Elevated Expression of Inhibitor of Apoptosis Proteins in Prostate Cancer

Maryla Krajewska,1 Stan Krajewski,1 Steven Banares, Xianshu Huang, Bruce Turner, Lukas Bubendorf, Olli-P. Kallioniemi, Ahmed Shabaik, Antonella Vitiello, Donna Peehl, Guo-Jian Gao, and John C. Reed2

The Burnham Institute, La Jolla, California 92037 [M. K., S. K., S. B., X. H., J. C. R.]; Department of Radiation Oncology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 [B. T.]; Institute of Pathology, University of Basel, Switzerland [L. B.]; Laboratory of Cancer Genetics, National Human Genome Research Institute, NIH, Bethesda, Maryland 20892-4470 [O-P. K.]; Department of Pathology, University of California, San Diego, San Diego, California 92103 [A. S.]; The R. W. Johnson Pharmaceutical Research Institute, San Diego, California [A. V.]; Department of Urology, Stanford University, Stanford, California 94305 [D. P.]; and BD Biosciences Pharmingen, San Diego, California 92121 [G-J. G.]

ABSTRACT

Purpose: Inhibitor of apoptosis (IAP) family proteins are suppressors of apoptosis that have been implicated in apoptosis resistance in some cancers. Their expression and relevance to the prognosis of prostate cancer were investigated.

Experimental Design: The expression of four members of the IAP family (cellular inhibitor of apoptosis protein 1, cellular inhibitor of apoptosis protein 2, X chromosome-linked IAP, and survivin) was examined by immunohistochemistry and immunoblotting in human prostate cancers and in prostate tissues from transgenic mice expressing SV40 large T antigen under control of a probasin promoter.

Results: Tumor-associated elevations in the levels of all four IAP family members were common in prostate cancers of both humans and mice, suggesting concomitant up-regulation of multiple IAP family proteins. Compared with normal prostate epithelium, increased IAP expression was often evident even in prostatic intraepithelial neoplasia lesions (carcinoma in situ), suggesting that deregulation of IAP expression occurs early in the pathogenesis of prostate cancer. IAP expression did not correlate with Gleason grade or prostate-specific antigen levels.

Conclusions: The findings demonstrate that tumor-associated elevations in the expression of several IAP family proteins occur as a frequent and early event in the etiology of prostate cancer.

INTRODUCTION

Prostate cancers are generally slow-growing malignancies that are characterized by an imbalance in the rates of cell division and cell death. Tissue kinetics studies indicate that insufficient programmed cell death represents the chief explanation for the gradual accumulation of prostate cancer cells in vivo in humans (1). Progression of localized hormone-dependent prostate cancers to metastatic, hormone-refractory disease is also associated with dysregulation of normal apoptotic mechanisms.

Apoptosis is executed by a family of cysteine proteases known as caspases. Caspases are produced in cells as inactive zymogens and generally must undergo proteolytic processing to become active proteases (reviewed in Ref. 2). The IAPs3 are the only known endogenous caspase inhibitors (3). They contain Baculovirus IAP repeat domains, and some of them bind and potently inhibit activated caspases, including, in mammals, the effector caspases-3 and -7 and the initiator caspase-9 (reviewed in Ref. 4). In addition to Baculovirus IAP repeat domains, several IAPs also contain a RING domain, which binds ubiquitin-conjugating enzymes that promote degradation of IAP caspase complexes (5). Eight human IAPs have been recognized, including XIAP, cIAP1, cIAP2, survivin, NAIP, apollon (BRUCE), ML-IAP (livin, KIAP), and ILP-2 (reviewed in Ref. 6). The antiapoptotic properties of IAPs have also been linked to the Rel/nuclear factor κB pathway and mitogen-activated protein kinase signal transduction (7, 8). In particular, cIAP1 and cIAP2 have been shown to activate nuclear factor κB (9); XIAP, NAIP, and ML-IAP have been reported to modulate apoptosis pathways via the TAK1/c-Jun-NH2-terminal kinase signaling cascade (10).

Some IAP family proteins are overproduced in cancers, suggesting that IAP-mediated suppression of apoptosis may contribute to tumor pathogenesis, progression, and resistance to drug treatment. For example, survivin is expressed abundantly in fetal tissues but scarcely present in most adult tissues. High levels of survivin protein have been reported in many types of human cancers, suggesting that reactivation of expression of this...

3 The abbreviations used are: IAP, inhibitor of apoptosis; PIN, prostatic intraepithelial neoplasia; PSA, prostate-specific antigen; XIAP, X chromosome-linked IAP; cIAP, cellular inhibitor of apoptosis protein; NAIP, neuronal apoptotic inhibitory protein; TRAMP, transgenic mouse model of prostate cancer; BPH, benign prostatic hyperplasia; DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase; NPE, nonmalignant prostate epithelium; RFS, relapse-free survival; HR, hazard ratio; CI, confidence interval; TAKI, TGFβ-activated kinase.

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1 Both authors contributed equally to this work.
2 To whom requests for reprints should be addressed, at The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037. Phone: (858) 646-3140; Fax: (858) 646-3194; E-mail: jreed@burnham.org.
gene represents a common event in tumorigenesis (reviewed in Refs. 11 and 12). Indeed, genome-wide transcription profiling suggests that survivin is among the most tumor-specific genes thus far identifiable (13). Similarly, the ML-IAP protein is not expressed at detectable levels in most normal adult tissues but is present in melanomas (14) and perhaps some other types of cancers. Likewise, whereas XIAP is broadly expressed in normal tissues, higher levels of this IAP family member have been demonstrated in patients with acute myelogenous leukemia (15). Thus, elevations in the levels of certain IAP family proteins may occur in tumors, conferring a selective survival advantage.

In this report, we analyzed the expression of several IAP family proteins, including survivin, XIAP, cIAP1, and cIAP2, in prostate cancers. Our data demonstrate that tumor-associated increases in the expression of several IAP family proteins occur commonly in prostate cancer and probably as an early event. Moreover, overexpression of these IAP family members was also documented in TRAMP, the model further suggesting an important role for deregulation of IAP expression in the pathogenesis of prostate cancer. IAP family proteins thus may be candidate drug discovery targets for restoration of apoptosis sensitivity in prostate cancer.

MATERIALS AND METHODS

Patient Specimens. Prostate cancer specimens for tissue microarray preparation were obtained from the archives of the Institute of Pathology, University of Basel (Basel, Switzerland), the Cantonal Institute of Pathology (Liestal, Switzerland), and the Tampere University Hospital (Tampere, Finland). The tissue microarrays included transurethral resections from 32 patients with BPH and 725 prostate cancer specimens, containing 646 primary tumors derived from 592 patients, and 79 metastases. The primary tumors included 225 specimens representing clinically inapparent (stage T1, according to International Union Against Cancer criteria (16)) tumors; 368 cancers from radical prostatectomies, including both locally confined (stage T2) and locally advanced disease (stage T3–T4); and an additional 53 local recurrences after hormonal therapy failure. Metastatic tumor specimens (n = 79) were collected at autopsy from 62...
In addition, prostate carcinoma specimens were obtained from a well-organized cohort of uniformly treated patients presenting to the Thomas Jefferson University, Department of Radiation Oncology (Philadelphia, PA) for whom clinical follow-up information was available. Needle biopsy specimens included 64 primary tumors derived from these patients, who presented with stage T2 peripheral zone carcinomas (T2 N0 M0) treated by external beam radiation. For 48 of these patients, additional biopsies lacking tumor tissue were also available for comparison. Cancer progression during a median follow-up of 66 months was defined as biochemical recurrence [three consecutive rises in PSA concentration]. Of the 16 of 62 (26%) patients who experienced rising PSAs, 15 patients developed metastatic disease as documented by bone scans (94%).

Additional normal prostatic tissues for immunohistochemical analysis were derived from human biopsy and autopsy material (Department of Pathology, University of California, San Diego).

**Tissue Preparation.** Tissues were fixed in either neutral-buffered formalin, zinc-buffered formalin (Z-fix; Anatech Inc.), or Bouin’s solution (Sigma, St. Louis, MO) and embedded in paraffin. Tissue microarrays were constructed as described previously (17).

**Antibodies.** Polyclonal antisera for survivin (AR-26) and XIAP (AR-27A) were generated in New Zealand white rabbits using recombinant protein immunogens. Survivin (full-length protein) was produced as a glutathione S-transferase-fusion protein and affinity purified essentially as described previously (18). Affinity-purified His6-tagged XIAP (BIR2) recombinant protein was produced as described previously (19) and used as an immunogen for producing XIAP-specific antiserum.

Fig. 2 Examples of immunohistochemical detection of IAP family proteins in normal and malignant human prostate. Prostate cancer biopsies from stage T2 patients (A–F, I, and J) and tissue microarrays (G, H, K, and L) were immunostained, applying IAP antisera followed by detection using a HRP-based method with DAB colorimetric substrate (brown). Nuclei were counterstained with hematoxylin (blue). Representative data for cIAP1 (A–D), cIAP2 (E–H), XIAP (I and J), and survivin (K and L) are shown. Immunostaining results in regions of invasive cancer are shown for cIAP1 (C and D, ×150–200), cIAP2 (H, ×20), and survivin (L, ×20). Examples of malignant and adjacent normal prostatic epithelium are presented for cIAP1 (A, ×100), cIAP2 (E–G, ×100, ×400, and ×5, respectively), XIAP (I and J, ×100–400), and survivin (K, ×20). An example of PIN immunostaining is presented for cIAP1 (B, ×250).
Table 1  IAP immunoexpression data for tissue microarray

<table>
<thead>
<tr>
<th>Marker</th>
<th>NPE</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>cIAP1</td>
<td>54</td>
<td>31 ± 11</td>
</tr>
<tr>
<td>cIAP2</td>
<td>40</td>
<td>47 ± 15</td>
</tr>
<tr>
<td>XIAP</td>
<td>17</td>
<td>18 ± 18</td>
</tr>
<tr>
<td>Survivin</td>
<td>29</td>
<td>3 ± 13</td>
</tr>
</tbody>
</table>

*Antisera are available from Biocarta, Inc. (www.biocarta.com) and Science Reagents, Inc. (www.sciencereagents.com).*

AR-27A). Polyclonal anti-cIAP1 and anti-cIAP2 antibodies were obtained from R&D Systems Inc.

The monoclonal antibody to human cIAP2, clone F30-2285, was generated according to routine procedures after fusion of hybridoma cell line F0 with spleenocytes from a mouse immunized with full-length recombinant cIAP2 protein. Clonal selection resulted in the isolation of the three specific clones, F30-2285, F30-2295, and F30-591, which reacted with human cIAP2 recombinant protein and were isotyped as IgG1. Only the F30-2285 clone was used in this study. Generation of this antibody was performed at BD Biosciences-PharMingen, Inc. (San Diego, CA), and the antibody will be distributed by the company.

The monospecificity of all antibodies for their intended protein targets was tested by SDS-PAGE/immunoblot analysis, using IAP family proteins in vitro translated from cDNAs or using recombinant proteins produced in bacteria (3, 15, 20, 21).

**Immunohistochemistry.** Dewaxed tissue sections were immunostained by using a DAB-based detection method as described previously, using the Envision-Plus HRP system (DAKO) and an automated immunostainer (Dako Universal Staining System; Ref. 22). Polyclonal rabbit antisera specific for survivin (AR-26) and XIAP (AR-27A) were applied at 1:10,000 and 1:5,000 (v/v), respectively, whereas rabbit antibodies against cIAP1 (R&D Systems Inc.) and cIAP2 (R&D Systems Inc.) were used at 1:600 (v/v). The cIAP2 monoclonal antibody was used at 0.4 μg/ml. For all polyclonal antisera used, the immunostaining procedure was performed in parallel using preimmune serum to verify specificity of the results. Initial confirmations of antibody specificity also included experiments in which antiserum was preabsorbed with 5–10 μg/ml of either synthetic peptide immunogen or recombinant protein immunogen. The scoring of tumor immunostaining was based on the percentage of immunopositive cells (0–100) multiplied by staining intensity score (0, 1, 2, or 3), yielding immunoscores of 0–300. For all biomarkers tested, immunoscores in cancers (CA) were compared with those in NPE, using an unpaired t test.

**Immunoblotting.** Human tissue lysates containing prostate cancer (n = 12) with high ratios of cancer cells relative to stroma (>70%), BPH and PIN (n = 5), or normal prostate (n = 4) were normalized for total protein content (100 μg/lane) and subjected to SDS-PAGE/immunoblot analysis, using a 1:2000 (v/v) dilution for XIAP (AR-27A), a 1:5000 dilution for survivin (AR-26), and a 1:1000 dilution for cIAP1 (R&D Systems Inc.) and cIAP2 antisera (R&D Systems Inc.) and using secondary HRP-conjugated goat antirabbit antibody [1:3000 (v/v) dilution; Bio-Rad]. Alternatively, the mouse anti-cIAP2 monoclonal antibody was used at 0.4 μg/ml in conjunction with secondary HRP-conjugated goat antimouse IgG (Bio-Rad). Tissue lysates containing normal, premalignant, or malignant prostatic epithelium derived from TRAMP mice (24) were subjected to the same procedure. Detection was accomplished using an enhanced chemiluminescence (Amersham-based) multiple antigen detection immunoblotting method that allows for multiple reprobing of blots without antibody stripping, as described previously (25).

**Animal Model.** Transgenic mice (C57BL/6) expressing the SV40 large T antigen controlled by rat probasin promoter regulatory elements were obtained from Baylor University (Houston, TX) and bred in accordance with institutional guidelines. Animals were genotyped for the Tag gene by PCR (24, 26). Additionally, Tag expression in prostate was confirmed by immunohistochemistry using monoclonal antibodies to Tag (Pab100 and Pab101; PharMingen Inc.). TRAMP male mice (n = 44) were examined at 12–28 weeks of age. Prostates were harvested from anesthetized animals after initial perfusion with 2% paraformaldehyde and postfixation in Bouin’s solution. For histological and immunohistochemical investigation, the specimens were embedded in paraffin, and 4-μm sections were cut.

**Statistical Analysis.** Data were analyzed using the STATISTICA software package (StatSoft). A log-rank test was used for correlation of immunostaining data with patient survival. Survival distributions were estimated using Kaplan-Meier curves. Multivariate Cox proportional hazards models were fitted to the data to assess which biomarkers were independently associated with disease-free survival. Ninety-five percent CIs for the HR were calculated by the formula exp (β ± 1.960 SE(β)), where SE(β) denotes the SE of the estimated regression coefficient. Gleason score of >7 and pretreatment PSA of >10 ng/ml were defined as “high” for purposes of dichotomizing data. Statistical significance of differences in IAP levels in normal versus malignant prostatic tissue was assessed using the unpaired t test.

**RESULTS**

**Characterization of IAP Antibodies and Immunoblot Analysis of Prostate Tissues.** To study expression of the IAP family proteins cIAP1, cIAP2, XIAP, and survivin, we used
various antibodies that were either generated in our laboratory or obtained from commercial sources. Immunoblot analysis was performed to characterize the specificity of these antibodies, using IAP family proteins produced by in vitro translation from cDNAs, recombinant IAP family proteins generated in bacteria, and lysates from cell lines and tissues. These included related reference controls comprised of various IAP family proteins (XIAP, survivin, cIAP1, cIAP2, NAIP, BRUCE, and Cp-IAP) and unrelated proteins such as Traf-3 and CD40. Moreover, surgical specimens from normal human spleen, brain, prostate, liver, and kidney were compared by immunoblotting with cancer cell lines and primary prostate cancer specimens. Representative data are presented in Fig. 1.

A commercially available rabbit polyclonal antibody from R&D Systems, Inc., was determined to be specific for cIAP1, reacting only with cIAP1 but not with cIAP2, XIAP, NAIP, or survivin (Fig. 1A). Rabbit polyclonal antibodies generated in our laboratory against fragments of recombinant IAP family proteins were determined to be specific for XIAP, survivin, cIAP1, cIAP2, NAIP, BRUCE, and Cp-IAP and unrelated proteins such as Traf-3 and CD40. Moreover, surgical specimens from normal human spleen, brain, prostate, liver, and kidney were compared by immunoblotting with cancer cell lines and primary prostate cancer specimens. Representative data are presented in Fig. 1.

Using the specific antibodies, immunoblot analysis was performed on another set of tissue lysates derived from normal prostate glands and BPH specimens consisting of histologically confirmed, nontransformed NPE, making comparisons with lysates from resected prostate cancers. This immunoblot analysis provided preliminary evidence of elevated expression of IAP family proteins in prostate cancers (Fig. 1, D–F). For example, levels of cIAP2 were higher in four of four cancer specimens tested, compared with NPE specimens (Fig. 1, D and E). Likewise, in another group of specimens, at least half of the eight cancer samples examined contained higher levels of cIAP1 and XIAP than normal prostate specimens (Fig. 1F). Elevations in survivin protein were also seen in some of these tumor tissue specimens (Fig. 1F). Reprobing the blots with antibodies recognizing β-tubulin confirmed loading of equivalent amounts of total protein from all samples (data not shown). In addition, a mouse monoclonal antibody raised against recombinant cIAP2 as an immunogen was determined to be specific for its intended protein, lacking cross-reactivity with cIAP1, Survivin, or XIAP (Fig. 1D). All of these anti-IAP antibodies reacted with the mouse homologues of cIAP1 (MIAP-2), cIAP2 (MIAP-1), XIAP (MIAP-3), and survivin (MIAP-4), reflecting the high percentage of amino acid sequence identity of these proteins in humans and in mice (84–90% of sequence identity for MIAP-2, MIAP-3, and MIAP-4 and 61% of sequence identity for MIAP-1; data not shown).

Using the specific antibodies, immunoblot analysis was performed on another set of tissue lysates derived from normal prostate glands and BPH specimens consisting of histologically confirmed, nontransformed NPE, making comparisons with lysates from resected prostate cancers. This immunoblot analysis provided preliminary evidence of elevated expression of IAP family proteins in prostate cancers (Fig. 1, D–F). For example, levels of cIAP2 were higher in four of four cancer specimens tested, compared with NPE specimens (Fig. 1, D and E). Likewise, in another group of specimens, at least half of the eight cancer samples examined contained higher levels of cIAP1 and XIAP than normal prostate specimens (Fig. 1F). Elevations in survivin protein were also seen in some of these tumor tissue specimens (Fig. 1F). Reprobing the blots with antibodies recognizing β-tubulin confirmed loading of approximately equal amounts of total proteins from all lysates tested (Fig. 1, E and F).
IAPs Are Commonly Overexpressed in Human Prostate Cancers. To further characterize the expression of IAPs in human prostate cancers, we used previously constructed tissue microarrays (17) containing archival prostate tumors reflecting the full range of neoplastic prostate disease, including clinically inapparent (stage T1) tumors and cancers from radical prostatectomies that were either locally confined (stage T2) or locally extensive (stage T3) as well as local recurrences after failed hormonal therapy and metastatic tumor specimens from patients obtained at autopsy. Gleason score data were available for 45% of these tumors, whereas clinical stage information (T1–T4) according to International Union Against Cancer criteria (16) was known for 40% of tumor specimens. In addition to invasive or metastatic cancer, these microarrays contained 32 separate cases of BPH for comparison with cancers. In addition, roughly 8% of tumor specimens on the arrays contained non-neoplastic prostate epithelium adjacent to tumor tissue, permitting additional comparisons of nontransformed epithelium with invasive cancer.

Microarrays were immunostained using the aforementioned antibodies specific for cIAP1, cIAP2, XIAP, or survivin (15). The specificity of these immunostaining results was confirmed by control stainings performed using either preimmune serum or immune antisera that had been preabsorbed with the relevant immunogens (data not shown). Immunostaining results were quantified according to the approximate percentage of immunopositive cells (0–100%) and immunointensity on a 0–3 scale, and then an immunoscore was calculated from the product of the percentage immunopositivity and immunointensity (0–300; Ref. 23).

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### Table 2
Comparison of IAP expression data with RFS in stage T2 prostate cancer patients

IAP immunostaining data were dichotomized using the median immunoscore for segregating patients into high versus low expression categories (cutpoint indicated). Data represent the proportion of patients (percentage in parentheses) with low versus high expression remaining relapse free after median follow-up of 66 months. Significance (\(P\)) was calculated based on a log-rank test.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Median score</th>
<th>Proportion (%)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cIAP1</td>
<td>160</td>
<td>19/24 (80%)</td>
<td>25/37 (68%)</td>
</tr>
<tr>
<td>cIAP2</td>
<td>225</td>
<td>24/29 (83%)</td>
<td>18/30 (60%)</td>
</tr>
<tr>
<td>XIAP</td>
<td>60</td>
<td>23/38 (61%)</td>
<td>22/24 (92%)</td>
</tr>
<tr>
<td>Survivin</td>
<td>100</td>
<td>25/32 (78%)</td>
<td>21/30 (70%)</td>
</tr>
</tbody>
</table>

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### Table 3
Summary of histopathology for prostate tissue from TRAMP mice

Summary of H&E histology. The prostate glands of a total of 44 TRAMP male mice and 10 age-matched control mice were investigated by H&E histology. The numbers of animals developing PIN or invasive cancer are indicated at various ages. PIN and cancer lesions were multifocal in nearly all animals in which transformation of prostate epithelium was found.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>TRAMP</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Animals/group PIN Cancer</td>
<td>Animals/group PIN (cases/foci) Cancer (cases/foci)</td>
</tr>
<tr>
<td>12–16</td>
<td>6 2 0</td>
<td>4 0 0</td>
</tr>
<tr>
<td>18–24</td>
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<td>3 0 0</td>
</tr>
<tr>
<td>28</td>
<td>30 22 12</td>
<td>3 1 0</td>
</tr>
<tr>
<td>Total</td>
<td>44 28 13</td>
<td>10 1 0</td>
</tr>
</tbody>
</table>

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Fig. 4 Correlations of biomarker immunostaining data with RFS for stage T2 prostate cancer patients. The log-rank test was used for correlation of immunoscore data with patient survival. Kaplan-Meier curves illustrate correlations of the investigated biomarkers with RFS for this cohort of patients. RFS curves for patients expressing high levels of markers are denoted in black symbols. White symbols indicate patients whose tumors contain lower protein levels, using the median immunoscore for dichotomizing data. Median immunoscores were 160 for cIAP1, 225 for cIAP2, 60 for XIAP, and 100 for survivin.
Fig. 5 Examples of immunohistochemistry data for IAPs in the TRAMP model. To characterize the expression of cIAP1, cIAP2, XIAP, and survivin in TRAMP mice, paraffin sections from male urogenital system were immunostained. Immunodetection of specific reactions was accomplished by a DAB-based colorimetric method (brown), and the sections were either not counterstained or counterstained with methyl green or hematoxylin (blue). Expression of cIAP1/MIAP-2 protein (A, B, and D) is demonstrated in the transformed prostatic epithelium. PIN lesions are indicated by arrows in A and B (∼10 and ∼80, respectively). Note elevated immunostaining in invasive adenocarcinoma (D, ∼200). Elevated cIAP2/MIAP-1 levels are shown during progression of malignant transformation of the prostate gland in E–I [E (∼200), normal prostatic epithelium (arrow) and PIN (brown); G (∼400), PIN; H (∼150), invasive cancer]. Survivin/MIAP-4 expression data are shown in J–L. Survivin immunostaining of prostate tissue of TRAMP mice reveals marked increase in immunointensity in PIN compared with nontransformed prostatic epithelium (J and K; arrow). Immuno-
Immunohistochemical analysis of tumor tissues on the microarrays revealed cancer-specific elevations in the expression of the four IAP family proteins tested. Fig. 2 shows representative examples of the immunostaining results for tumor specimens, demonstrating higher intensity immunostaining in invasive cancer compared with NPE for all IAP proteins investigated. Calculated immunoscores were also higher for all four IAPs, comparing cancer with NPE (Table 1).

Using the tissue microarray data, we compared IAP immunoscores with a variety of variables, including Gleason grade, clinical stage (T1–T4), and hormone-refractory disease. IAP family immunoscore data did not correlate with Gleason grade. However, higher cIAP2 immunoscores correlated with higher T stage, suggesting that higher levels of this IAP family protein tend to occur in larger or more invasive tumors. For example, cIAP2 immunoscores were 83 ± 5 (mean ± SE) for T1–T2 (n = 221) tumors compared with 168 ± 18 for T3–T4 tumors (n = 18; P < 0.0001). Survivin immunoscores also tended to be higher in more invasive tumors, with immunoscores of 65 ± 6 for T1–T3 tumors (n = 152) versus 97 ± 16 for T3–T4 tumors (n = 19), but this difference did not reach statistical significance (P = 0.06).

Comparisons of untreated tumors with hormone-refractory specimens on the microarray revealed significant correlations of refractory disease with higher cIAP2 [139 ± 4 (n = 563) versus 202 ± 8 (n = 144)] and with lower cIAP1 [104 ± 4 (n = 516) versus 67 ± 7 (n = 137)] and lower survivin [77 ± 4 (n = 319) versus 34 ± 13 (n = 32)]. Further evidence of a correlation of higher cIAP2 expression with aggressive disease was found by comparing the immunoscores of 79 metastases collected at the autopsies from 62 patients, who had undergone androgen deprivation by orchiectomy and had subsequently died of end-stage metastatic prostate cancer, revealing higher levels of cIAP2 protein (mean immunoscore, 215 ± 10) compared with primary tumors (mean immunoscore, 142 ± 4; n = 591; P < 0.0001).

Analysis of IAP Expression in a Cohort of Uniformly Treated Patients with Early-Stage Prostate Cancer. To more precisely contrast the levels of IAP family protein expression in tumor versus normal prostate tissue, we analyzed skinny-needle biopsies from a small cohort of men (n = 64) with early-stage disease (T2N0M0) who were uniformly treated with external beam irradiation. During needle biopsy before radiotherapy, several cores of tissue were obtained from each patient, providing case-matched tissue samples containing only normal prostatic epithelium for 48 of the 64 tumor biopsies available.

Using the normal and tumor specimens derived from these early-stage patients, we evaluated the expression of the cIAP1, cIAP2, XIAP, and survivin proteins by immunohistochemistry and scored the results as described above (23). Whereas immunostaining results varied widely among specimens examined, the overall immunoscores for the cancers displayed clear elevations in immunoreactivity when compared with histologically normal specimens (Fig. 3; Table 1). For example, whereas 98% of normal prostate specimens had cIAP1 immunoscores of <100, 50 of 61 (82%) invasive cancer specimens had immunoscores of ≥100 (P < 0.0001), thus suggesting that many prostate cancers develop pathological elevations in the levels of this antiapoptotic protein. Similarly, levels of cIAP2 protein in 60 of 61 (99%) of cancers exceeded cIAP2 immunoscores representative of 98% normal prostate specimens (H-score ≥ 100; P < 0.0001). Likewise, XIAP immunoscores were ≤100 for nonmalignant epithelium, in contrast to invasive cancers, where 15 of 64 (23%) had immunoscores of >100 (P < 0.0001). Finally, all normal prostatic epithelium samples possessed immunoscores of ≤40 for survivin, whereas 44 of 62 invasive cancers (71%) had survivin immunoscores of >40 (P < 0.0001).

We also looked at the data an alternative way, where only case-matched samples of normal and tumor tissue from the same patient were evaluated (n = 48), thus excluding the 14 tumor specimens for which normal tissue was unavailable. In pairwise analyses, expression of IAP family proteins as defined by immunoscore was greater in tumor compared with matching normal prostate tissue for cIAP1 in 35 of 36 (97%) cases, cIAP2 in 32 of 32 (100%) cases, XIAP in 26 of 40 (65%) cases, and survivin in 36 of 44 (82%) cases (all Ps < 0.001). Thus, for all IAP family members examined, evidence of tumor-associated up-regulation of expression was observed by comparisons of normal versus tumor tissues from these early-stage prostate cancer patients. These patient materials thus provide independent confirmation of the impressions derived from archival tissue microarrays that multiple IAP family proteins are often simultaneously overexpressed in prostate cancers.

In addition to frequent overexpression of IAPs in invasive cancers, we also observed increases in immunostaining for cIAP1, cIAP2, XIAP, and survivin in many precancerous PIN lesions. Based on comparisons with NPE, levels of cIAP1 protein in 12 of 21 (57%) PIN lesions exceeded cIAP1 immunoscores representative of normal prostate epithelium (H-score ≥ 100; P < 0.0001), suggesting that elevations of cIAP1 protein occur early in the process of malignant transformation. Similarly, 9 of 22 (41%) PIN specimens had elevated cIAP2 immunoscores compared with NPE (P < 0.0001). Also, whereas XIAP immunoscores were ≤100 for all NPE specimens, 8 of 26 (31%) PIN lesions showed increased levels of XIAP protein (P < 0.0001). Similarly, compared with NPE, 45% of PIN specimens (10 of 22) had elevated survivin immunoscores (P < 0.0001).

Staining of invasive cancer with anti-survivin antibody (L) or with preimmune serum (M) is also shown, revealing intense survivin immunoreactivity in nuclei and cytosol of tumor cells and confirming specificity of the immunostaining, respectively. N–Q show XIAP/MIAP-3 staining results. Elevated XIAP levels were observed in PIN (N × 80; P × 200) and invasive cancer (Q × 250), whereas normal prostatic epithelium contained barely detectable XIAP/MIAP-3 immunostaining (N × 80; P × 200; arrows). The specificity of the immunostaining results was confirmed by control stainings performed using either preimmune serum or immune antiserum that had been preabsorbed with the relevant immunogens [C (×80), preabsorbed MIAP-2; F (×200), preabsorbed MIAP-1; I (×150), preimmune serum; M (×250), preabsorbed survivin/MIAP-4; O (×80), preabsorbed XIAP/MIAP-3].
Table 4 Summary of IAP expression data for prostate tissue from TRAMP mice

<table>
<thead>
<tr>
<th>Marker</th>
<th>PIN POS/total foci</th>
<th>CA POS/total foci</th>
<th>PIN POS/total foci</th>
<th>CA POS/total foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>cIAP1/MIAP-2</td>
<td>148/164 (90.3%)</td>
<td>27/30 (90%)</td>
<td>1/3 (33%)</td>
<td>0</td>
</tr>
<tr>
<td>cIAP2/MIAP-1</td>
<td>164/164 (100%)</td>
<td>25/30 (83%)</td>
<td>2/3 (67%)</td>
<td>0</td>
</tr>
<tr>
<td>XIAP/MIAP-3</td>
<td>135/164 (82.3%)</td>
<td>22/30 (73%)</td>
<td>3/3 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Survivin/MIAP-4</td>
<td>158/164 (96.3%)</td>
<td>14/30 (47%)</td>
<td>1/3 (33%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Although representing a relative small cohort, attempts were made to correlate expression of IAP family proteins with clinical outcome using the results obtained for the 64 early-stage patients. To dichotomize data into higher versus lower expression categories, the median immunoscore result for each IAP family protein tested was used to split the data set, thus comparing RFS (PSA relapse) for the patients with immnoscores above versus below the median value. Kaplan-Meier curves and log-rank tests demonstrated a trend of patients with higher cIAP1 and higher cIAP2 to relapse with greater frequency during the follow-up period (median follow-up, 5.5 years), but the results did not reach statistical significance (P = 0.06 for cIAP1 and cIAP2; Fig. 4, Table 2). In contrast, higher levels of XIAP were unexpectedly associated with longer RFS (P = 0.0001). Survivin immunostaining data were not correlated with RFS (Fig. 4).

Immunostaining data did not correlate with Gleason scores or PSA nadir for all IAPs tested (data not shown). However, low levels of XIAP protein were associated with higher pretreatment PSA (mean, 26.4 ng/ml; Hybritech assay) compared with tumors containing higher XIAP protein (mean PSA, 11.7 ng/ml; P = 0.009), which may be related to the unexpected association of higher XIAP with longer RFS. Gleason grade also was significantly correlated with RFS (P = 0.01) in this patient cohort. When stepwise multivariate analyses were conducted using a Cox proportional hazards regression model (variables included cIAP1, cIAP2, XIAP, survivin, Gleason score, and pretreatment PSA), the factors that remained predictive of relapse in this cohort were high cIAP1 immunoscore (HR, 3.8; 95% CI, 1.16–12.53; P = 0.03), lower XIAP (HR, 0.06; 95% CI, 0.01–0.31; P = 0.0007), and high Gleason score [HR, 2.8; 95% CI, 1.1–7.25; P = 0.03 (see supplemental data)].

Analysis of IAPs in a Mouse Transgenic Model of Prostate Cancer. The SV40 T-antigen TRAMP closely resembles the progression of human prostate cancer (28).

Histological analysis of prostate tissue from TRAMP transgenic mice revealed age-dependent appearance of PIN and invasive cancer, with 73% of male mice developing multifocal PIN (22 of 30, 73%), and approximately half of mice developing invasive cancer [also often multifocal (12 of 30, 40%) by 28 weeks of age (Table 3). In contrast, only 1 of 10 control littermates developed PIN, and no prostate cancers were detected in nontransgenic mice.

We then explored the expression of IAP family proteins in the prostates of male transgenic and aged-matched littermate control mice, using immunohistochemistry and immunoblotting methods (Fig. 5, A–Q; see supplemental data). Although barely expressed in the normal prostatic epithelium, high levels of MIAP-1 and MIAP-2 immunostaining (assessed using antibodies raised against human cIAP2 and cIAP1), respectively, were found in transformed prostatic epithelium of SV40 large T-antigen transgenic mice (Fig. 5, A, B, and E–G; summarized in Tables 3 and 4). In some animals, overexpression of these IAP family proteins was evident in carcinoma in situ (PIN), in addition to invasive adenocarcinomas (Fig. 5, B, D, G, and H; Table 4). Similarly, survivin/MIAP-4 immunostaining was not detected in normal prostate epithelium but was found in PIN and in many invasive cancers (Fig. 5, J–L). Interestingly, whereas most normal cells were immunonegative for survivin, positive immunostaining was found in occasional nontransformed prostatic epithelial cells of both TRAMP and normal mice, associated with chromosomes or mitotic structures in cells apparently undergoing division. In contrast, intense survivin immunoreactivity was found in interphase nuclei and in the cytosol of most transformed cells within PIN lesion and invasive cancers in TRAMP mice. Heterogeneity in the percentage of transformed cells expressing survivin, however, resulted in some cases scoring as negative, where at least 50% immunopositivity was set as the threshold (see legend of Table 4 for details). The intensity of XIAP/MIAP-3 immunostaining also increased during malignant transformation (Fig. 5, N–Q), although more aggressive tumors that had infiltrated seminal vesicles rarely contained high levels of this protein. The specificity of these immunostaining results was confirmed by control stainings performed using either preimmune serum or immune antisera that had been preabsorbed with the relevant immunogens (Fig. 5, C, F, I, M, and O).

Immunoblot analysis at least partially corroborated these findings. Expression of M IAP-1 (orthologue of human cIAP2), MIAP-2 (orthologue of human cIAP1), and XIAP/MIAP-3 was detected in every tumor specimen evaluated (n = 8; data not shown). Elevated levels of MIAP-2 protein (compared with normal prostate tissue) were readily documented by immunoblotting for all tumor specimens evaluated. Cancer-associated increases in MIAP-1, XIAP/MIAP-3, and survivin/MIAP-4 were more heterogeneous, as measured by comparisons of normal and tumor tissue by immunoblotting, possibly because of admixture of normal and malignant cells. Nevertheless, at least half the tumor specimens analyzed contained elevated levels of...
MIAP-1, XIAP/MIAP-3, and survivin/MIAP-4, as determined by immunoblotting. Thus, we conclude that tumor-associated overexpression of several IAP family proteins occurs during prostate cancer development in TRAMP mice.

DISCUSSION

In the normal prostate, tissue homeostasis is achieved by matching the 1–2% daily rate of cell proliferation with an equal rate of cell death (29). Deregulation of apoptosis contributes to tumor initiation, progression to the androgen-insensitive state, and metastasis. IAP family proteins represent critical regulators of apoptosis that serve as endogenous inhibitors of caspase family cell death proteases (3). Our immunohistochemical analysis demonstrates for the first time that alterations in the expression of several IAP family proteins (cIAP1, cIAP2, XIAP, and survivin) occur commonly and often simultaneously in prostate cancers.

Previously, survivin was reported to be aberrantly overexpressed in most cancers, including prostate, lung, colon, breast, pancreatic, and gastric cancer and others (reviewed in Ref. 11). However, little is known about the expression of other members of the IAP family in cancers. Expression of cIAP1, cIAP2, XIAP, survivin, and NAIP has been examined in the National Cancer Institute collection of 60 human tumor cell lines, revealing widespread expression of cIAP1, XIAP, and survivin in these tumor lines of diverse tissue origins and demonstrating cIAP2 expression primarily in lymphoid malignancies (15). Importantly, these studies also demonstrated poor correlation of cIAP1, cIAP2, and XIAP protein levels with mRNA levels, consistent with emerging knowledge that expression of many IAPs is regulated predominantly at the level of protein stability (15). Higher levels of XIAP protein have been correlated with shorter remission duration after chemotherapy and shorter survival in patients with acute myelogenous leukemia (15). The IAP family member ILP-2 (livin, ML-IAP, KIAP) reportedly is overexpressed in melanoma (14).

Thus, when combined with these prior reports, our data provide additional evidence that pathological elevations in the expression of antiapoptotic IAP family proteins represent a common event in many types of cancer. Not only were elevations in IAP family proteins seen in archival human prostate cancer specimens, but we also observed increases in the levels of cIAP1/MIAP-2, cIAP2/MIAP-1, XIAP/MIAP-3, and survivin/MIAP-4 during disease progression in a transgenic mouse model of prostate cancer. Up-regulation of IAP expression concomitant with the emergence of PIN implicates a role of these proteins in the early stages of the pathogenesis of prostate cancer and emphasizes the utility of TRAMP for identifying molecular changes in early disease. This finding suggests that overexpression of IAP family proteins is a general concomitant of transformation of prostate epithelium and reinforces the idea that multiple IAP family members become dysregulated in their expression during the pathogenesis of malignancy in the prostate gland. It remains to be seen whether the simultaneous overexpression of several IAP family proteins reflects a commonality in the mechanisms controlling the levels of IAP family proteins in cells versus the possibility that multiple independent signaling pathways that control individual IAP family members become simultaneously deregulated in these neoplasms.

Based on the data provided here, we cannot conclude whether differences in the expression of one or more IAP family proteins (individually or in combination) are of prognostic significance for men with prostate cancer. In the tissue microarray data set, higher cIAP2 levels were associated with larger tumor size (T stage), hormone-refractory disease, and metastatic disease, but these patients were heterogenous with respect to Gleason grade, pretreatment PSA, and therapy. In the small cohort of early-stage patients treated uniformly with external beam radiation, higher cIAP2 correlated in multivariate analysis with shorter RFS. In contrast, higher XIAP was unexpectedly associated with longer RFS in this small cohort, based on both univariate and multivariate analyses. The association of higher cIAP1 and cIAP2 levels with more aggressive disease is consistent with the documented function of these antiapoptotic proteins as suppressors of caspases (20). As for XIAP, whereas this protein is a potent caspase inhibitor and suppressor of apoptosis (3), XIAP has also recently been reported to inhibit cell proliferation by down-regulating levels of cyclins A and D1 and inducing expression of the cyclin-dependent kinase inhibitors p21/Waf1 and p27/Kip1 (30). Thus, XIAP may provide a selective survival advantage while simultaneously impairing division of cancer cells. Other explanations for the paradoxical association of XIAP with the shorter RFS could be related to expression in the same tumors of endogenous antagonist proteins (which were not measured here), such as SMAC, XAF1, and the serine protease Omi/HtrA2, which can negate apoptosis suppression by XIAP (31–35).

The functional importance of IAPs in cancer is beginning to be clarified. Involvement of IAP family proteins in tumor resistance to chemotherapeutic drugs and other apoptotic agents has been demonstrated by use of antisense techniques, helping to validate certain IAPs as potential drug targets for cancer (36–42). Also, heptameric peptides from the NH2 terminus of the IAP antagonist protein SMAC have also been applied to reverse caspase inhibition by IAPs in vitro (43–49) and to overcome IAP-mediated suppression of apoptosis in leukemia cell lines (45), suggesting a strategy for cancer therapy. Furthermore, a recent observation that cytochrome c microinjection fails to induce apoptosis in prostate cancer LNCaP cells without SMAC also implies a crucial role for proteins of the IAPs in this organ (48).

Given emerging data suggesting an important role for IAP family proteins in sustaining tumor cell survival and suppressing apoptosis induced by anticancer drugs (38–42, 44–50), our results demonstrating that overexpression of several IAP family proteins occurs frequently in prostate cancers in humans and transgenic mice provide further validation of IAPs as potential drug discovery targets for the improved treatment of prostate cancer. The observation that more than one IAP family member is often overexpressed simultaneously in prostate cancers, however, raises the possibility that effective strategies will require IAP antagonists that are capable of inhibiting multiple members of this family of apoptosis-suppressing proteins.
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Elevated Expression of Inhibitor of Apoptosis Proteins in Prostate Cancer

Maryla Krajewska, Stan Krajewski, Steven Banares, et al.


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