The Inhibitor of Apoptosis Protein Livin (ML-IAP) Plays a Dual Role in Tumorigenicity

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Abstract

The inhibitor of apoptosis protein (IAP) family can inhibit apoptosis induced by a variety of stimuli. We and others previously described the IAP Livin (ML-IAP). We found that Livin is unique among the IAP members as, on a strong apoptotic stimulus, it is specifically cleaved by caspases to produce a truncated protein with paradoxical proapoptotic activity (tLivin). We also showed that Livin encodes two splicing variants, termed Livin α and β, with diverse antiapoptotic effects in vitro. In this study, we compared the Livin isoforms in vivo. An animal model was established and the effects of Livin α and β on the initiation and development of tumors were compared. In the animal model, Livin α promotes tumor initiation in comparison with control. Interestingly, the growth of tumors originating from cells expressing Livin β was inhibited. In these tumors, Livin β was cleaved and produced a high level of the proapoptotic tLivin β that repressed tumor development. When we eliminated the proapoptotic effect of Livin β by point mutations, the resulting antiapoptotic Livin β mutants contributed to tumor progression. In terms of mechanism, we show that Livin β tumors develop only in mice lacking natural killer (NK) cell activity. Thus, from the animal model, we can conclude that Livin plays a major role in tumorigenicity and that NK cells induce cleavage of Livin to its proapoptotic truncated protein that in turn inhibits tumor growth. Therefore, Livin and tLivin may serve as potential targets for cancer therapy. [Cancer Res 2009;69(13):5475–80]

Introduction

Apoptosis is a major barrier to cancer that must be circumvented. Indeed, cancer cells can acquire resistance to apoptosis through a variety of strategies. The resistance of tumor cells to drug-induced apoptosis is a major category of cancer treatment failure. The process of apoptosis is tightly controlled. In the last decade, a complex network of proapoptotic and antiapoptotic proteins that governs this tight regulation has been revealed and it is now possible to lay out a provisional apoptotic signaling circuitry. A novel family of intracellular antiapoptotic proteins that has become increasingly prominent in the field of cancer is the inhibitor of apoptosis protein (IAP) family. IAP family members are able to inhibit apoptosis induced by a variety of stimuli mainly by binding and inhibiting specific caspases, primarily 3, 7, and 9. IAPs can also suppress apoptosis through caspase-independent mechanisms (1, 2).

Eight human IAPs have been identified: c-IAP1, c-IAP2, NAIP, Survivin, XIAP, Bruce, ILP-2, and Livin (ML-IAP). IAP family members contain one or more repeats of a highly conserved 70–amino acid domain termed the baculovirus IAP repeat (BIR) located at the NH2 terminus. Some human IAPs also contain a conserved zinc binding motif known as really interesting new gene (RING) domain that is located at the COOH terminus (1).

The IAP Livin contains a single BIR domain at the NH2 terminus as well as a COOH-terminal RING domain (3). We found that Livin encodes two splicing variants, termed Livin α and Livin β. The two proteins are highly similar, except for 18 amino acids located between the BIR and the RING domains, which are present in the Livin α but not the Livin β isoform. Both isoforms share similar antiapoptotic properties and can protect Jurkat T cells from apoptosis triggered via tumor necrosis factor and FAS receptors. However, despite the high similarity, we showed also different antiapoptotic properties of the two isoforms. Only Livin α isoform protects cells from apoptosis triggered by staurosporine, whereas only Livin β protects cells from etoposide-induced cell death (3).

We further found that both Livin isoforms are specifically cleaved at the Asp52 residue to produce truncated proteins, p30-Livin α and p28-Livin β. The truncated Livin protein (tLivin) not only loses its antiapoptotic activity but also acquires a proapoptotic effect (4). Thus, Livin bears the ability both to protect cells from cellular death and to promote it once it is cleaved.

In this work, we developed an animal model to study the role of Livin in tumorigenesis. We compared between the effects of Livin α and Livin β on the initiation and development of tumors in mice. We found that Livin α promotes tumor initiation. In contrast, tumors originating from cells expressing Livin β did not develop due to cleavage of Livin and accumulation of high levels of the proapoptotic truncated Livin β. We also showed in the animal model that tumors expressing Livin β develop only in mice lacking natural killer (NK) cell activity.

Materials and Methods

Cells. L428, a Hodgkin’s disease (HD) cell line, was grown in RPMI 1640. Transformed human melanocytes (MLTR; kindly provided by P.B. Gupta and R.A. Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA; ref. 5) were grown in DMEM. Media were supplemented with 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 mmol/L l-glutamine.

Plasmids and transfection. L428 cells were transfected with the pIRE2-EGFP plasmid (Qiagen) encoding the various constructs of Livin, which was carried out by electroporation. Following transfection, clones were isolated by limiting dilutions followed by fluorescence-activated cell sorting (FACS) for green fluorescent protein (GFP) to achieve 95%
GFP-positive populations. Clones that express similar levels of Livin α and β were selected for further studies.

**Retrovirus vectors and infections.** We generated retroviral constructs by subcloning the Livin cDNA into the pWZL-ires-blasticidin (subcloned into EcoRI-BglII sites) and pBabe-neomycin (subcloned into EcoRI-SalI sites) vector systems. Livin mutants were described previously (4, 6, 7). We created amphotropic retroviruses by transient cotransfection of 10-cm plates of 293T cells with 3 μg of appropriate packaging plasmid pCl-10A, 1 μg of mobile-based virus infectious and CMV-BSR81, CMV-MSV/GIT for lentiviral-based virus infections and CMV-B8.91, CMV-MSV/S for lentiviral infections (P lentilox 3.7), and 5 μg of carrier vector using FuGene 6 (Roche). We collected viral supernatants 48 and 72 h after transfection, passed them through a 0.45-μm filter, and supplemented them with 8 μg/mL polybrene (Sigma) before infection. Together with the infection of retroviruses expressing Livin cDNA, we coinfected MLTR cells with P lentilox 3.7 that encodes for GFP. We achieved ~85% infection as judged by GFP and blasticidin resistance. Two days after infection, cells were split and placed under selection using either blasticidin (8 μg/mL) in the case of pWZL-blasticidin vector or neomycin (500 μg/mL) in the case of pBabe-neomycin vectors. Following drug selection, the polyclonal populations were sorted by FACS to achieve >95% GFP-positive cell population.

**Tumorigenicity studies.** All tumorigenicity studies were approved by the institute animal ethics committee (MD 103,18-4). Nonobese diabetic (NOD)/severe combined immunodeficient (SCID) and SCID/bg immunodeficient 7- to 8-wk-old male mice (Harlan) NOD/scid IL2Rγnull and NOD/SCID (The Jackson Laboratory) were used in this study; mice were housed and maintained under specific pathogen-free conditions in facilities under an institute-approved animal protocol. In the animal model, mice were injected s.c. with tumorigenic cells in the right flank. L428 cells are poorly tumorigenic in NOD/SCID mice as only 60% of NOD/SCID mice developed tumors at 12 d after injection of 25 × 10^6 L428 HD cells (8). We injected mice with 15 × 10^6 L428 cells (in 200 μL of PBS; n = 16) or with 1 × 10^6 of the highly tumorigenic MLTR cells (in 100 μL of PBS; n = 4–20; ref. 5). Weight and tumor measurements were obtained every 2 to 3 d. Tumors were measured with a caliper, and the volume was calculated using the formula cm^3 = 4/3πr^3 (r = tumor radius). At different time points, the mice were sacrificed, and the tumors were harvested and analyzed. Mice were sacrificed when one-dimensional tumor diameter reached 1 to 2 cm (according to the guidelines of the animal core facilities).

**Western blot analysis.** Tumors were removed and homogenized in lysis buffer (20 mM/L Tris-HCl, 2 mM/L EDTA, 6 mM/L β-mercaptoethanol, 1% NP40, 0.1% SDS, and protease inhibitors [1 mM/L phenylmethylsulfonyl fluoride, protease inhibitor cocktail Sigma, and complete inhibitor cocktail (Roche)]) at 1°C for 20 min, with vigorous vortexing. Whole-cell lysates were prepared from 0.25 to 1 × 10^6 cultured cells using 100 μL lysis buffer (as described above), and cells were lysed at 4°C for 20 min, with vigorous vortexing. Protein content was measured by the detergent-compatible protein assay (Bio-Rad), according to the manufacturer’s instructions. Samples were resolved on a 10% Bis-Tris precast gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was exposed to the antibodies in a blocking solution (PBS, 1% casein, 0.05% Tween 20) for 1 h followed by three 5-min washes with PBS. A monoclonal antibody against Livin (clone 88C570, Imgenex) was diluted 1:3,000. Envision horseradish peroxidase (DAKO) was used as a secondary antibody for enhanced chemiluminescence reaction.

**Immunohistochemistry.** Tumors were formalin fixed, embedded in paraffin, and sectioned. Cleavage of caspase-3 in vivo was evaluated by immunohistochemistry with an anti–cleaved caspase-3 antibody (Cell Signaling Technology).

**Statistical analysis.** Data were expressed as mean ± SE values, and the results were analyzed using a two-tailed Student’s t test to assess statistical significance. Values of P < 0.05 were considered statistically significant.

**Results**

**Differential effects of Livin α and β on tumorigenicity.** To study the role of Livin in tumorigenicity in vivo, we used L428, a HD cell line that is poorly tumorigenic in NOD/SCID mice, requiring a very high number of cells to generate tumors in 60% of injected mice (8). L428 cells do not express the Livin protein (Fig. 1A).

To begin with, we compared the tumorigenicity of L428 cells stably expressing Livin α or Livin β to cells transfected with empty vector (EV). We injected the tumor cells s.c. to NOD/SCID mice and followed tumor development. Tumors originating from cells expressing Livin α developed earlier than cells expressing Livin β or control cells: 11 days after injection, 47% (7 of 15) of mice injected with cells expressing Livin α developed tumors and none (0 of 15) of the control mice. Surprisingly, only 6% (1 of 16) of mice injected with cells expressing Livin β developed tumors. Thirteen days after injection, 40% of mice injected with control cells developed tumors and 87% (13 of 15) of mice injected with cells expressing Livin α developed tumors. Surprisingly, only 13% of mice injected with cells expressing Livin β developed tumors (Fig. 1B). Moreover, tumors expressing Livin α grew much faster as measured by tumor volume compared with cells expressing Livin β (P = 0.012 and 0.001 at 11 and 13 days after injection, respectively) or control cells (P = 0.01 and 0.007 at 11 and 13 days after injection, respectively; Fig. 1C). As the tumors developed, tumors of control cells grew closer in size to tumors of cells expressing Livin α, and ~22 days after injection, the size of the control tumors was insignificant from the Livin α tumors (Fig. 1D). However, Livin β tumors grew slowly throughout the experiment. Twenty-five days after injection, tumors expressing Livin β were significantly smaller in volume from Livin α (P = 0.0003) and control tumors (P = 0.006; Fig. 1D).

These results indicate that Livin α plays a role in tumor initiation and promotes tumor progression. Interestingly, these results indicate that Livin β plays a different role in tumorigenicity and inhibits tumor development.

We previously showed the important role of Livin in melanoma and a role for Livin in drug resistance of melanoma tumor cells (4). Therefore, we were interested to study this role of Livin in the animal model. We used normal human melanocytes that underwent transformation using defined genetic elements (SV40, hTERT, and ras-v12; ref. 5). The transformed melanocytes are highly tumorigenic and form melanomas that frequently metastasize to multiple secondary sites in NOD/SCID mice (5). We tested the effect of Livin α and Livin β on the progression of tumors of these transformed melanocytes by injecting cells stably expressing Livin α, Livin β, and control cells (Fig. 2A) s.c. to NOD/SCID mice (n = 6). Similarly to the HD model, transformed melanocytes expressing Livin α grew faster, as measured by tumor volume, than cells expressing Livin β (P = 0.0018) and control cells (P = 0.013) at day 4 after injection (Fig. 2B). However, very quickly tumors that arose from control cells grew closer in size to tumors of cells expressing Livin α and their size was insignificant from the Livin α tumors at days 5 and 9 after injection (Fig. 2B). In addition, similarly to the HD model, we found that the tumors of transformed melanocytes expressing Livin β grew slowly throughout the experiment (50 days; Fig. 2C). These tumors were significantly smaller than both Livin α tumors (P = 0.002, 0.0006, or 0.01 at 5, 9, or 36 days after injection, respectively) and control tumors (P = 0.02, 0.029, or 0.003 at 5, 9, or 36 days after injection, respectively; Fig. 2B and C). Thus, the same effect of Livin α and Livin β on development of tumors was shown in both the HD and melanoma models.

Because most human tumors that express the Livin protein express both α and β isoforms (3, 9–19), we next explored the interaction between Livin α and β by coexpression of both isoforms in the melanoma cells (n = 8; Fig. 3A). Once again, Livin β
inhibited tumor development compared with Livin α and control tumors (Fig. 3B). Tumors expressing both α and β isoforms behaved similarly to tumors expressing Livin β alone in comparison with control tumors (P = 0.012 and 0.009, respectively; Fig. 3C).

Truncated proapoptotic Livin is responsible for inhibition of tumor development. In our previous work, we showed that both Livin isoforms are cleaved to produce truncated proteins that lead to cell death (4). To test if Livin is cleaved during tumorigenesis, we performed a time course experiment of the cleavage of Livin during tumor development. Transformed melanocytes stably expressing Livin α, Livin β, and control cells were injected s.c. to NOD/SCID mice (n = 20). During tumor development, three mice from each group were sacrificed at days 3, 8, 12, 17, and 44 after injection (Fig. 4A). Tumors were removed and Livin protein in tumors bearing Livin α or Livin β was analyzed by Western blot. In Livin α tumors, we did not observe cleavage of Livin (Fig. 4B, top), but in Livin β tumors, the protein underwent cleavage throughout the experiment (Fig. 4B, bottom). At the end of the experiment, sections of the tumors were examined for apoptotic cell death (20) by staining of cleaved (activated) caspase-3 (Fig. 4C). In Livin β tumors, we observed necrotic areas that were not observed in Livin α or control tumors. The majority of the transformed melanocytes in the periphery of these necrotic areas stained positive for cleaved (activated) caspase-3. This observation indicates that tLivin β leads to induction of cell death and destruction of tumor cells. Because Livin α was not cleaved in tumors (Fig. 4B), we tested directly the effect of the tLivin α on tumorigenicity. tLivin α was introduced into transformed melanocytes that were injected to mice and allowed to develop tumors. Unlike Livin α and similarly to Livin β, truncated Livin α inhibited tumor development (P = 0.0034 at 40 days after injection; n = 6; Fig. 4D). Thus, expression of the proapoptotic tLivin protein, either by ectopic expression (in the case of tLivin α) or by cleavage of full-length Livin (in the case of tLivin β), leads to tumor cell death and inhibition of tumor growth.

To abrogate the cleavage of Livin β and the accumulation of proapoptotic tLivin in the tumor cells, we introduced a point mutation in Livin β at the cleavage site that does not allow cleavage of Livin (Livin βD→A; ref. 6). We also used mutated Livin β that carries a point mutation in the RING domain (Livin βRING) inhibited tumor development compared with Livin α and control tumors (Fig. 3B). Tumors expressing both α and β isoforms behaved similarly to tumors expressing Livin β alone in comparison with control tumors (P = 0.012 and 0.009, respectively; Fig. 3C).

Figure 1. The effect of Livin on HD tumor development in mice. L428 cells (15 × 10⁶) stably expressing Livin α, Livin β, or EV control cells were injected s.c. into the right flank of NOD/SCID mice (n = 16). A, Western blot of Livin in L428 cells expressing Livin α, Livin β, and control cells. B, percentage of mice bearing tumors (radius > 0.15 cm) at 11 and 13 d after injection of tumor cells (percentage noted above each bar). C, tumor volumes at 11 and 13 d after injection of tumor cells (tumors developed in 8 of 15 EV mice, 14 of 15 Livin α mice, and 5 of 16 Livin β mice). D, time course of tumor development for 27 d. *, P < 0.012.

Figure 2. The effect of Livin on melanoma tumor development in mice. MLTR cells (1.5 × 10⁶) stably expressing Livin α, Livin β, or EV control cells were injected s.c. into the right flank of NOD/SCID mice (n = 6). A, Western blot of Livin in MLTR cells expressing Livin α, Livin β, and control cells. B, tumor volumes at 4, 5, and 9 d after injection of tumor cells (all mice developed tumors). C, time course of tumor development for 48 d. *, P < 0.03.
that was shown to abolish the proapoptotic activity of tLivin β (7). Both Livin β mutants that hold only the antiapoptotic activity lost the inhibition of tumor development that was observed in Livin β tumors (Fig. 5). In fact, these tumors grew faster than control tumors \((P = 0.0055\) for Livin \(β_{D-A}\) and 0.0004 for Livin \(β_{RING}; n = 7;\) Fig. 5). Thus, we propose that accumulation of the truncated proapoptotic protein in Livin β tumors is responsible for the inhibition of this tumor cell proliferation.

NK cell activity is required for the apoptotic activity of Livin β. We described above that tumor development of transformed melanocytes expressing Livin β was inhibited as a result of cleavage of Livin and the accumulation of proapoptotic tLivin. Because no exogenous apoptotic stimuli were applied to induce cleavage of Livin, we suspected that cleavage was induced by antitumor activity of the immune system that plays a major role in tumor development. SCID mice used in this study are immunocompromised and lack T and B lymphocytes. However, NK cell activity in these mice remains intact (21). NK cells, as part of the innate immune system, can recognize and eliminate tumor cells. We therefore investigated the role of NK cells in the development of tumors expressing Livin β. We compared tumor development of transformed melanocytes stably expressing Livin β between two mouse strains, NOD/SCID mice and SCID/bg mice, which have defective NK cell activity (22). SCID/bg mice carry a mutation in the \textit{beige} gene that leads to defects in NK cell function (22). Indeed, in SCID/bg mice, Livin β lost the inhibitory effect on tumor development that was observed in NOD/SCID mice \((P = 0.017, n = 4;\) Fig. 6.). In fact, the SCID/bg mice tumors developed rapidly and these mice had to be sacrificed 20 days after injection. We used an additional strain of mice with defective NK cell activity, NOD/SCID IL2Rα\textsuperscript{null} mice, which lack a functional common interleukin-2 chain (IL2Rα\textsuperscript{null}) resulting in inhibition of NK cell development (23). Similarly to the SCID/bg mice, transformed melanocytes expressing the \textit{beige} gene that leads to defects in NK cell function (22). Indeed, in SCID/bg mice, Livin β lost the inhibitory effect on tumor development that was observed in NOD/SCID mice \((P = 0.017, n = 4;\) Fig. 6.). 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Livin β developed only in IL2Rγnull mice that are deficient in NK cells and not in NOD/SCID mice (\(P = 0.003, n = 4\); Fig. 6B).

**Discussion**

Many studies revealed links between members of the IAP family and cancer. Although some studies imply that IAPs participate in tumor progression rather than initiation (24), other studies show high levels of IAPs in early stages and even premalignant lesions, indicating an early role in these tumors (2). One possible mechanism by which IAPs can promote tumorigenesis is by keeping mutated cells alive, increasing the risk of malignant transformation (25). Survivin and XIAP are expressed in many malignancies. Their expression is correlated with unfavorable cancer characteristics, such as aggressive behavior, shorter overall survival, resistance to therapy, and increased risk for recurrences (26, 27). Livin is overexpressed in many cancer cells, including melanoma, breast, cervical, colon, prostate, lung, bladder, neuroblastoma, leukemia, and lymphoma cells (3, 9–19). *In vitro* silencing of Livin β but not Livin α inhibits the proliferation of HeLa cells (28). In addition, small interfering RNAs, targeting both Livin isoforms, inhibited human cancer growth in mice (29). Given the role of Livin in controlling apoptosis and its selective up-regulation in malignancies make this IAP a potential therapeutic target for anticancer strategies.

We and others showed that XIAP (30), cIAP1 (31), and Livin (4) are specifically cleaved by effector caspases during apoptosis induced by various apoptotic stimuli. Caspase-mediated cleavage converts Livin and cIAP from antiapoptotic to proapoptotic factors. However, the truncated proapoptotic cIAP does not contain the BIR domain that plays a role in the antiapoptotic function of IAPs. In contrast, truncated Livin acts as a proapoptotic factor, although it contains an intact BIR domain (4).

In this work, we introduced Livin to tumorigenic cell lines and studied tumorigenicity in immunodeficient NOD/SCID mice. We found that Livin α plays a role in tumor initiation and promotes tumor development. The effect of Livin α on initiation and development of tumors was shown in both HD cells (Fig. 1) and melanoma model (Fig. 2). The relatively poor tumorigenicity of the L428 HD cells allows better demonstration of the growth advantage provided by Livin α and its role in tumor initiation and progression that is maintained for 20 days after tumor cell injection. Interestingly, in both animal models, Livin β tumors grew slowly compared with Livin α and EV. These results indicate that Livin β plays a diverse role in tumor development and slows tumor progression that is maintained for 20 days after tumor cell injection. Thus, full-length antiapoptotic Livin protects tumor cells from cellular death and facilitates tumor formation. These results indicate that the balance between the level of the full-length protein and its cleaved product determines whether Livin inhibits or promotes apoptosis and thereby tumor development.

We next sought to determine why Livin β is cleaved in tumors. We previously showed that noncleavable Livin protects cells from human NK cell–induced cytotoxicity (6). Here, we show *in vivo* using NK-deficient mice (Fig. 6) that NK cells induce inhibition of tumor development. Thus, Livin β is a target for NK cell–mediated tumor destruction. Here, we show the different contribution of Livin α and β to tumor development through their response to NK cell–induced cytotoxicity. Immune-mediated tumor destruction is emerging as a modality to cure cancer patients and several immunotherapeutic strategies have shown that immune manipulation can induce the regression of tumors.

Our animal model agrees with our and other clinical findings. Previously, we showed differential expression pattern of Livin in primary cultures derived from melanoma tumors. We also showed...
a direct correlation between the expression level of Livin and the resistance of the cells to chemotherapy both in vitro and in melanoma patients receiving chemotherapy. High levels of expres-
sion were also correlated with a lower survival rate (2). Results from
our lab show that high Livin protein expression in melanoma
patients is correlated with poor prognosis. However, low level of
Livin protein was correlated with better prognosis compared with
patients with no Livin expression.3 Our animal model for Livin
proves that Livin is a regulator of apoptosis rather than an
inhibitor of apoptosis and can explain the observations of our
clinical data: full-length, antiapoptotic Livin is responsible for the
poor response of patients to chemotherapy. However, cleavage of
Livin in response to chemotherapy and the accumulation of
truncated proapoptotic Livin lead to tumor cell death and better
response to chemotherapy. Our results in the animal model,
combined with the clinical data, agree with recent published results
in childhood acute lymphoblastic leukemia (ALL; ref. 19) that show a
positive correlation between Livin protein expression and favorable
prognostic factors. As shown by Choi and colleagues (19) in the ALL
responding patients, Livin underwent cleavage and there was accumula-
tion of the truncated proapoptotic Livin. In neuroblastoma
patients, high level of Livin protein correlated with better survival in
comparison with low, intermediate, or no Livin expression (18).

In summary, Livin is unique among the IAP members, exerting
both antiapoptotic and proapoptotic activities while retaining both
functional domains, making it a regulator of apoptosis rather than
antiapoptotic protein. This study provides a better understanding
of the contribution of Livin to the development of cancer. Our
animal model has similar behavior to our melanoma primary cultures and patients (4).3 Livin with the antiapoptotic activity
promotes tumor development and confers resistance against
apoptosis. However, upon cleavage of Livin, mediated by NK cell
activity, tumor cells undergo apoptosis, leading to inhibition of
tumor development. Truncated Livin is a highly potent proapop-
totic agent that would be a most useful therapeutic tool for the
treatment of malignancies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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