VE-Cadherin Expression and Clustering Maintain Low Levels of Survivin in Endothelial Cells

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Survivin is strongly expressed in embryonic organs and in tumor cells but is low or absent in differentiated normal tissues. Resting endothelium expresses low levels of survivin but can up-regulate its synthesis on activation to proliferate. The mechanisms responsible for survivin down-regulation in resting conditions are still unknown. We report here that confluence and vascular endothelial-cadherin (VE-cadherin) expression induce contact inhibition of cell growth and survivin down-regulation in the endothelium. Using β -catenin null and positive isogenic endothelial cell lines we found that the effect requires β -catenin expression and its association to VE-cadherin cytoplasmic tail. Furthermore, in allantois organ cultures, survivin expression is up-regulated in areas of growing vessels where VE-cadherin is partially dismantled from junctions or in VE-cadherin $-/-$ specimens. Overall, these data indicate that VE-cadherin and β -catenin may negatively regulate survivin synthesis in endothelial cells. Consistently, in epidermal and pancreatic cell lines or ovarian tumors, epithelial-cadherin (E-cadherin) and survivin expression is inversely related, suggesting a non-cell-specific role of cadherins in reducing survivin synthesis. (*Am J Pathol* 2004, 165:181–189)

Cadherins are transmembrane adhesive proteins at intercellular adherens junctions (AJ).^{1–4} Classic cadherins promote homophilic adhesion and are linked, through their cytoplasmic tail, to intracellular partners including β -catenin, plakoglobin, and p120. β -catenin and plakoglobin, through their binding to α -catenin, mediate the anchorage of cadherins to the actin cytoskeleton.^{5,6} The endothelial-specific cadherin, vascular endothelial-cadherin (VE-cadherin) is required for a normal development of the vasculature in the mouse embryo or for new vessel formation in the adult.^{7–9}

A prominent feature of β -catenin and, in some conditions, of plakoglobin and p120 is their ability to translocate to the nucleus and, in association with other transcription factors, modulate gene expression.^{6,10–12} In general, over-expression of β -catenin in different cell systems leads to increased cell proliferation and reduced sensitivity to apoptosis, so that β -catenin stabilization or permanent signaling may facilitate tumor progression. There is a long list of β -catenin target genes, which includes genes important not only for cell division and apoptosis but also for cell differentiation.^{6,11,12} When β -catenin is bound to cadherins, it is stabilized and retained at the cell membrane. It has been hypothesized that the absence or mutation of cadherins may increase the pool of free β -catenin in the cytosol and its signaling activity.^{13,14} An extensive literature is available showing inverse correlation between cadherin expression and tumor progression.¹⁵

In addition, different groups including ours found that expression and clustering of cadherins in complex with β -catenin may trigger responses at the cell membrane such as PI3-kinase and Rac activation.^{16–19} Comparing long-term confluent cultures of VE-cadherin $+/+$ and $-/-$ cells, we found that the presence of this protein contributes to contact inhibition of cell growth.^{18,19} This

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effect requires constitutive expression and clustering of VE-cadherin at junctions and likely involves continuous intracellular signaling.

Survivin is a member of the family of inhibitors of apoptosis called IAP (Inhibitor of Apoptosis Protein).²⁰ Survivin is a structurally unique IAP protein that is expressed at mitosis in a cell cycle-dependent manner and bound to components of the mitotic apparatus and notably to spindle fibers.^{21–23} Survivin has been implicated in both preservation of cell viability and control of cell division and is present at low levels of expression in normal tissues but strongly up-regulated in human cancers where it is positively correlated with unfavorable prognosis.^{24,25}

Recent evidence indicates that in human colorectal cancer β -catenin can transcriptionally up-regulate survivin.²⁶ Consistently, *wtAPC* (adenomatous polyposis coli) down-regulates survivin expression likely by inducing β -catenin ubiquitination and destruction in proteosomes.²⁷ This suggests that when APC is mutated as in colon cancer, β -catenin would be stabilized and increase survivin levels.

In this paper, we report that in endothelial cells survivin is negatively regulated by confluence. This effect requires VE-cadherin and β -catenin complex formation suggesting that cadherins play an important role in maintaining survivin at low levels in normal tissues. Mutations or lack of expression of cadherins may increase survivin synthesis and promote its function. This effect may be important both in vascular homeostasis and tumor progression.

Materials and Methods

Cell Preparation and Characterization

Endothelial cells were derived from murine embryonic stem cells and embryos with homozygous null mutation of the *VE-cadherin* gene (*VE-cadherin* $-/-$) as described in detail by Carmeliet et al and Balconi et al.^{7,28} Wild-type and mutant forms of VE-cadherin were introduced in these cells using the retroviral vector pINCO. Cell types that expressed the following proteins were generated: wild-type VE-cadherin (*VE-cadherin* $+/+$), $\Delta p120$ (amino acid deletion 621–702, corresponding to the binding region of p120), and $\Delta \beta$ -catenin (amino acid deletion 703–784, corresponding to the binding region of β -catenin). The details of the production and characterization of these cells were described by Lampugnani et al.¹⁶

β -catenin $+/+$ and $-/-$ cells, wild-type, and homozygously null mutated in *β -catenin* gene,²⁹ respectively, were isolated from 9.5-day post-coitum (dpc) littermate embryos, as described in detail by Balconi et al.²⁸ Human umbilical vein endothelial cells (HUVECs) were prepared as described.³⁰ For all of the endothelial cells of murine origin, culture medium was Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Paisley, UK) with 10% fetal calf serum (FCS; HyClone, Logan, UT), heparin (100 μ g/ml from porcine intestinal mucosa; Sigma Chemical, St. Louis, MO), and endothelial cell growth supplement (ECGS) (50 μ g/ml, Sigma). Culture

medium for HUVECs was MCDB-131 (Life Technologies) with the same supplements.

In some experiments, confluent VE-cadherin $+/+$ cells were exposed to: inhibitors of PI3-kinase, wortmannin (WM, Cell Signaling Technology, Beverly, MA, USA) and LY294002 (Calbiochem, San Diego, CA, USA); an inhibitor of Akt phosphorylation, 1L-6-hydroxymethyl-*chiro*-inositol2- β -2-O-methyl-3-O-octadecylcarbonate (Calbiochem);³¹ an inhibitor of p42/44MAP-kinase pathway, PD98059 (Cell Signaling Technology). Cells were prepared as described below for the proliferation assay, and on day 10 were exposed to the inhibitors (WM 0.1 μ mol/L, LY294002 20 μ mol/L, 1L-6-hydroxymethyl-*chiro*-inositol2- β -2-O-methyl-3-O-octadecylcarbonate 10 μ mol/L, PD98059 50 μ mol/L) for 8 hours at 37°C, then lysed and the total cell extract processed by Western blot.

The non-endothelial cell lines used in this study were the following: human pancreatic cells including normal primary pancreatic cultures (NP)³² and a panel of tumor cell lines: SKPC-1, AsPC-1 and Hs766T,³³ IMIMPC2,³⁴ BxPC3,³⁵ and murine keratinocyte cell lines: MCA3D, HaCa4,³⁶ MSC11A5.³⁷ Clones E 24 and E62 derive from transfection of E-cadherin in HaCa4 parental cell line as described.³⁶ Cells were grown in DMEM (Life Technologies) (NP, SKPC-1, AsPC-1, Hs766T, MSC11A5), RPMI medium (Life Technologies) (BxPC3), Ham's F-12 medium (Life Technologies) (MCA3D, PDV, HaCa4, E24, E62) supplemented with 10% FCS.

Proliferation Assay

For growth curves, murine endothelial cells were seeded (30×10^3 cell/cm²) in 24-well plates in DMEM (Life Technologies) with 10% FCS. For synchronization, 24 hours later cells were serum-starved for 24 hours in serum free MCDB-131 medium (Life Technologies) supplemented with 1% bovine serum albumin (BSA; Sigma). Fresh DMEM with 10% FCS was then added and cells were counted at different days thereafter. Medium was changed only 3 days after the beginning of the experiment.

Immunofluorescence

The HUVECs (30×10^3 cell/cm²) were cultured for 24 hours in MCDB-131 with 10% FCS on fibronectin-coated (7 μ g/ml; Sigma) glass coverslips. Cell layers were washed once in MCDB-131 (Life Technologies) and cultured for 24 hours in MCDB-131 medium (Life Technologies) supplemented with 1% BSA (Sigma). Fresh DMEM with 10% FCS was then added and cells were fixed at day 2 (sparse), 4 (subconfluent), 7 (confluent) in 3% formaldehyde from paraformaldehyde (PAF) for 15 minutes and permeabilized with 0.5% Triton X-100 before staining. After incubation with anti-VE-cadherin BV9 antibody³⁸ for 1 hour at 37°C, cells were labeled with appropriate Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Fluorescence was detected with a fluorescence microscope (Leica DMR, Wetzlar, Germany) and images acquired by the Hamamatsu 3CCD camera (Hamamatsu Photonics,

Hamamatsu City, Japan) before processing with Adobe Photoshop software for MacIntosh.

Western Blot

Western blots were performed according to standard protocols, and the proteins were visualized by enhanced chemoilluminescence (Amersham Biosciences, Little Chalfont, UK). The signals on film (Hyperfilm ECL; Amersham Biosciences) were scanned and quantified using the NIH Image 1.62 program for the MacIntosh computer, developed at the National Institutes of Health and freely available at <http://rsb.info.nih.gov/nih-image/web> site. The primary antibodies used were: the rabbit anti-human survivin polyclonal Ab kindly provided by Dr. D. Altieri (Department of Cancer Biology and the Cancer Center, University of Massachusetts Medical School, Worcester, MA) the anti-mouse monoclonal Ab against C-terminal fragment of the epithelial-cadherin (E-cadherin; Transduction Laboratories, BD Biosciences, San Jose, CA), and the monoclonal anti- β -actin Ab (Sigma).

Northern Blot

Total RNAs from confluent VE-cadherin +/+ and -/- were obtained as described³⁹ and further processed for Northern blot hybridization with ³²P-dCTP random primer-labeled survivin cDNA or control β -actin probe, as described.²¹

Allantois Cultures

For experiments with allantoises, mice heterozygous for VE-cadherin allele⁷ were intercrossed to generate VE-cadherin +/+ and -/- embryos. The gestational age of embryos was determined by setting 0.5 dpc at noon on the morning that a vaginal plug was observed. Genotyping of embryos was done by PCR amplification of DNA isolated from yolk sac using previously described primers.⁷

Allantoises were isolated as previously described.^{40,41} Briefly, embryos at 8.5 dpc were dissected in phosphate-buffered saline (PBS, 4°C), the allantoises excised and seeded into NUNC 4-chambered culture slides (Nalgene Nunc International, Rochester, NY, USA) containing 0.4 ml DMEM with 10% FCS and 1% penicillin-streptomycin. Explants were cultured at 37°C in a 5% CO₂ incubator for 18 hours, fixed, and permeabilized as shown previously in detail,⁴⁰ exposed to anti-survivin and VE-cadherin (clone BV13)⁴² or PECAM-1 antibodies (MEC13.3),⁴³ and processed according to standard immunofluorescence procedure (see above). Allantoises were analyzed by a Leica TCS SP2 AOBs Filter-Free Spectral Confocal and Multiphoton microscope and dedicated Leica Confocal Software (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). Images were processed using ImageJ 1.30k freely available at <http://rsb.info.nih.gov/ij/java1.3.1/> web site and Adobe Photoshop 7.0 (Adobe Systems, Inc., San Jose, CA).

E-Cadherin and Survivin Expression in Ovarian Cancer Specimens

Expression levels of E-cadherin and survivin genes were measured in 25 ovarian cancer samples (stage III or IV) belonging to different histotypes (11 serous, 9 endometrioid, 2 undifferentiated, 2 mucinous, and 1 clear cell). The samples were part of a project of gene expression profiling of ovarian carcinomas and ovary cell lines (manuscript in preparation). RNAs were hybridized on cDNA microarrays containing 4451 unique clones selected from the human sequence verified I.M.A.G.E. clone collection (Research Genetics/Invitrogen, Carlsbad, CA), using a common reference sample consisting of a pool of 10 different human tumor cell lines. Total RNAs were reverse transcribed and labeled with Cy5-dCTP (ovarian cancers) or Cy3-dCTP (reference sample) (Amersham Biosciences, Amersham, UK). Slides were scanned using the GenePix 4000A Microarray Scanner and the resulting images were analyzed using the GenePix v.3.0 software (Axon Instruments, Union City, CA) to quantify the levels of Cy3 and Cy5 fluorescence. The GenePix Pro raw data files were processed using the GenePix post-processing program to filter and normalize data. Expression levels of E-cadherin and survivin genes (corresponding to clones IMAGE: 251019 and IMAGE: 796694, respectively) were measured as log₂ ratio of Cy5/Cy3.

Results

Survivin Is Decreased by Cell Confluency and VE-Cadherin Expression

Endothelial cells are contact inhibited in their growth as other types of cells forming epithelium-like linings. As reported in Figure 1A, we compared two isogenic endothelial cell lines differing for expression of VE-cadherin only. As previously reported,¹⁸ we found that confluent VE-cadherin positive cells stop replicating while VE-cadherin null cells maintain sustained growth and reach significantly higher cell density.

As reported in Figure 1B, cell density decreased survivin expression only in the presence of VE-cadherin, while the protein (Figure 1B) and mRNA (Figure 1C) remained high in VE-cadherin null endothelial cells. Similarly, in freshly isolated HUVECs, survivin markedly declined in relation to cell density and VE-cadherin clustering at cell-to-cell contacts (Figure 2, A and B).

β -Catenin Association to VE-Cadherin Is Required for Survivin Down-Regulation

We then tested whether the binding of VE-cadherin to β -catenin/plakoglobin or to p120 was required for survivin down-regulation. We infected VE-cadherin null endothelial cells with two different deletion mutants of VE-cadherin (Figure 3A): Δ p120 and Δ β -catenin lacking the cytoplasmic domains responsible for p120 or β -catenin/plakoglobin binding, respectively. These mutants have

Figure 1. Effect of VE-cadherin expression and cell confluence on endothelial cell proliferation and survivin expression. **A:** Growth curve of endothelial cells expressing (VE-cadherin $+/+$) or not (VE-cadherin $-/-$) VE-cadherin. Cells (seeding $30 \times 10^3/\text{cm}^2$) were cultured in complete culture medium. At the indicated time point, cells were detached and counted. In absence of VE-cadherin cells were not contact inhibited in their growth and reached a higher density than VE-cadherin positive cells. **B:** Western blot analysis of survivin expression in VE-cadherin $+/+$ and $-/-$ endothelial cells. Cells were seeded at the same density ($30 \times 10^3/\text{cm}^2$) as for the growth curve; the cell extracts collected at day 1, 3, 5, and 10. The columns represent the intensity of the bands measured as optical density (O.D.) arbitrary units. Survivin expression in VE-cadherin $+/+$ cells decreased as the cells became confluent, while remaining stable in the VE-cadherin $-/-$ cells. Western blot of β -actin confirmed that equal amounts of proteins were seeded (not shown). **C:** Northern blot analysis of survivin mRNA in VE-cadherin $+/+$ and $-/-$ cells. The position of molecular weight markers is indicated on the left. The mRNA was collected from cells at confluence (day 10). Expression of VE-cadherin in endothelial cells is associated to a 1.6-fold decrease after normalization to β -actin in survivin mRNA. Each type of experiment was repeated at least three times using four different VE-cadherin $+/+$ and $-/-$ cell lines.

been characterized previously in detail for construct expression, β -catenin, and p120 association and distribution at junctions.¹⁶

We found (Figure 3B) that transfecting the $\Delta\beta$ -catenin mutant did not significantly reduce survivin expression while the $\Delta p120$ mutant was comparable to VE-cadherin $+/+$ cells, strongly suggesting that VE-cadherin binding to β -catenin/plakoglobin is required for down-regulation of survivin.

To further investigate the role of β -catenin, we compared β -catenin $+/+$ and $-/-$ endothelial cells. These cell lines have been previously characterized in detail for

Figure 2. VE-cadherin clustering correlates with the survivin expression in HUVECs. **A:** Western blot analysis of survivin expression in HUVECs. Equal amounts of total extracts ($50 \mu\text{g}$) were collected from cells at different time points corresponding to sparse (day 2, $10 \times 10^3/\text{cm}^2$), intermediate (day 4, $30 \times 10^3/\text{cm}^2$), and confluent (day 7, $50 \times 10^3/\text{cm}^2$) conditions. Survivin in HUVEC is down-regulated at confluence. A typical experiment is reported in each panel. Each type of experiment was repeated at least three times using three separate cultures of HUVECs. **B:** VE-cadherin expression was studied by immunofluorescence in human endothelial cells in three different conditions corresponding to sparse (day 2), subconfluent (day 4), and confluent (day 7). The staining was initially (day 2) limited to cell-cell contacts, as the cells grow more confluent (day 4 and 7) and the intercellular contacts extend to the entire cell edge, VE-cadherin signal associated to the entire cell margin. Magnification, $\times 63$.

expression of specific markers and functional properties.^{18,29} As shown in Figure 4A, β -catenin expression was required for the decline of survivin levels at confluency.

Cadherin engagement has been reported to trigger PI3-kinase and MAP-kinase activation.^{7,16,17} As reported in Figure 4B we found that wortmannin, LY294002 (inhibitors of PI3-kinase activation) and Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol2- β -2-O-methyl-3-O-octadecylcarboxylate) but not PD98059, an inhibitor of MAP-kinases phosphorylation (not shown), could prevent down-regulation of survivin in VE-cadherin $+/+$ cells at confluency.

Survivin Up-Regulation in Growing Vessels

To test the relevance of the observations made *in vitro* in a more complex model of vasculogenesis and angiogenesis we used the isolated allantois model. As previously reported in detail⁴⁰ at 24 hours after isolation and culture of allantoises it is possible to evidence a well organized vascular network which can be stained by PECAM-1⁴³ and VE-cadherin antibodies (Figure 5A). At the tips of growing vessels (Figure 5A, arrowheads) VE-cadherin staining at junctions is somehow less intense⁴⁴ while survivin is up-regulated. In allantoises obtained from VE-cadherin $-/-$ mice⁷ the vascular organization is severely impaired. In the regions where endothelial cells can still form tubular structures (Figure 5B, arrows; see staining with anti-PECAM-1 antibodies) the levels of survivin appear high in all of the cells, indicating lack of its down-

Figure 3. Association of β -catenin to VE-cadherin is required for survivin down-regulation. **A:** VE-cadherin $-/-$ cells were transfected with VE-cadherin wild-type or truncated mutants lacking β -catenin ($\Delta\beta$ -catenin) or p120 ($\Delta p120$) binding domains. Intra, intracellular region; extra, extracellular region. **B:** Western blot analysis of survivin expression in VE-cadherin mutants. Equal amounts of cell extracts were collected 10 days after cell seeding ($30 \times 10^3/\text{cm}^2$). The columns represent the intensity of the bands measured as optical density (O.D.) arbitrary units. In absence of VE-cadherin or when β -catenin binding domain was truncated, survivin expression was not down-regulated by cell confluence. Levels of survivin remained higher by eight (VE-cadherin $-/-$) and sevenfold ($\Delta\beta$ -catenin) over the control (VE-cadherin $+/+$). A typical experiment out of three is reported.

regulation. These data support the idea of an inverse relationship between expression of VE-cadherin and survivin.

Expression of Cadherins Inversely Correlates with Survivin Synthesis in Tumors

The data presented above point to an important role for cadherins in regulation of survivin synthesis. In tumors, down-regulation of cadherins positively correlates with their invasive potential.¹⁵ We tested whether, similarly to endothelial cells, there may be an inverse relationship between cadherin and survivin expression in a series of tumor or transformed cell lines. We analyzed normal primary pancreatic (NP) cultures, five pancreatic tumor cell lines (SKPC-1, AsPC-1, Hs766T, IMIMPC2, BxPC3), one line of immortalized non-tumorigenic keratinocytes

Figure 4. β -catenin is required for VE-cadherin-induced down-regulation of survivin. **A:** Western blot analysis of survivin expression in β -catenin $+/+$, and $-/-$ cells. Equal amounts of extracts ($50 \mu\text{g}$) were collected at day 2 (sparse) and 7 (confluent) days after seeding the cells at the same density ($30 \times 10^3/\text{cm}^2$). The columns represent the intensity of the bands measured as optical density (O.D.) arbitrary units. Survivin was down-regulated at confluence only in β -catenin positive cells. **B:** Western blot analysis of survivin expression in VE-cadherin $+/+$ cells at day 1 (sparse), day 2 (subconfluent), and day 10 (confluent) with or without the inhibitors of PI3-kinase WM ($0.1 \mu\text{mol/L}$) and LY294002 ($20 \mu\text{mol/L}$), the inhibitor of the AKT phosphorylation 1L-6-hydroxymethyl-chiro-inositol2- α -2-O-methyl-3-O-octadecylcarbonate (AKT inhibitor, $10 \mu\text{mol/L}$). Equal amounts of cell extracts ($50 \mu\text{g}$) were tested. The inhibitors WM, LY294002 and 1L-6-hydroxymethyl-chiro-inositol2- α -2-O-methyl-3-O-octadecylcarbonate, significantly prevented the reduction of survivin expression at confluence resuming the level of survivin expressed by sparse cells. The figure reports a typical experiment out of three performed.

(MCA3D), and two lines of transformed keratinocytes derived from carcinomas (MSC11A5 and HaCa4) (Figure 6A).³²⁻³⁷ These cell lines have been selected for these studies since they have been characterized previously for their marked differences in E-cadherin expression.³⁶ We found that in pancreatic tumor cell lines there was an inverse correlation between E-cadherin and survivin expression. The tumor keratinocyte-derived cell lines, MSC11A5 and HaCa4, expressed little if any, E-cadherin but were positive for survivin. The two non-transformed lines, MCA3D and NP, had high levels of E-cadherin but no survivin. Transfection of E-cadherin cDNA in HaCa4 resulted in clones (E24 and E62) expressing different levels of the protein.³⁶ Survivin was down-regulated in a way inversely related to E-cadherin levels.

Figure 5. Survivin up-regulation in growing vessels. Allantoises isolated from VE-cadherin (VE-cad +/+) and VE-cadherin null (VE-cad -/-) embryos were cultured at 37°C in 5% CO₂ incubator for 18 hours and immunostained for survivin and the endothelial markers VE-cadherin or PECAM-1, as indicated. **A:** In VE-cad +/+ mice-derived allantoises, survivin is specifically up-regulated at the tips of angiogenic vessels (arrowheads). **Bar,** 40 μ m. **B:** In VE-cad -/- mice-derived allantoises survivin is uniformly expressed in all of the endothelial cells of the developing vessels (arrows). **Bar,** 20 μ m.

To further investigate the relationship between E-cadherin and survivin, a large set of ovarian cancer samples from different histological subtypes was studied. Gene profile analysis⁴⁵ (Figure 6B) showed that, in comparison to the reference sample, (see Materials and Methods) the levels of E-cadherin inversely correlated with survivin in 23 of 25 tumor specimens analyzed. The reference sample consists of a pool of 10 highly malignant human tumor cell lines of different origin which present low or absent levels of E-cadherin and high levels of survivin. For this reason, in the comparison presented in the figure, the ovarian cancer specimens present higher levels of E-cadherin and lower levels of survivin.

Discussion

In optimal *in vivo* conditions, endothelial cells are resting and contact inhibited in their growth. Junctional adhesion proteins may contribute to this quiescent condition by transferring intracellular signals and modifying gene expression.^{44,46,47}

VE-cadherin is required for endothelial cell homeostasis. Administration of VE-cadherin blocking antibodies *in vivo*, causes dramatic changes in vascular organization and increase in permeability.⁴²

Survivin is very low in terminally differentiated normal tissues including resting endothelial cells but is strongly increased in angiogenic vessels or in endothelial cells exposed to growth and anti-apoptotic agents such as vascular endothelial growth factor (VEGF) or angiopoietin-1.^{48–50}

The mechanisms which maintain the levels of survivin low in resting cells are unknown. In this paper we show that in endothelial cells VE-cadherin expression and its clustering at intercellular junctions significantly reduces survivin synthesis and keeps its levels low in confluent cells.

VE-cadherin is important in contact inhibition of cell growth.^{18,19,51,52} Other groups using isogenic tumor cell lines differing for expression of other types of cadherins found that inhibition of growth was mediated, at least in part, by the induction of cell cycle arrest at G1 phase due to de-phosphorylation of retinoblastoma protein, elevation of cyclin D1-dependent kinase inhibitor p27kip1 and late reduction of cyclin D1.^{13,14} These effects are likely mediated by the capacity of cadherins to link β -catenin limiting its nuclear translocation. β -catenin up-regulates cyclin D1 and *c-myc* transcription and therefore inhibition of its activity would indirectly limit growth.^{6,10,53,54}

In addition, it was found that when VE-cadherin is clustered at junctions, it forms a complex with VEGF

Figure 6. Inverse correlation between E-cadherin and survivin expression in tumor cell lines and ovarian tumors. **A:** Western blot analysis of E-cadherin and survivin in normal and tumor cell lines. The columns represent the intensity (optical density) of the bands expressed as percentage of the highest value of survivin (■) and E-cadherin (□) in the indicated cell lines. Survivin and E-cadherin expression showed an opposite trend: survivin was higher in cell lines expressing lower amounts of E-cadherin. Transfection of E-cadherin cDNA in the cell line HaCa4 resulted in two clones E62 and E24 expressing different levels of E-cadherin that inversely correlated with survivin expression. **B:** Survivin (△) and E-cadherin (○) gene expression profile in ovarian carcinomas. Twenty-five ovarian cancer specimens at stage III or IV were analyzed by cDNA microarrays. A pool of 10 tumor cell lines was used as reference sample. Gene expression values are given as $\log_2$ ratio of signal intensity of each specimen with respect to the reference. In 23 of 25 tumors, the trend of the E-cadherin expression is opposite to survivin.

receptor-2 (VEGFR-2) and the phosphatase DEP-1/CD148.¹⁸ The presence of the phosphatase causes dephosphorylation of the receptor and reduction of cell proliferation.¹⁸ When VE-cadherin is absent, cells respond in an uncontrolled way to the growth factor and continue to proliferate also at high density. β -catenin expression and association to VE-cadherin is required for the formation of the complex with VEGFR-2 and for its dephosphorylation.¹⁸

Reduction of survivin synthesis may therefore be related to inhibition of cell growth induced by VE-cadherin. Survivin has cell cycle regulated expression and is associated to the mitotic apparatus.^{20,23,55} Homologous recombination of the survivin gene induces marked modifications in microtubule assembly, absence of mitotic spindle, and generation of multinucleated cells.⁵⁶ In the absence of VE-cadherin, survivin is up-regulated and this is likely to be related to deregulation of cell mitotic index and normal growth control. However, in preliminary experiments, overexpression of survivin in confluent, contact inhibited, endothelial cells did not restore cell proliferation suggesting that up-regulation of survivin alone cannot abrogate contact inhibition of cell growth. It is therefore likely that other factors are simultaneously required to release the cells from growth inhibition.

VE-cadherin may reduce survivin synthesis in different ways. It may retain β -catenin at the membrane and reduce its transcriptional activity. This is supported by data with VE-cadherin mutants able to bind different intracellular partners. We found that $\Delta\beta$ -catenin VE-cadherin did not reduce survivin levels suggesting that VE-cadherin association to β -catenin is required for this effect; consistently, $\Delta p120$ behaved like wild-type VE-cadherin.

However, not all of the observations available fit in this picture. In contrast to human cells,²⁶ mouse survivin promoter does not contain β -catenin/T-cell factor (TCF)-binding elements. In addition, survivin is reduced only when cells reach confluence while β -catenin association to VE-cadherin is comparable at low or high cell density.¹⁸ Finally in absence of β -catenin, survivin is not reduced but, on the contrary, remains high in confluent cells indicating that β -catenin is not needed to maintain high survivin levels in sparse cultures.

An alternative explanation would be that cadherins signal for reduction of survivin through a different mechanism. Cadherins may activate PI3-kinase and this process requires β -catenin association to them.⁷ We found that wortmannin, LY294002 and the Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol2- β -2-O-methyl-3-O-octadecylcarbonate) prevent the reduction of survivin expression induced by cell density suggesting that this pathway is implicated in the process. If this is the case, β -catenin may have a signaling role at the membrane, independently from its transcriptional activity, by promoting membrane localization and activation of PI3-kinase by cadherin expression.⁷

Papapetropoulos et al⁵⁰ reported that angiopoietin-1 induction of survivin required PI3-kinase activation and Akt phosphorylation. These data appear inconsistent with this study. However, down-regulation of survivin by cadherins is a slow and lasting phenomenon that requires continuous engagement and signaling of these proteins at junctions. In contrast, angiopoietin-1 induction of survivin peaks at 12 hours and then declines within 24 hours. It is conceivable that a low but lasting activation of PI3-kinase is required for maintenance of low levels of survivin in resting cells.⁴⁴ Acute induction of PI3-kinase by angiopoietin or other growth factors may up-regulate survivin following different downstream pathways.⁵⁷

In conclusion, this paper shows that in endothelial cells survivin is down-regulated by VE-cadherin expression

and clustering at intercellular junctions. This would suggest that, in the case of vascular wound or angiogenesis when junction organization is impaired,⁴⁴ survivin is up-regulated. Consistently, in the allantois, at the tips of growing vessels the junctional staining of VE-cadherin is reduced and survivin is up-regulated. Other authors found increased survivin expression in endothelial cells undergoing tubulogenesis *in vitro* or during angiogenesis *in vivo*.⁴⁸ The role of cadherins in regulating survivin synthesis may be of interest also in other cell types. In tumors, cadherins are frequently down-regulated and a strong inverse correlation between cadherin expression and tumor invasion has been documented.¹⁵ From data shown here using a set of pancreatic and ovarian tumors, it appears that an inverse relation exists between E-cadherin and survivin expression, suggesting that regulation of survivin by cadherins and β -catenin is not cell specific and may occur also in tumors.

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