Malignant Lymphomas

AKT plays a role in the survival of the tumor cells of extranodal NK/T-cell lymphoma, nasal type

Phosphorylated AKT has been detected in extranodal NK/T-cell lymphoma, nasal type (ENTL). Either interleukin-2 (IL-2) or interleukin-15 (IL-15) could prevent AKT dephosphorylation and apoptosis in the NK-92 cell line model. IL-15, but not IL-2, was preferentially elevated in patients' serum. AKT and IL-15 may be important in ENTL tumor survival.

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Extranodal natural killer/T-cell lymphoma, nasal type (ENTL) is an aggressive malignant tumor, characterized by a distinctive immunophenotype of $CD2^+$, surface $CD56^+$, cytoplasmic $CD3^+$ and strong association with Epstein-Barr virus (EBV). A small proportion of cases show an EBV^+, $CD56^-$ cytotoxic T-cell phenotype with T-cell receptor rearranagement. ENTL has a unique geographic distribution, occurring mainly in Asia and South America but being rare in the United States and Europe.[1] The pathogenesis of this rare lymphoma is not fully understood. Allelic loss in chromosome 6q and tumor suppressor gene silencing by promoter hypermethylation have been reported.[2-4] In this study, the role of AKT in tumor survival was investigated.

Genomic DNA from 4 samples of frozen tissues from patients with primary ENTL were screened for genomic anomalities by comparative genome hybridization-array chip analysis (AmpliOnc I microarray, Vysis, Downers Grove, IL, USA; *see supplementary data*). Low level amplification of *AKT1* was detected in 3 out of 4 cases of ENTL (75%). An average tumor:normal ratio of 1.245 was obtained for *AKT1*.

The functional significance of AKT was further investigated in 6 additional cases of primary ENTL by immunohistochemical analyses with an antiphospho-AKT (Ser 473) antibody following a standard microwave-sodium citrate antigen retrieval protocol. Strong positive nuclear staining of phosphorylated AKT was seen in 5 out of the 6 cases of primary ENTL (Figure 1A). The signals were negative in the surrounding stroma and the reactive lymphoid infiltrates. Control experiments on lymphoid cells from benign lesions of the nasal mucosa were also negative. These results indicate that AKT was phosphorylated and in a functionally activated state in ENTL. The role of AKT in ENTL was further studied in the cell line model, NK-92. Comparative genome hybridization analysis and real-time quantitative PCR of the genomic DNA of NK-92 detected amplification of the *AKT1* locus (*AKT1*: β-globin = 1.46, *see supplementary data*). The level of total AKT protein in NK-92 cells was also higher than in normal natural killer (NK) cells from healthy individuals, as shown by Western blotting (*see supplementary data*). Furthermore, AKT was phosphorylated (Figure 1B). AKT is a serine/threonine kinase activated by phosphoinositide 3-kinase (PI3K). Inhibition of PI3K by LY294002 resulted in a reduction of phosphorylated AKT and apoptosis of NK-92 (*see supplementary data*). Amplification and overexpression of AKT are frequently detected in different types of tumors, including gastric, breast, prostate and ovarian carcinomas. Active AKT has been shown to promote anti-apoptosis through phosphorylating pro-apoptotic proteins including Bad, caspase-9, forkhead transcription factors and IκB. AKT also participates in cell-cycle progression by inducing E2F activity and transcription of c-myc. Proteins related to cell-cycle arrest, such as p21 and p27, can also be phosphorylated by AKT, leading to exclusion of these proteins from the nucleus.[5] Our data in primary ENTL and in the NK-92 cell culture model highlighted the importance of AKT activation in cell survival of ENTL. In order for NK-92 cells to proliferate, interleukin-2 (IL-2) must be added to the culture medium. Interleukin starvation resulted in dephosphorylation of AKT and apoptosis. Laddering of genomic DNA and an increase in the sub-G1 cells were observed (Figure 2). Remarkably, apoptosis of NK-92 can be prevented by supplementa-

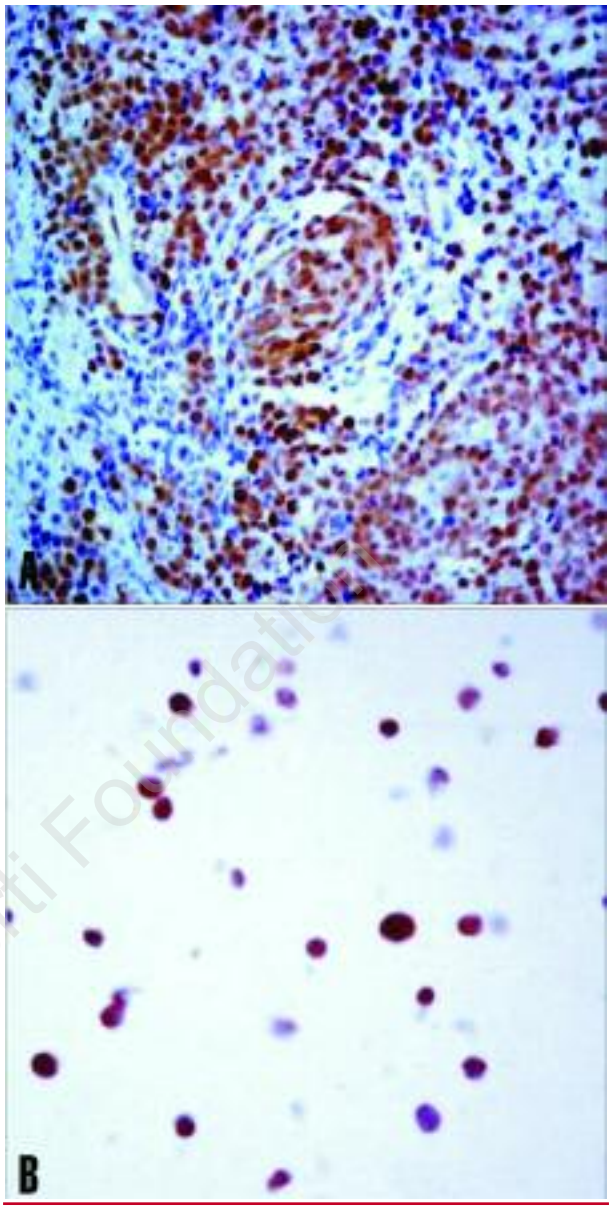

Figure 1. AKT of primary ENTL (A) and NK-92 (B) was activated. Activated AKT was analyzed by an immunohistochemical method in formalin-fixed paraffin-embedded primary ENTL tissue and cell blocks of NK-92 with rabbit antiphospho-AKT (Ser 473) antibody. Strong positive staining was predominantly noted in the nuclei of the tumor cells.

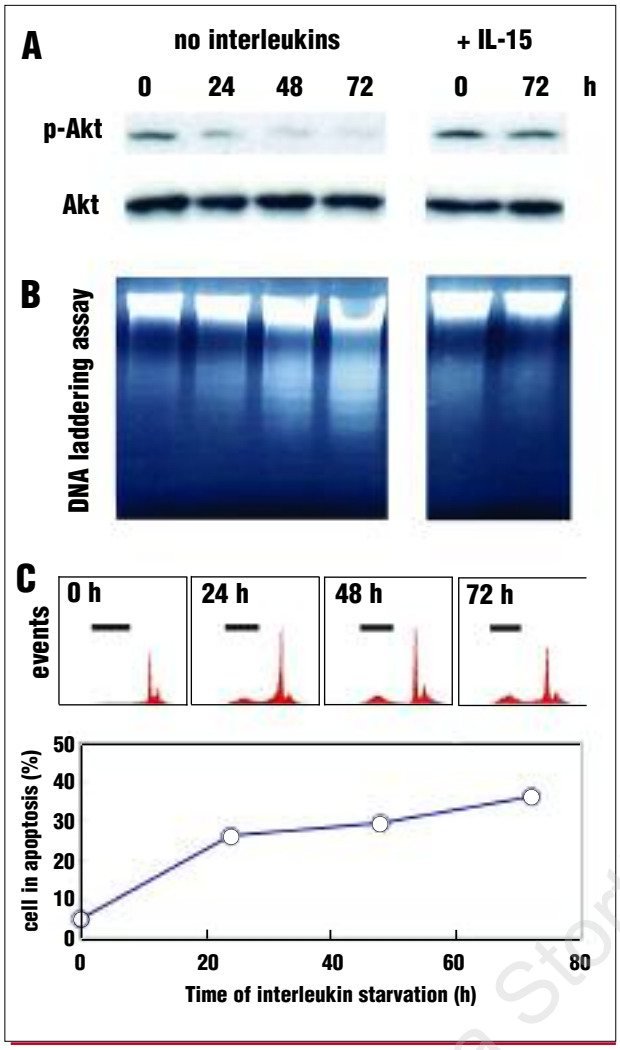

Figure 2. Apoptosis was induced after starving NK-92 cells of interleukins (IL-2 and IL-15). Western blotting analysis (A) for NK-92 deprived of interleukins revealed progressive dephosphorylation of AKT (A, upper panel, p-Akt) at different time points (0, 24, 48 and 72 hours, respectively). Total AKT (A, lower panel, Akt) remained unchanged. Starving NK-92 cells of interleukins led to apoptosis as shown by the progression to laddering of genomic DNA (B) and the progressive increase in the portion of cells in the sub-G1 peaks (C) in flow cytometry analyses. Supplementing NK-92 with IL-15 maintained phosphorylation of AKT and prevented cells undergoing apoptosis (right column, A and B, +IL-15 at 0 and 72 hours, respectively).

tion with interleukin-15 (IL-15, 5 ng/mL). AKT phosphorylation was also preserved by the IL-15 supplementation (Figure 2). These results suggest that, besides IL-2, IL-15 can also maintain the survival of NK-92 cells and AKT activation. The serum levels of IL-15 were significantly higher in ENTL patients (mean±standard deviation [SD]=3.22±0.53 pg/mL, n=8) than in normal healthy individuals (mean±SD=1.46±0.12 pg/mL, n=6; Student's t-test, p=0.011). The serum level of IL-2, on the other hand, was not significantly different (mean ± SD=0.159±0.012 IU/mL and 0.154±0.013 IU/mL in ENTL and normal subjects, respectively). These data suggest that while both IL-2 and IL-15 can maintain ENTL cell survival, IL-15 might play a significant role in ENTL patients *in vivo*. IL-2 and IL-15 share 2 common βγ receptors.[6] The mechanisms of IL-15 stimulation *in vivo* and the downstream pathways are, however, not clear. In a mouse model, prolonged stimulation with IL-15 resulted in the development of NK lymphoma.[7] Our data suggest that AKT may be one of the downstream pathways mediating the action of IL-15 in ENTL. The systemic increase of IL-15 in ENTL patients suggests that IL-15 may be important for the increase in tumor load of ENTL. All our cases are EBV positive, but the causes of raised IL-15 remain to be determined. Investigation along these pathways in ENTL may allow further understanding of this rare lymphoma.

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Multiple Myeloma

Lack of receptor activator of nuclear factor-κB ligand (RANKL) expression and functional production by human multiple myeloma cells

The direct expression and production of the critical osteoclastogenic factor, the receptor activator of NF-κB ligand (RANKL), is a matter of controversy. In this study we definitively demonstrate by both qualitative and quantitative polymerase chain reaction analysis that human myeloma cells do not express significant levels of RANKL mRNA or produce RANKL able to stimulate osteoclast formation.

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