, our findings were consistent with previous reports demonstrating nuclear localization of phosphorylated-AKT following activation.^{4,5} In addition to its well recognized role at the plasma membrane, AKT is also known to be important in nuclear transduction.³ Furthermore, AKT has been shown to migrate to the nucleus following receptor activation as seen with the B-cell antigen receptor in B lymphocytes.⁶ Once in the nucleus, activated AKT is believed to influence the functions of several regulatory proteins, such as AFX/Forkhead transcription factors, primarily through regulation of their subcellular localization.^{7,8}

Because IL-6 is known to be an important cytokine for myeloma cell survival and is known to mediate phosphorylation of AKT, we analyzed the effect of AKT inhibitors (wortmannin and LY294 002) on the IL-6-dependent human myeloma cell line U266. Similar to the analysis of other MM cell lines, both AKT inhibitors caused marked apoptosis as detected by annexin/7-AAD staining (Figure 2).² These results indicate that IL-6-mediated activation of AKT/PKB signaling is important for cellular survival of plasma cells in MM.

In summary, our study confirms that AKT plays a significant role in MM cell survival. However, the predominant immunohistochemical localization pattern of phosphorylated-AKT differs from that reported by Hsu et al. This is most likely related to the differences in the techniques used for pSer473-AKT detection,

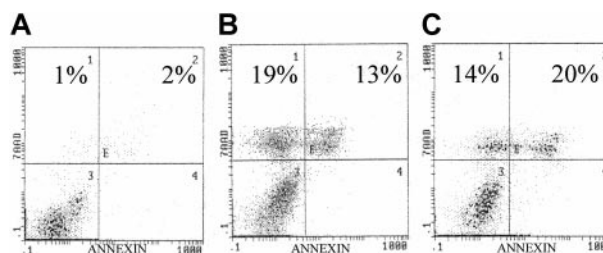


Figure 2. Effects of AKT inhibitors on U266 cell line. (A) Control. Exposure to the AKT inhibitors (panel B, wortmannin, 10 μ M/L; panel C, LY294002, 10 μ M/L) resulted in significantly increased apoptosis as indicated in each histogram. U266 cells were incubated with/without the presence of each inhibitor for 72 hours. Similar to our previous study, apoptosis was quantified by annexin/7AAD staining, indicating early and late apoptosis, respectively.⁹

including the source of antibody, fixation (Bouin vs formalin), and utilization of antigen retrieval. Overall, our data and those reported by Hsu et al indicate that overexpression and activation of AKT plays a significant role in MM cell survival. Thus selective inhibitors that specifically target the AKT signaling pathway may have important future therapeutic implications in the treatment of patients with MM.

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Response:

Anticoagulants for prophylaxis and treatment of thromboembolic events

The Food and Drug Administration (FDA) wishes to correct misleading statements published recently in *Blood*.¹ Dr David K. Cundiff asserts that several decision makers at FDA are "considering the withdrawal of the indication for anticoagulants (heparin, LMWH, and vitamin K antagonists) in prophylaxis and treatment of VTE."^{1(p723)} Dr Cundiff's assertion is incorrect: FDA is not, and has not been, considering withdrawal of these indications for these drugs.

Rather, FDA merely evaluated materials submitted to the agency by Dr Cundiff, who questioned the value of heparin (and, by extension, of other anticoagulants) in the treatment of deep venous thrombosis and pulmonary embolism. Having reviewed these submitted materials, FDA notified Dr Cundiff in a letter dated December 19, 2001, that the agency was satisfied with the quality and quantity of data supporting heparin's indications.

In addition, Dr Cundiff states that heparin is a “grandfathered” drug,^{1(p723)} implying that its efficacy has never been reviewed by FDA. Dr Cundiff’s statement is incorrect; heparin is not a grandfathered drug. FDA first approved heparin sodium injection on June 22, 1939, for prevention and treatment of postoperative thrombosis and embolism. A review of heparin’s efficacy was conducted for FDA by the National Research Council of the National Academy of Sciences (NRC/NAS) under implementation of the drug efficacy study (DESI). In 1970, NRC/NAS concluded that heparin is effective for the treatment and prevention of all venous and arterial thrombosis, thromboembolic disease, and the prevention and treatment of pulmonary embolism. As has been explained to Dr Cundiff, FDA evaluated and concurred with the efficacy conclusions of the NRC/NAS report.² In summary, FDA is

satisfied with the quality and quantity of data available in support of heparin’s stated indication.

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