

Nuclear-cytoplasmic localization of IAPs as novel marker for tumor progression

Barbara Vischioni

Apoptosis or programmed cell death is the complex and regulated process responsible for the maintenance of tissue homeostasis by balancing cell proliferation with cell death. It is essential for proper development and functioning of multicellular organisms, and for the removal of damaged or infected cells operated by the immune system. In the execution of the apoptotic process the key molecules are a family of aspartic-specific cysteine-proteases, called caspases (1).

Deregulation of this process has been related to several diseases, including cancer. Intensive research during the past decades has resulted in the identification of several proteins, which may promote tumorigenesis by inhibiting caspases (2).

The IAP (inhibitor of apoptosis) proteins constitute a family of negative regulators of programmed cell death, characterized by the presence of one to three copies of a ~ 70 amino acids BIR (baculoviral inhibitory repeat) motif, which is highly conserved from viruses to mammals. In humans, four IAPs (XIAP, cIAP1, cIAP2 and survivin) have been shown to restrain cell death in cancer cells through a mechanism of inhibition of both initiator and effector caspases (3).

The dynamic localization of proteins within different sub-cellular compartments is progressively being recognized as a fundamental process for cell function and regulation (4). In particular, nuclear-cytoplasmic shuttling has been shown to play an important role for apoptosis-related proteins, such as p53 or nuclear factor kappa-B (NFkB), whose respective function of cell cycle regulators or transcription factor depends strictly upon nuclear localization. The aim of this study is to investigate the localization of human IAPs in cancer cells and tumor samples, in order to understand whether their possible nuclear-cytoplasmic shuttling may be a mechanism of regulation for their function.

Our group recently reported that survivin is a nuclear shuttling protein that is actively exported from the nucleus through the CRM1-export receptor (5). By extending the study on localization to other IAPs, we were able to identify also cIAP1 and cIAP2 as nuclear-cytoplasmic shuttling proteins, whose functions and involvement in different pathways of apoptosis control and TNF (tumor necrosis factor) signal transduction may be differentially regulated in different cell compartments. We hypothesized that changes in the sub-cellular localization of IAPs might be important for different regulatory pathways involved in sensitivity of cancer cells to therapy and might be regarded as tumor marker. Blocking CRM1-mediated export with the specific drug leptomycin B (LMB) induced accumulation of both endogenous cIAP1 and epitope-tagged cIAP1 and cIAP2 in the nucleus of a panel of human cancer cells. Time course experiments showed that the kinetics of active nuclear import and CRM1-mediated nuclear export of these proteins lead to different rates of nuclear accumulation. By PCR and standard cloning techniques, we carried out a deletion analysis of YFP-cIAP1, and the shuttling capacity of a series of deletion mutants was compared with that of full-length cIAP1. This analysis identified the amino acid 184-275 of cIAP1 as the region responsible for shuttling. With an *in vivo* export assay, we have identified in this region a novel leucine-rich nuclear export signal (NES) that mediates cIAP1 export through its binding to the CRM1 exporter. The essential residues for export activity were characterized by site-directed mutagenesis and we used these findings to increase the nuclear accumulation of wild type cIAP1 by inactivating the NES activity. Forced relocation of cIAP1 to the nucleus did not significantly alter its ability to prevent apoptosis. Interestingly, co-expression experiments showed that the cIAP1 and 2-interacting protein TRAF2 (TNF-R-associated factor 2) plays an important role as regulator of the shuttling of both these cIAPs, by preventing their nuclear translocation. TRAF2-mediated cytoplasmic retention of cIAP1 was reduced upon TNF treatment. Our results identify molecular mechanisms that contribute to regulate the subcellular localization of cIAP1 and cIAP2. Translocation between different cell compartments may add a further level of control for cIAP1 and cIAP2 activity. The results briefly presented here were awarded the AACR-Bristol-Myers Squibb Oncology Scholar-in-Training Award by the 2003 AACR Annual Meeting Committee (6) and were written in a manuscript, which has now been published in *Experimental Cell Research* (7).

To translate the results from the lab to a clinical setting using the expertise in combination studies available in the Department of Oncology of the VU medical Center in Amsterdam (The Netherlands), we have assessed with immunohistochemistry the expression of survivin on tumor from 53 patients with advanced non-small cell lung cancer (NSCLC), and correlated that with the observed response to chemotherapy, time to progression and overall survival (8). Our group has already published the data about cIAP1, cIAP2 and XIAP expression in the same series of samples in 2001 (9). Expression of survivin is commonly detected in cancers but not in normal differentiated tissues. Survivin is usually localized in the cytoplasm of cancer cells, but nuclear localization has been also described and correlated with patient survival (10). Survivin was present only in malignant tissues, and 47/53 (89%) of the specimens were positive.

Three patterns of localization were observed: 42% of the cases (22/53) showed reactivity confined to the nucleus, 17% (9/53) only in the cytoplasm, and 30% (16/53) both in the nucleus and in the cytoplasm. Nuclear survivin levels predicted longer overall survival and relapse-free survival, by univariate and multivariate analyses. Expression and differential localization of survivin did not correlate with response to chemotherapy. Our results indicate that differential localization of survivin may be a prognostic factor for NSCLC.

In order to complete the study on IAPs expression and localization, we have performed an immunohistochemical study in a complete panel of normal human tissues. While survivin is usually not expressed in normal adult tissues, cIAP1, cIAP2 and XIAP have been found broadly expressed at mRNA levels within normal cells. We confirmed at the protein level the broad expression of IAPs, with few exceptions for specific cell types within each tissue. Moreover, we demonstrated that not only IAPs expression but also localization patterns differ according to cell lineage and stage of cell differentiation. As in cancer cells, cIAP1 expression was not only detected within the cytoplasm, but also in the nucleus of specific cell types. The results of this study are consistent with a critical role of IAP proteins in protecting both normal and cancer cells from death stimuli. The variety of their localization within different cell types may suggest a certain level of compartmentalization for their functions. These findings have been written in a paper recently submitted for publication (11).

In summary, all results of the molecular and translational studies supports the hypothesis of a possible role of IAPs localization as a marker for tumor progression and encourages further investigation.

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