Temporal and Spatial Patterns of Expression of Inhibitors of Apoptosis in Human Placentas

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The apoptosis cascade that plays a central role in normal and pathological processes is strictly controlled, in part by newly described members of the inhibitor of apoptosis (IAP) family (HIAP-1, HIAP-2, XIAP, NAIP, Survivin, and Livin). Here, we report the expression of IAP mRNAs and proteins in early and late gestation human placentas, term cytotrophoblast cells, and two choriocarcinoma cell lines, JEG-3 and Jar. Reverse transcriptase-polymerase chain reaction identified mRNAs derived from all of the currently known IAP genes in all samples. Analysis by immunoblotting revealed that IAP proteins are present in early and late gestation human placentas and that levels of IAPs are not identical in normal and transformed trophoblast cells. Immunohistochemical experiments performed on paraformaldehyde-fixed tissue sections taken from early and late stages of pregnancy demonstrated that expression patterns differed according to cell lineage and stage of cell differentiation. The results of this study are consistent with the postulate that IAP proteins have critical roles in placental cell survival and suggest that specific apoptosis inhibitors may protect normal and transformed trophoblast cells from cell death. (*Am J Pathol* 2003, 163:413–422)

Apoptosis, also known as programmed cell death, is a central feature of organ development, homeostasis, and immune defense in multicellular organisms.¹ Several types of molecules are known to interfere with apoptosis. Some act at the plasma membrane, some on mitochondria, and others on the caspases.^{1,2} For example, anti-apoptotic members of the Bcl-2 family such as Bcl-2, Mcl-1, and Bcl-X_L act on mitochondria to inhibit cell death by maintaining mitochondrial function, limiting the effects of reactive oxygen species, and preventing the release of proapoptotic factors such as cytochrome c and apoptosis-inducing factor.²

Apoptosis may also be inhibited by recently identified molecules that include members of the inhibitors of apoptosis (IAP) family.^{2,3} The IAPs restrict apoptosis using baculovirus IAP repeat domain(s) at the NH₂-terminus to

interfere with caspases.^{4,5} Seven human IAPs have been reported: human IAP-1 (HIAP-1, also called cIAP-2 and hMIHC), HIAP-2 (also called cIAP-1 and hMIHB), X-linked IAP (XIAP, also called hMIHA and hILP), neuronal apoptosis inhibitory protein (NAIP), Survivin, Livin (also called ML-IAP and KIAP), and Apollon.^{4–7}

Apoptosis is believed to have a major role in development of the normal human placenta,^{8,9} and appears also to play a role in pathologies of this organ, resulting in preeclampsia and intrauterine growth retardation.^{10–13} Subversion of the cell death cascade may also be critical to survival of placental tumor cells such as choriocarcinomas, but this has not as yet been investigated.

Of the well-described apoptosis inhibitors, only Bcl-2 and Mcl-1 have been identified in human placentas; both have been postulated to inhibit apoptosis during syncytialization^{14,15} and trophoblast invasion.¹⁰ With the exception of XIAP,¹⁶ transcription and translation of the IAPs have not been reported in human placentas. To initiate studies on the roles of these molecules in normal and disease states, we established cell type-specific and temporal expression of IAP mRNAs and proteins in early and late gestation placentas, term cytotrophoblast cells, and two choriocarcinoma cell lines, JEG-3 and Jar. Apollon was not tested because reagents were unavailable.

Materials and Methods

Cell Lines and Tissue Samples

Trophoblast-derived choriocarcinoma cell lines, JEG-3 (HTB-36) and Jar (HTB-144) cells, were purchased from the American Type Culture Collection (Manassas, VA). The cell lines were grown in tissue culture flasks at 37°C in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and antibiotics (Sigma). Placentas from first trimester ($n = 6$, ranging from 6 to 12 weeks of gestation) were obtained from patients undergoing elective pregnancy termination, and term placentas ($n = 6$) were obtained from patients at term who underwent cesarean section to alleviate fetal distress. These acquisitions were done in

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Table 1. Summary of PCR Primer Sequences, Annealing Temperatures, and Expected Product Sizes

Primer	Sequence of forward and reverse primers (5' → 3')	Annealing temperature (°C)	Product size (bp)	Number of cycles	GenBank accession number
HIAP-1	GCCTGATGCTGGATAACTGG GGCGACAGAAAAGTCAATGG	56	349	35	U45878
HIAP-2	GCCTGATGCTGGATAACTGG GCTCTTGCCAAATCTGATGG	60	361	35	U45879
XIAP	GTGACTAGATGTCCACAAGG CTTGAGGAGTGTCTGGTAAG	56	368	35	U45880
NAIP	TTATACCAGCGCCAGTTTCC TGGTGGAAGTAAGGGAGAGG	56	299	35	U19251
Survivin	CTTGAAAGTGGCACCAGAGG TGCAGCTCAGATTCAACAGG	60	290	35	AF077350
Livin	CTGGTCAGAGCCAGTGTTCC TCATAGAAGGAGGCCAGACG	60	311	35	AF311388
β -Actin	CACCCCGTGCTGCTGACCGAGGCC CCACACGGAGTACTTGCCTCAGG	60	719	25	X00351

accordance with protocols approved by the institutional Human Subjects Committee. Tissues were dissected manually and samples were taken randomly from villous placenta and reflected amniochorion. Samples were either frozen in liquid nitrogen and stored at -80°C or fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, for 24 hours at 4°C and embedded in paraffin.

Cytotrophoblast Cell Purification

Cytotrophoblast cells were isolated from term placenta ($n = 3$) as described previously.^{17,18} Briefly, ~ 30 g of villous tissue was dissected from placenta, washed with sterile 0.9% (w/v) saline to remove excess blood, thoroughly minced, and repeatedly washed with sterile saline. Tissue was then digested three times at 37°C in a shaking water bath in Hanks' balanced salt solution (Sigma) containing 0.25% trypsin (Sigma), 300 U/ml DNase I (Sigma), and 25 mmol/L HEPES (pH 7.4; Sigma). Cells were centrifuged from digestion supernatants, washed with culture medium (Dulbecco's modified Eagle's medium, Waymouth 1:1, Mediatech, Herndon, VA) and filtered through 100- μm nylon mesh (Becton Dickinson, Franklin Lakes, NJ). Cells were then separated by Percoll (Sigma) gradient centrifugation. Cytotrophoblast cells were further purified by removal of HLA-A-, -B-, and -C-positive cells using the mouse monoclonal antibody to HLA class I antigens, W6/32,¹⁹ and goat anti-mouse immunoglobulin-conjugated magnetic microbeads (Miltenyi Biotec Inc., Auburn, CA) according to the manufacturer's protocol. The purity was assessed by centrifuging the cells onto glass slides using a Shandon Cytospin (Pittsburgh, PA), and analyzing the cells by immunohistochemical staining reactions using mouse anti-pan cytokeratin (Lu-5; Bio Genex, San Ramon, CA) and anti-CD14 (Zymed, San Francisco, CA), which identify trophoblast cells and macrophages, respectively, as previously described.²⁰ We further qualified the purity by immunostaining using mouse anti- βhCG (CG05; Neomarker, Fremont, CA) to detect any contaminating syncytial fragments. Average

purity was 99% cytotrophoblast cells after the magnetic bead purification procedure.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Expression of HIAP-1, HIAP-2, XIAP, NAIP, Survivin, and Livin mRNAs in placentas, cytotrophoblast cells, and choriocarcinoma cell lines, JEG-3 and Jar, was examined by RT-PCR. Total cellular RNA was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD). Two μg of total RNA were treated with DNase I (Sigma) and reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Grand Island, NY) to obtain cDNAs. The cDNA templates were then diluted 1:5 with sterile water and amplified by PCR using *Taq*DNA polymerase (Life Technologies, Inc.). The specific primers and the number of PCR cycles used in this procedure are listed in Table 1. PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. The identity of each amplified PCR product was verified by sequence analysis after cloning into the pCRII vector (Invitrogen, San Diego, CA). The optical density of each IAP and β -actin band was quantified by scanning densitometry using Epson1680 (Epson, Long Beach, CA) and GelPro Analyzer (Media Cybernetics, Silver Spring, MD). Values are presented as the ratio of each IAP-integrated optical density to β -actin-integrated optical density.

Protein Isolation and Immunoblot Analysis

HIAP-1, HIAP-2, XIAP, NAIP, Survivin, and Livin proteins in placentas, cytotrophoblast cells, and choriocarcinoma cell lines, JEG-3 and Jar, were analyzed by immunoblotting. To obtain cellular protein, cytotrophoblast cells, JEG-3 cells, and Jar cells were lysed by incubating in lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.2 mmol/L Na_3VO_4 , 0.2 mmol/L phenylmethylsul-

Table 2. Primary Antibodies Used in Immunoblotting and Immunohistochemistry

Antibody	Catalog number	Type	Concentration (or dilution)	Source*
For immunoblotting				
HIAP-1	sc7944	Rabbit polyclonal	1 μ g/ml	Santa Cruz Biotechnology
HIAP-2	AF818	Rabbit polyclonal	1 μ g/ml	R&D Systems
XIAP	610763	Mouse monoclonal	1 μ g/ml	BD Biosciences
NAIP	AF829	Rabbit polyclonal	1 μ g/ml	R&D Systems
Survivin	AF886	Rabbit polyclonal	1 μ g/ml	R&D Systems
Livin	LIVN11A	Rabbit polyclonal	2 μ g/ml	Alpha Diagnostic International
Actin	A2066	Rabbit polyclonal	1:5000	Sigma
Rabbit IgG	I-1000	Normal rabbit IgG	1 or 2 μ g/ml	Vector Laboratories
Mouse IgG ₁	554121	Normal mouse IgG ₁	1 μ g/ml	Pharmingen
Rabbit serum	R9133	Normal rabbit serum	1:5000	Sigma
For immunohistochemistry				
HIAP-1	AF817	Rabbit polyclonal	1 μ g/ml	R&D Systems
HIAP-2	PC596	Rabbit polyclonal	1:2000	Oncogene Research Products
XIAP	610763	Mouse monoclonal	8 μ g/ml	BD Biosciences
NAIP	PC245	Rabbit polyclonal	1 μ g/ml	Oncogene Research Products
Survivin	AF886	Rabbit polyclonal	1 μ g/ml	R&D Systems
Livin	LIVN11A	Rabbit polyclonal	1 μ g/ml	Alpha Diagnostic International
Rabbit IgG	I-1000	Normal rabbit IgG	1 μ g/ml	Vector Laboratories
Mouse IgG ₁	554121	Normal mouse IgG ₁	8 μ g/ml	Pharmingen
Rabbit serum	R9133	Normal rabbit serum	1:2000	Sigma

*Alpha Diagnostic International, San Antonio, TX; BD Biosciences, San Diego, CA; Oncogene Research Products, San Diego, CA; Pharmingen, San Diego, CA; R&D Systems, Minneapolis, MN; Santa Cruz Biotechnology, Santa Cruz, CA; Sigma, St. Louis, MO; Vector Laboratories, Burlingame, CA.

fonyl fluoride, 50 mmol/L NaF, 30 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin) for 30 minutes at 4°C. Cell lysates were passed through a 26-gauge needle and then clarified by centrifugation ($16,000 \times g$ for 15 minutes at 4°C). Samples of villous placenta were thawed and immediately homogenized in lysis buffer at a ratio of 1 g tissue per 5 ml buffer, and cellular debris was removed by centrifugation ($16,000 \times g$ for 15 minutes at 4°C). The concentrations of protein in cell lysates and placental tissue extracts were determined using a Bradford protein assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin used as the standard. Proteins in cell lysates (30 μ g/lane) or placental tissue extracts (40 μ g/lane) were denatured in sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer, separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and transferred to nitrocellulose membranes. Separate blots were prepared for each antibody. To assess consistent loading, each blot was re-blotted with antibody against actin. Blots were blocked for 1 hour at room temperature with 5% (w/v) nonfat milk-TBST (Tris-buffered saline with 0.1% Tween-20). Primary antibodies, described in Table 2, were diluted according to the manufacturer's recommendations in 2% milk-TBST. Blots were incubated with each primary antibody overnight at 4°C, and rinsed for 30 minutes at room temperature with TBST. Blots were then incubated with the peroxidase-conjugated goat anti-rabbit (for HIAP-1, HIAP-2, NAIP, Survivin, Livin, and actin; Jackson ImmunoResearch Laboratories, West Grove, PA) or goat anti-mouse (for XIAP, Jackson ImmunoResearch Laboratories) secondary antibody for 1 hour at room temperature, and rinsed again for 30 minutes at room temperature with TBST. Immunoreactive proteins were detected by chemiluminescence (SuperSignal West Pico; Pierce Chemical Co., Rockford, IL) according to the manufacturer's rec-

ommendations using Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ). The optical density of each IAP and actin band in the immunoblots was quantified by scanning densitometry using Epson1680 (Epson) and GelPro Analyzer (Media Cybernetics). Values are presented as the ratio of each IAP-integrated optical density to actin-integrated optical density.

Immunohistochemical Analysis

HIAP-1, HIAP-2, XIAP, NAIP, Survivin, and Livin proteins in placenta were localized by immunohistochemistry. Sections (5 μ m) were deparaffinized and rehydrated in an ethanol gradient. Tissue sections were blocked with 10% normal goat or normal horse serum for 30 minutes at room temperature, each primary antibody was added, and the tissue sections were incubated overnight at 4°C in a humidified chamber. The primary antibodies that were used in this procedure are listed in Table 2. For each tissue tested, purified normal rabbit IgG (for HIAP-1, NAIP, Survivin, and Livin), normal rabbit serum (for HIAP-2), or normal mouse IgG₁ (for XIAP) was substituted for each antibody and served as a negative control. For peptide inhibition experiments, NAIP and Livin antibodies were preincubated with synthetic peptides specific for the protein sequences of NAIP (Ab-1 control peptide, 25 μ g/ml; Oncogene Research Products, San Diego, CA), Livin (LIVN11-P, 20 μ g/ml; Alpha Diagnostic International, San Antonio, TX), respectively, for 1 hour at room temperature. Tissue sections were washed with PBST (PBS with 0.3% Tween-20) three times and the peroxidase block was performed with 0.5% H_2O_2 in methanol for 30 minutes. The biotinylated goat anti-rabbit or horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA) was added and incubated for 1 hour at

Figure 1. RT-PCR analysis of IAP mRNAs in human placentas. **A:** Placental tissue samples from first trimester (TM) ($n = 6$) and term placentas ($n = 6$) were tested. β -actin was used as a loading control. Molecular weight markers (M) are noted to indicate bp of PCR products. Sequencing experiments demonstrated authenticity of PCR products. **B:** The ratio of each IAP mRNA density to β -actin mRNA density obtained by scanning densitometry. *, $P < 0.05$ when compared with first trimester.

room temperature. After washes with PBST, the streptavidin peroxidase conjugate (Zymed) was added and the tissue sections were incubated for 10 minutes at room temperature. The sections were washed with PBST and the 3-amino-9-ethylcarbazole in *N,N*-dimethylformamide color development substrate (Zymed) was added to the tissue sections, which were then incubated for 10 minutes at room temperature. The tissue sections were washed in water, counterstained with Mayer's hematoxylin, and coverslipped.

Statistical Analysis

Densitometry data from RT-PCR and immunoblotting were subjected to Student's *t*-test to determine the difference of IAP message and protein levels between first trimester and term placentas. Results were considered to be significant when P was < 0.05 . Data are presented as means with SE.

Results

Identification of mRNAs Encoding IAPs in Human Placentas Using RT-PCR

In the first series of experiments, the goal was to determine whether or not mRNAs encoding members of the IAP family were detectable in human placentas and to establish expression in early and late gestation.

As shown in Figure 1, analysis by RT-PCR demon-

strated that placentas from first trimester and term contained specific messages encoding HIAP-1, HIAP-2, XIAP, NAIP, Survivin, and Livin. In the densitometry analysis for this semiquantitative RT-PCR, signal intensity for Survivin was significantly ($P < 0.05$) weaker in term than in first trimester placentas, but other IAP members were not different. Controls in which reverse transcriptase was omitted were negative for all sets of primers (data not shown). Each amplified PCR product was sequenced and identified as authentic.

Identification of IAP Proteins in Human Placentas by Immunoblotting

Having determined that specific messages for all of the apoptosis inhibitors under study were present in human placentas at both early and late stages of pregnancy, the next set of experiments was done to establish translation. Antibodies that identify proteins in immunoblots were available commercially for all of the proteins (Table 2) and were tested separately on blots of placental proteins. To assess consistency of loading, each blot was reblotted with an antibody against actin. Isotype-matched non-specific IgG and preimmune serum control blots were analyzed for each IAP, and there was no detectable signal (data not shown).

Figure 2 shows that with the exception of Livin, all of the apoptosis inhibitory proteins were detected in all samples of placental protein. In the densitometry analyses, signal intensities for IAP proteins were not signifi-

Figure 2. Immunoblot analysis of IAP proteins in human placentas. **A:** Placental tissue samples from first trimester (TM) ($n = 6$) and term placentas ($n = 6$) were tested. Molecular weight (kd) of each protein is indicated on the left. Actin was used as a loading control. **B:** The ratio of each IAP protein density to actin density obtained by scanning densitometry.

cantly different ($P > 0.05$) between first trimester and term placentas. Contrary to expectations, Livin protein was not detectable despite the presence of specific mRNAs (Figure 1).

Localization of IAP Proteins in Villous Placentas by Immunohistochemistry

To determine which among the multiple types of cells in human placentas contained the IAP proteins identified by immunoblotting, immunohistochemical studies were done on early and late gestation placentas, using six first trimester and six term placentas. Immunoreactivity was undetectable in control tissue sections where the primary antibodies were substituted with respective nonspecific rabbit IgG, mouse IgG, or preimmune rabbit serum. Further controls consisting of preincubation of the anti-NAIP and anti-Livin antibodies with specific peptide were also negative (data not shown).

Figure 3 shows that as predicted by the RT-PCR and immunoblotting experiments, all IAPs were detectable in both early and late gestation placentas with cell type-specific expression.

HIAP-1

Immunoreactive HIAP-1 was present in the cytoplasm of villous cytotrophoblasts, syncytiotrophoblast, and vil-

lous mesenchymal cells in both first trimester and term placentas. A tendency for more intense immunostaining in cytotrophoblast than in syncytiotrophoblast was noted in first trimester placentas.

HIAP-2

Although HIAP-2 was clearly identified in most samples of early and late gestation placentas by immunoblotting, the immunohistochemical experiments failed to detect strong signals. Immunoreactive HIAP-2 was detectable in the cytoplasm of villous cytotrophoblast cells and syncytiotrophoblast but not in the villous mesenchymal cells of either first trimester or term placentas.

XIAP

As with HIAP-2, immunoreactive XIAP was detectable in the cytoplasm of villous cytotrophoblast cells and syncytiotrophoblast, but not in the villous mesenchymal cells of either first trimester or term placentas. Staining was patchy and inconsistent in villi. In first trimester placentas, signals were more intense in villous cytotrophoblast cells than syncytiotrophoblast whereas in term placentas, similar signal intensities were observed in these two subpopulations of trophoblast cells.

Figure 3. Photomicrographs illustrating representative localization of IAP proteins by immunohistochemistry in human placentas. Specific immunostaining signals were not detected when the primary antibodies were substituted with their respective normal rabbit IgG, mouse IgG, or preimmune rabbit serum (control). TM, trimester; vCTB, villous cytotrophoblast cells; STB, syncytiotrophoblast layer; FM, fetal mesenchyme; IVS, intervillous space. Original magnifications, $\times 400$.

NAIP

This protein was strongly expressed in both first trimester and term placentas. The cytoplasm of villous cytotrophoblast cells, syncytiotrophoblast, villous mesenchymal cells, and villous endothelial cells all contained immunoreactive NAIP.

Survivin

Immunostaining for Survivin showed a cell type-specific pattern. In first trimester placentas, signals were intense in the villous cytotrophoblast cell layer. In term placentas signals continued in both cytotrophoblast cells and syncytiotrophoblast, but staining was patchy. Mesenchymal cells were positive for Survivin protein and signal was also detectable in villous endothelial cells.

Livin

The pattern of protein expression for this apoptosis inhibitor was distinctly different from the patterns of the other proteins. As expected from our failure to detect Livin by the less sensitive immunoblotting technique, Livin was not abundant in placentas. Unexpectedly, this protein was localized exclusively to cell nuclei. Immunoreactive protein was detectable in the nuclei of villous cytotrophoblast cells and mesenchymal cells and, to a lesser degree, in syncytiotrophoblast.

Localization of IAP Proteins to Extravillous Cytotrophoblast Cells in First Trimester Placentas and in the Amniochorion Membrane by Immunohistochemistry

Figure 3 demonstrated that to a greater or lesser degree, essentially all of the apoptosis inhibitory proteins tested in this study could be found in villous cytotrophoblast cells. Further efforts were made to learn whether the proteins showed any new patterns as the cells formed the columns that generate the invasive phenotype of early gestation or formed the chorion membrane, the quiescent cell layer of extravillous cytotrophoblast cells in term membranes.

Figure 4 demonstrates that extravillous cytotrophoblast cells in cell columns observed in four different first trimester placentas contained all of the proteins (HIAP-1, XIAP, NAIP, Survivin, Livin), except HIAP-2, that were detected in first trimester villous placenta. Intracellular localization remained the same, with the exception of Survivin, which, as with Livin, was localized mainly to the nuclei rather than the cytoplasm in cytotrophoblast cells comprising the cell columns. Interestingly, there was a decrease of XIAP staining intensity from proliferative to invasive extravillous cytotrophoblast cells.

Figure 4 also illustrates representative immunostaining patterns obtained using six different term amniochorion. As with extravillous cytotrophoblast cells in early gestation placentas, chorionic cytotrophoblast cells contained

Figure 4. Photomicrographs illustrating representative localization of IAP proteins by immunohistochemistry in extravillous cytotrophoblast cells present in cell columns of first trimester placentas and amniochorion membranes of term placentas. Specific immunostaining signals were not detected when the primary antibodies were substituted with their respective normal rabbit IgG, mouse IgG, or preimmune rabbit serum (control). vCTB, villous cytotrophoblast cells; exvCTB, extravillous cytotrophoblast cells; IVS, intervillous space; A, amniotic epithelia; FM, fetal mesenchyme; C, chorionic membrane; D, decidua capsularis. Original magnifications, $\times 400$ for cell columns; $\times 200$ for amniochorion.

all of the proteins although HIAP-2 was weakly expressed. By contrast, the amnion epithelium contained immunoreactivity for only four of the six apoptosis inhibitors; no signal was detectable for HIAP-2 or XIAP. In the decidua, five of the six proteins were detectable (HIAP-2, XIAP, NAIP, Survivin, and Livin). HIAP-1 was undetectable. As with villous placentas, respective normal IgG or preimmune serum did not bind to these samples, and pretreatment of the specific antibodies with specific peptides for NAIP and Livin inhibited binding. The immunohistochemistry results for IAP proteins are summarized in Table 3.

Expression of IAPs in Villous Cytotrophoblast Cells Isolated from Term Placentas

The experiments reported above indicated that villous and extravillous cytotrophoblast cells contain all six of the apoptosis inhibitor proteins. These precursor cells are scarce and difficult to identify in term placentas, and, as a consequence, their expression of the apoptosis inhibitor proteins was uncertain. To obtain more information on this subpopulation we conducted RT-PCR and immunoblotting studies on highly purified preparations of cytotrophoblast cells isolated from term placentas. Figure 5A shows that all six mRNAs encoding the apoptosis inhibitors under study (HIAP-1, HIAP-2, XIAP, NAIP, Survivin, and Livin) were detectable in term cytotrophoblast cells. Immunoblotting experiments (Figure 5B) readily identified HIAP-1, HIAP-2, XIAP, and NAIP proteins, but Survivin and Livin were in low abundance.

Expression of IAPs in Choriocarcinoma Cell Lines

To determine whether trophoblast-derived tumor cells might use the same or different molecules as their normal counterparts to avoid cell death, RT-PCR and immunoblotting experiments were conducted on two trophoblastic tumor cell lines, JEG-3 and Jar. All six specific messages were also present in both JEG-3 and Jar cells (Figure 5A). As shown in Figure 5B, JEG-3 and Jar cells contained all of the proteins. Survivin and Livin protein signals were markedly more intense in the cell lines than in the normal cytotrophoblast cells.

Discussion

The results of this study show for the first time that 1) IAP messages and proteins are present in human placentas at both early and late stages of gestation; 2) the proteins are localized to specific types of cells; 3) specific messages and proteins derived from the IAP genes are detectable in both cytotrophoblast cells isolated from term placentas and trophoblast-derived choriocarcinoma cells; and 4) patterns of expression are not identical in normal and transformed trophoblast cells.

The observations made here, which record the presence of IAP proteins in early and late gestation placentas,

Table 3. Summary of Immunostaining of IAP Proteins in Human Placentas*

IAPs	Syncytio- trophoblast	Villous cytotropho- blast cells	Extravillous cytotropho- blast cells†	Villous mesenchyme	Villous endothelium	Amnion epithelium	Chorion	Decidua capsularis
HIAP-1								
1st	+	+	+	+	+	ND	ND	ND
TM								
Term	+	+	+	+	+	+	+	0
HIAP-2								
1st	+	+	0	0	0	ND	ND	ND
TM								
Term	+	+	+	0	0	0	+	+
XIAP								
1st	+	+	+	0	0	ND	ND	ND
TM								
Term	+	+	+	0	0	0	+	+
NAIP								
1st	+	+	+	+	+	ND	ND	ND
TM								
Term	+	+	+	+	+	+	+	+
Survivin								
1st	+	+	+	+	+	ND	ND	ND
TM								
Term	+	+	+	+	+	+	+	+
Livin								
1st	+	+	+	+	+	ND	ND	ND
TM								
Term	+	+	+	+	+	+	+	+

*Immunostaining for IAPs was evaluated as (0) not detectable or (+) present. TM, trimester; ND, not determined.

†Extravillous cytotrophoblast cells were observed in cell columns in first trimester placentas and in the chorion membrane in term tissues.

are of great potential importance to understanding how cell death is tightly regulated during placental organogenesis. Placentas are rich sources of apoptosis-inducing ligands such as tumor necrosis factor (TNF)- α , FasL (Fas ligand), TRAIL (TNF-related apoptosis inducing ligand), and LIGHT (homologous to lymphotoxin, exhibits inducible expression, competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes) as well as their receptors.^{20–26} IAPs, which can inhibit caspases,^{2–4} may play a critical role in preventing death ligand/receptor-induced apoptosis in this organ. Many placental cell types, ie, villous and extravillous cytotrophoblast cells, syncytiotrophoblast, and mesenchymal cells, express TNF superfamily death receptors,^{14,20,27,28} and we here report that these cell types also express IAPs. This coincidence of expression suggests that IAPs may serve a protective function.

Although clearly a matter of speculation, it is entirely possible that these inhibitory proteins may act by redirecting potential death pathways into cell survival pathways. In some types of cells the death-dealing TNF superfamily ligands activate nuclear factor- κ B,²⁹ and cells that respond in this manner do not undergo apoptosis. Recent reports document that TNF- α induces HIAP-1, HIAP-2, and XIAP expression through this nuclear factor- κ B pathway, thus forming a positive feedback loop against apoptosis.^{30–34} However, in placentas, protection may be overcome by high concentrations of death-dealing ligands; villous cytotrophoblast cells can be killed by 25 ng/ml of recombinant TNF- α , and are even more susceptible when interferon- γ is also present.³⁵ Finally, it is worth noting that other members of the TNF family can activate nuclear factor- κ B, and all of these are

synthesized in human placentas.²⁰ It therefore seems reasonable to propose that IAPs may also protect against the apoptotic activity of some of these TNF family members.

Cell type-specific expression of IAPs was observed. Essentially all subpopulations of trophoblast cells contained the IAP proteins, but expression appeared to vary among the subpopulations. For example, Survivin was weak in syncytiotrophoblasts, but strong in villous and extravillous cytotrophoblasts. Other differences that could impact function were also observed. One instance is Livin, which was localized exclusively to cell nuclei whereas all of the other inhibitors were cytoplasmic. Interestingly, Survivin was cytoplasmic in villous cells but moved to the nucleus in early gestation extravillous cytotrophoblast cells.

Survivin and Livin were particularly interesting in their cell expression and localization. Villous cytotrophoblast cells in first trimester are highly proliferative and express high levels of cell-cycle regulators, such as cyclins and cyclin-dependent kinases.³⁶ In other types of cells, evidence has been accumulated indicating that Survivin not only plays a role in inhibiting apoptosis, but also associates with microtubules of mitotic spindles and mediates cell-cycle progression of G₂/M phase transition as well as S phase shift.^{37,38} This suggests that in villous cytotrophoblast cells, Survivin may preserve the integrity of the mitotic apparatus and act as a significant regulator of cytotrophoblast cell proliferation. Livin may also be associated with cell-cycle regulation in a manner similar to that of Survivin, inhibiting apoptosis by inhibiting the ability of apoptotic signals to act on nuclear proteins, as previously suggested.⁶

Figure 5. RT-PCR (A) and immunoblot (B) analyses of IAP expression in cytotrophoblast cells (CTB) and choriocarcinoma cell lines, JEG-3 and Jar. Cytotrophoblast cells for immunoblot analysis were from three different term placentas. Molecular weight markers (M) are noted to indicate bp of PCR products, and the molecular weight (kd) of each protein is indicated. Note that messages for IAPs are present in cytotrophoblast cells and choriocarcinoma cell lines, but proteins are detectable in an inhibitor type-specific manner. RTase +/–, with (+) or without (–) reverse transcriptase.

Interestingly, Survivin and Livin were significantly more abundant in choriocarcinoma cells than in normal cells. This might reflect similar strategies for avoiding apoptosis in the poorly differentiated, highly proliferative, and invasive normal subpopulation of trophoblast and their transformed counterparts. Lehner and colleagues³⁹ have recently shown that Survivin expression is increased in hydatidiform mole, indicating that this condition may be preneoplastic.

In cytotrophoblast cells, the initial stages of apoptosis are clearly observed, ie, the phosphatidylserine flip occurs, but further progress of apoptosis is inhibited.¹⁴ It has been suggested that anti-apoptotic members of the Bcl-2 family, including Bcl-2 and Mcl-1, which are present in the syncytiotrophoblast layer, block the progress of apoptosis during syncytialization.^{14,15} Our results suggest that IAPs, which are present in the syn-

cytotrophoblast layer, may also serve central roles in the inhibition of apoptosis that accompanies syncytialization.

The amniochorion membrane, which consists of the amnion epithelial layer, the chorionic cytotrophoblast layer, and the maternal decidua, expresses death receptors and their ligands. Apoptosis occurs in the amniochorion membrane during gestation with an increased level in chorion and decidua parietalis at term.⁴⁰ Our identification of IAPs in these membranes suggests that these inhibitors might protect against the high levels of apoptosis-inducing ligands that characterize the final stages of pregnancy.⁴¹

In conclusion, our results show that IAPs are present in human placenta and that their expression is regulated in a cell type-specific manner, suggesting that IAPs may play critical roles in placental function. These findings add substantively to our understanding of the mechanisms by which apoptosis is tightly regulated in various types of cells during placental development.

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