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Increased Levels of Survivin, via Association With Heat Shock Protein 90, in Mucosal T Cells From Patients With Crohn's Disease

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Abstract

BACKGROUND & AIMS—Defective apoptosis of lamina propria T cells (LPTs) is involved in the pathogenesis of Crohn's disease. Survivin, a member of the inhibitors of apoptosis family, prevents cell death and regulates cell division. Survivin has been studied extensively in cancer, but little is known about its role in Crohn's disease.

METHODS—LPTs were isolated from mucosal samples of patients with Crohn's disease or ulcerative colitis and healthy individuals (controls). LPTs were activated with interleukin-2 or via CD3, CD2, and CD28 signaling, and cultured at 42°C to induce heat shock. Survivin expression was assessed by immunohistochemistry, confocal microscopy, and immunoblotting; survivin levels were reduced by RNA interference. Cell viability, apoptosis, and proliferation were measured by trypan blue exclusion, annexin-V/7-Aminoactinomycin D staining, and uptake of [3]thymidine, respectively.

RESULTS—LPTs from patients with Crohn's disease had higher levels of survivin than LPTs from patients with ulcerative colitis or controls. RNA knockdown of survivin in LPTs inhibited their proliferation and promoted apoptosis. Levels of survivin were low in LPTs from patients with ulcerative colitis and controls as a result of ubiquitin-mediated proteasome degradation. In LPTs from patients with Crohn's disease, survivin bound to the heat shock protein (HSP)90, and therefore was resistant to proteasome degradation. Incubating LPTs with 17-N-allylamino-17-demethoxygeldanamycin, an inhibitor of HSP90, reduced levels of survivin and induced apoptosis.

CONCLUSIONS—Levels of survivin are increased in LPTs from patients with Crohn's disease (compared with ulcerative colitis and controls) because survivin interacts with HSP90 and

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prevents proteasome degradation. This allows LPTs to avoid apoptosis. Strategies to restore apoptosis to these cells might be developed to treat patients with Crohn's disease.

Keywords

Inflammatory Bowel Disease; IAP; 17-AAG; Immune Regulation

Although Crohn's disease (CD) may be triggered by innate immunity defects,¹ inflammation is characterized by an increase of activated mucosal T cells even when clinically quiescent.² When compared with T cells from ulcerative colitis (UC), the other major form of inflammatory bowel disease (IBD), CD T cells proliferate more, express higher levels of interleukin (IL)-2 receptor α , and are more cytotoxic.^{3,4} Production of IL-2, interferon- γ , and IL-21 is greater by CD than UC T cells,⁵⁻⁷ and IL-12 and IL-18 are increased in CD but not UC intestine.^{8,9} In addition, mucosal T cells express lower levels of Fas ligand in CD than UC,¹⁰ and CD T cells are more resistant to apoptosis.^{11,12} Finally, CD T cells cycle faster, display higher telomerase activity, and undergo more vigorous cell expansion than UC mucosal T cells.¹³

These observations indicate that abnormalities of T-cell proliferation and apoptosis are involved in CD pathogenesis, but the underlying mechanisms are still unclear. Multiple molecules control cell proliferation or death, such as survivin, a member of the inhibitors of apoptosis gene family.¹⁴ Initially described as a protein expressed in human cancers,¹⁵ survivin is a key component of the chromosomal passenger complex, which is essential to cell division, and an apoptosis inhibitor.^{16,17} Survivin orchestrates intricate cellular networks and now is considered a nodal protein with a vital homeostatic role.^{18,19} Quantitative or qualitative alterations in survivin may have crucial consequences, as evidenced in human cancers, in which survivin invariably is overexpressed.²⁰ The involvement of survivin in inflammatory and autoimmune disorders, such as pleuritis, multiple sclerosis, and rheumatoid arthritis, also has been suggested.²¹⁻²³ Considering that cell proliferation and apoptosis are altered in CD,^{3,4} we inquired whether abnormalities of survivin might explain the excessive number of activated T cells in the mucosa.

Materials and Methods

RNA Silencing

To silence survivin gene expression by short interference RNA (siRNA),²⁴ lamina propria T cells (LPTs) were suspended in Human T Cell Nucleofector Solution (Amaxa Biosystems, Köln, Germany) containing the following siRNAs: 10 μ g (pre-optimized) of silencer BIRC5 (survivin), silencer glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or nonsilencer scrambled as a negative control (Ambion, Austin, TX); pre-optimized 1 μ g (pre-optimized) of silencer heat shock protein (HSP)90AA1 (HSP90), silencer GAPDH, or a nonsilencer scrambled siRNA as a negative control (Thermo Scientific, Waltham, MA). Mixtures were incubated for 10 minutes at 37°C, cells were transferred to a 2-mm cuvette at 50×10^6 cells/mL, and submitted to electroporation (Nucleofector II Device program U-14; Amaxa). After pulse (electroporation fraction time), cells were transferred to medium with 20 IU/mL of IL-2, and incubated at 37°C for 24–96 hours before analysis of apoptosis, cell cycle studies, protein, and messenger RNA (mRNA).

Proteinase K Sensitivity Assay

To study survivin susceptibility to proteolysis, cytoplasmic and nuclear fractions of IL-2-stimulated control, CD, and UC LPTs were isolated using the NE-PER kit (Pierce, Rockford, IL), exposed to 0, 5, 15, and 30 ng/mL of proteinase K (Sigma, St. Louis, MO)

for 10 minutes, and the reaction was stopped with 5 mmol/L phenylmethanesulfonyl fluoride (Sigma). Protein lysates were analyzed by immunoblotting.²⁵

Measurement of Proteasomal Activity

Proteasomal activity in cell extracts was measured using the 20S Proteasome Activity Assay Kit (Chemicon International, Temecula, CA). Cell lysates (5 μ g) were mixed with buffer and proteasome substrate in a 96-well fluorometer plate (read at 380/460 nm), and incubated for 2 hours at 37°C. As control, the proteasome was inhibited with lactacystin for 15 minutes before adding the substrate. Cell lysate and buffer backgrounds were subtracted from the final proteasomal activity.²⁶

Heat Shock

Fresh LPTs were exposed to 42°C for 1 hour, collected, and cultured at 37°C^{27,28}; cells were harvested and extracted at 3 and 6 hours after heat shock, and analyzed by immunoblotting and immunofluorescence of cytospin preparations.

Results

Survivin-Expressing Cells in Normal and IBD Mucosa

In normal mucosa, survivin was found in crypt epithelial cells, with sporadic survivin-positive mononuclear cells in the lamina propria (Figure 1A). In CD, numerous survivin-positive cells were present in the lamina propria and fewer in the epithelium (Figure 1A). Fewer survivin-positive cells also were noted in UC epithelium, the lamina propria contained more positive cells than controls, but far less than CD (Figure 1A). Digitized analysis revealed a significant ($P < .001$) increase of survivin-positive cells in inflamed CD compared with normal control mucosa; this increase was also significantly greater when compared with noninflamed CD ($P < .01$) and inflamed UC ($P < .001$) mucosa (Figure 1B). Survivin-positive cells were also significantly more frequent in noninflamed CD mucosa than normal control mucosa ($P < .004$).

The morphology of survivin-positive cells was compatible with T cells (Supplementary Figure 1A), as confirmed by co-localization with CD3-positive cells in control, CD, and UC mucosa (Figure 1C), and most CD3-positive T cells were survivin-positive in CD (Figure 1C). CD3-positive T cells infiltrating colonic cancer were negative for survivin, which was abundant in CD3-negative neoplastic cells (Figure 1C).

Quantification of Survivin in Freshly Isolated LPTs

Reverse-transcription polymerase chain reaction (RT-PCR) showed variable levels of survivin mRNA in control, CD, and UC LPTs, without significant differences among them (Supplementary Figure 2A). In contrast, immunoblotting showed that protein levels were significantly higher in freshly isolated CD compared with control and UC LPTs ($P < .001$ and $P < .04$, respectively) (Figure 2A). Phosphorylated (p) survivin was not detected in control LPTs, whereas p-survivin was present in CD and UC LPTs (Figure 2A). To investigate survivin's subcellular localization, cytosolic, nuclear, and total whole LPT lysates were probed, and most survivin was found in the nuclear fraction, whereas cytosolic survivin was absent in control but present in CD and UC cells (Figure 2B). P-survivin was found in the nuclear fraction, and significantly more in CD and UC than control LPTs (Figure 2B).

Modulation of Survivin in Activated LPTs

Control, CD, and UC LPTs were exposed to pro-inflammatory factors or activated through T-cell-receptor pathways. LPTs were cultured with IL-2 and survivin mRNA and protein levels were measured at various time points. Fourteen-day-old cultures showed consistently increased levels of survivin mRNA, without significant differences among control, CD, or UC cells (Supplementary Figure 2B). Comparable results were observed at 7 and 10 days, including when IL-15 was used (not shown). Protein amounts also increased in response to IL-2 (Figure 2C), both in the cytoplasm and nucleus (Figure 2D). Engagement of the CD3 or CD2 receptors, with/without CD28, enhanced survivin levels in control and IBD LPTs (Figure 2E). In contrast, 3-day exposure to IL-1 γ , tumor necrosis factor- α , interferon- γ , IL-6, IL-10, transforming growth factor- γ 1, and lipopolysaccharide failed to modulate survivin expression (not shown). Because survivin activity depends on phosphorylation,²⁹ we investigated changes with IL-2 activation. Levels of p-survivin paralleled those of total survivin in IL-2-activated LPTs (Figure 2C).

Changes in Survivin Expression in Proliferating and Resting LPTs

Upon exposure to IL-2, LPT number increased progressively in all groups, but significantly more in CD compared with control and UC cultures (Supplementary Figure 3A). Survivin protein levels peaked at 4 days in control and UC cultures, although it continued to climb in CD cultures (Supplementary Figure 3C).

Survivin and cell numbers also were assessed in fresh control, CD, and UC LPTs cultured alone (without IL-2) for 7 days. In all cultures, survivin mRNA and cell number declined (Supplementary Figures 2C and 3B, respectively), but cell number decreased significantly less in CD cultures (Figure 3B). In the absence of IL-2, protein levels declined in control and UC cultures but, surprisingly, increased significantly in CD cultures (Figure 2F and Supplementary Figure 3D). These changes were confirmed by confocal microscopy (Supplementary Figure 4).

Effect of mRNA Silencing on Survivin Level, LPT Proliferation, and Apoptosis

The effect of survivin mRNA silencing was shown by inhibition of survivin protein expression, but not Bcl-2 or GAPDH expression, in IL-2-stimulated cultures (Figure 3A). In anti-CD3/CD28 plus IL-2-stimulated cultures, siRNA treatment markedly suppressed control, CD, and UC LPT proliferation (Figure 3B, upper panel), and induced apoptosis in all groups (Figure 3C and D).

Survivin Degradation by the Ubiquitin-Proteasome Pathway in LPTs

The earlier-described results indicate that survivin is linked to LPT function, and its production or degradation is altered in CD. In unstimulated CD LPTs survivin increases whereas mRNA declines, therefore post-translational modifications could be present. Because survivin is degraded by the ubiquitin-proteasome pathway,³⁰ ubiquitylation, proteasomal degradation, or both could be altered in CD, prompting us to investigate these functions in LPTs.

First, we investigated whether survivin is susceptible to protein synthesis inhibition, proteasomal activity, and nuclear transport.^{30,31} Treatment of IL-2-activated control LPTs with cycloheximide removed survivin from the cytoplasm (Supplementary Figure 5). Treatment with the proteasomal inhibitor lactacystin stabilized survivin levels even in the presence of cycloheximide (Supplementary Figure 5B), resulting in its accumulation in the cytoplasm. Cycloheximide and leptomycin B, a nuclear export inhibitor, led to survivin accumulation in the nucleus (Supplementary Figure 5B), suggesting that exportin

(chromosome region maintenance 1) mediates transport of survivin from the nucleus to the cytoplasm in LPT.^{32,33}

Treatment with the proteasomal inhibitor MG132 time-dependently increased survivin at the 16-kilodalton size and induced several slower migrating bands in fresh and IL-2-activated LPTs (Figure 4). Interestingly, Bcl-2, another protein involved in LPT apoptosis, did not change after proteasomal inhibition. Reciprocal immunoprecipitation of survivin and ubiquitin followed by immunoblotting with anti-ubiquitin and anti-survivin antibodies, respectively, confirmed that survivin is ubiquitinated in LPTs (Figure 4).

Increased Ubiquitylation and Proteasomal Activity in CD LPTs

Survivin immunoprecipitation followed by ubiquitin immunoblotting in fresh control and IBD LPTs revealed that survivin ubiquitylation was much greater in CD than UC LPTs, and essentially absent in control LPTs (Figure 5A and Supplementary Figure 6A). Therefore, excess survivin in CD LPTs appeared to represent accumulation of ubiquitylated forms, implying a normal ubiquitylation function. Because neither lactacystin nor MG132 interfere with ubiquitylation, high survivin levels in CD LPTs could represent a defect of proteasomal function. We therefore measured intracellular proteasomal activity by degradation of a labeled Leu-Leu-Val-Tyr substrate.²⁶ By using LPT lysates, significantly increased proteasomal activity was detected in CD compared with control cells, whereas activity of control and UC lysates were comparable (Figure 5B). Moreover, the proteolytic activity of CD lysates was more resistant to inhibition by the proteasomal inhibitor lactacystin than control or UC lysates (Figure 5B).

Differential Sensitivity of Survivin to Proteolysis in Control, CD, and UC LPTs

Increased accumulation of ubiquitylated survivin in the face of increased proteasomal activity suggested that alternate mechanisms were responsible for the impaired survivin degradation in CD. Because survivin is degraded in the cytoplasm, we exposed lysates of IL-2-activated control, CD, and UC LPTs to proteinase K to assess survivin sensitivity to proteolysis in the cytosolic and nuclear compartments. As shown in Figure 5C and D, only survivin in the CD cytosolic lysates failed to be degraded, whereas in control and UC lysates degradation occurred dose-dependently. In the same lysates Bcl-2 degradation occurred in all groups (Figure 5C). Survivin in nuclear extracts was susceptible to proteolytic degradation in all groups (Figure 5C).

Association of Survivin With HSP90 in LPT

An explanation for the resistance of survivin to proteolysis in CD could be its association with the chaperone HSP90.²⁸ We therefore performed a reciprocal immunoprecipitation for survivin and HSP90. Immunoprecipitates of survivin from CD LPTs showed strong reactivity with anti-HSP90 antibodies, whereas those from control and UC cells showed weak and no reactivity, respectively (Figure 6A and Supplementary Figure 6B). The same pattern was observed when HSP90 immunoprecipitates were probed with anti-survivin antibodies (Figure 6A and Supplementary Figure 6B). Confocal microscopy of IL-2-activated LPTs showed association of survivin with HSP90 in the nucleus but not the cytoplasm (Supplementary Figure 7).

We next exposed control, CD, and UC LPTs to heat shock to induce HSP90 and assessed protein levels at various times. In control cells, survivin and HSP90 increased in parallel and the same pattern was observed with CD LPTs, although a strikingly different response was seen with UC LPTs, in which survivin failed to be induced by heat shock and HSP90 levels remained unchanged (Figure 6B). The association of survivin with HSP90 in LPTs was confirmed by confocal microscopy (Figure 7 and Supplementary Figure 7). When Bcl-2

immunoblotting was performed as control, the opposite was observed, Bcl-2 levels diminishing rather than increasing with time (Figure 6B).

To further explore the survivin/HSP90 relationship, IL-2-activated control, CD, and UC LPTs were treated with 17-N-Allylamino-17-Demethoxygeldanamycin (17-AAG) to disrupt HSP90 chaperone function,^{34,35} and making client proteins susceptible to proteolytic degradation. Immunoblotting of 17-AAG-treated LPTs showed a dose-dependent decrease of survivin in all cells (Figure 6C). This was accompanied by a parallel dose-dependent increase in LPT apoptosis as assessed by annexin V (Figure 6D). Because the decrease could be owing to survivin proteasomal degradation after HSP90 disengagement, we added MG132 to 17-AAG-treated cultures and observed higher levels of survivin than those resulting from treatment with 17-AAG alone (Figure 6C), a finding compatible with the protective function of HSP90 on survivin. Finally, we explored the effect of HSP90 RNA silencing on LPT function. Although effective in inhibiting protein expression (Supplementary Figure 8A), and contrasting with survivin RNA silencing (Figure 3), HSP90 silencing failed to significantly alter control, CD, and UC LPT proliferation (Figure 3B, lower panel) or induce LPT apoptosis (Figure 3C and D, and Supplementary Figure 8B).

Discussion

Dysplastic and neoplastic colonic epithelium contains numerous survivin-positive cells,^{36,37} but their presence in the lymphoid compartment has not been addressed. We detected a marked increase in survivin-positive, CD3-positive, T cells in CD. This increase concurs with the augmented proliferation and decreased apoptosis of T cells in CD^{11,12} and increased levels of total and p-survivin in fresh CD compared with control and UC LPTs. Thus, survivin is involved in the development, clonal expansion, cycling, and survival of T cells,^{38–40} functions that depend on its shuttling between cytosol and nucleus^{28,41} and phosphorylation status.²⁹

Survivin's nuclear pool appears involved in cell proliferation whereas the cytoplasmic pool controls cell survival.⁴² If so, the more abundant nuclear compartmentalization suggests that survivin plays a predominant role in promoting proliferation rather than survival of LPT, and its increased expression could explain the vigorous proliferation of LPTs in CD.¹³ Because p-survivin was present mostly in LPTs from inflamed mucosa, survivin may drive the progression of mucosal T cells through mitosis during inflammation, a conclusion supported by detection of survivin at the spindle midzone of dividing LPTs.¹⁶

Little is known about survivin modulation in human T cells.^{43,44} Anti-CD3/CD28- or anti-CD2/CD28-activated LPTs up-regulated its expression, but more so in CD. IL-2 or IL-15 also up-regulated survivin and induced its phosphorylation, whereas other cytokines or lipopolysaccharides failed to do so. Thus, survivin up-regulation is a selective response induced by T-cell-receptor engagement or growth factor stimulation, events leading to cell division. In fact, LPT numbers and survivin levels increased in parallel, particularly in CD. This increase cannot be explained by higher IL-2 production by CD LPTs because IL-2 production actually is decreased in CD compared with control cultures.^{4,5} However, CD LPTs respond more vigorously to IL-2 than control and UC cells, a response perhaps dependent on high survivin level. Differences among control, CD, and UC also could be explained by different LPT composition⁴³; however, CD45RO+, CD4+, and CD8+ subsets are similar among the 3 groups and do not change with disease activity (Supplementary Materials). Whether differences in T helper 1, T helper 2, and T helper 17 composition may impact survivin expression remains to be investigated. Silencing by RNA interference inhibited cell proliferation and induced apoptosis, showing that survivin controls these key functions both in normal and inflammatory T cells.^{16,20,44}

Survivin expression declined in control and UC LPTs cultured without growth stimuli, but its protein levels surprisingly increased in CD cultures even when its mRNA was no longer detectable. This startling observation led us to explore abnormalities of survivin post-translational modifications in CD.

Correct compartmentalization is essential for cell-cycle-associated proteins such as survivin.⁴⁵ We confirmed that survivin not only translocates to the nucleus in response to T-cell-receptor- or IL-2-mediated activation, but is exported to the cytoplasm via CRM-1-dependent nuclear export signal.⁴⁶ No differences in the dynamics of subcellular distribution of CD or UC were found compared with control T cells, indicating that defective compartmentalization cannot explain the selective accumulation of survivin in CD.

Survivin is regulated by the ubiquitin-proteasome pathway,³⁰ which controls cell cycling, growth, and apoptosis,⁴⁷ and protein persistence despite mRNA disappearance suggested defective processing by this pathway.^{47,48} Survivin was ubiquitinated in LPTs regardless of their origin, and accumulated in the cytoplasm after treatment with proteasome inhibitors, showing that survivin proteasomal degradation was normal. Moreover, accumulation of various survivin-ubiquitinated forms spoke against defective ubiquitination as a reason for increased expression in CD LPTs. Defective proteasomal activity also was ruled out because the activity in LPT lysates was greater and more resistant to lactacystin inhibition in CD than control or UC cell lysates. These results concur with an increased proteasomal activity in CD,⁴⁹ but fail to explain its high LPT expression.

Exposure of LPT lysates to PK revealed that only cytosolic, but not nuclear, survivin in CD LPTs was resistant to proteolysis. Because protein degradation can be prevented by association with chaperone proteins such as HSP90,^{50,51} we first confirmed by immunoprecipitation and immunofluorescence that survivin was associated with HSP90,²⁸ and particularly in CD LPTs. Second, we heat-shocked LPTs to up-regulate survivin expression: this occurred in control LPTs, but the response was remarkably stronger in CD and essentially absent in UC cells. Stress-induced up-regulation of survivin is independent of de novo gene transcription or cell cycling,⁵² suggesting that enhanced stability of survivin in CD T cells could be owing to the action of HSP90. If so, dissociation from HSP90 should make freed survivin available for degradation. Treatment of control, CD, and UC LPTs with 17-AAG, an inhibitor of HSP90,³⁴ decreased survivin in parallel with increased apoptosis, compatible with survivin release from HSP90 and subsequent proteasomal degradation. Interestingly, inhibition of HSP90 expression by RNA silencing failed to alter LPT proliferation or apoptosis significantly.

In addition to cancer,⁵³ nodal proteins such as survivin could be involved in immunity¹⁸ and in our study survivin emerges as a master regulator of T-cell-mediated mucosal immunity and inflammation. In CD, survivin function appears to be modulated by HSP90, another nodal protein.⁵⁴ Hyperthermia-induced HSP90 up-regulation dramatically increased survivin expression in CD T cells, possibly contributing to their high anti-apoptotic threshold and proliferation.⁵⁵ Although a weaker response was seen in normal T cells, the failure to up-regulate survivin and HSP90 in UC T cells was striking, underscoring a fundamentally different response of CD and UC T cells to stress. Endoplasmic reticulum stress is linked to IBD,⁵⁶ and stress-induced survivin changes may underlie loss of T-cell regulation in inflammation.

Based on these data, remarkably similar regulatory mechanisms appear operative in cancer cells and inflammatory T cells. Inhibition of cyclin-dependent kinases and cyclin D1 are effective anti-inflammatory approaches, including experimental IBD.^{57,58} Gene targeting of survivin to induce apoptosis and inhibit proliferation is effective in cancer cells,⁵⁹ and an

analogous approach could inhibit inflammatory T cells. Survivin gene silencing in CD is conceptually appealing as a molecular target, but destabilizing survivin-HSP90 interaction may be more practical. This can be accomplished with geldanamycin or its analogues 17-AAG and 17-DMAG,⁶⁰ shepherdin,⁶¹ or gamitrinibs.⁶² 17-AAG destabilized the interaction between HSP90 and survivin in LPTs, resulting in survivin degradation accompanied by reduced viability and proliferation. Thus, survivin networks may not necessarily be “tumor specific,”¹⁸ but actually applicable to chronic inflammation as well. This concept has been used successfully in experimental autoimmune encephalomyelitis and uveitis,^{35,63} and our results provide proof of concept for targeting survivin as a novel therapeutic option in CD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

AAG	N-Allylamino-17-Demethoxygeldanamycin
CD	Crohn's disease
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HSP	heat shock protein
IBD	inflammatory bowel disease
IL	interleukin
LPT	lamina propria T cells
mRNA	messenger RNA
p	phosphorylated
siRNA	short interference RNA
RT-PCR	reverse-transcription polymerase chain reaction
UC	ulcerative colitis

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Figure 1.

Distribution and quantification of survivin-expressing cells in colonic mucosa and colon cancer. (A) Survivin-positive cells (dark brown) in the crypts of normal colonic epithelium but not the lamina propria; marked increase of survivin-positive cells (*arrows*) in CD lamina propria; modest increase in survivin-positive cells (*arrows*) in UC lamina propria. (B) Increased numbers of survivin-positive cells in inflamed CD, noninflamed CD, and inflamed UC mucosa compared with normal control mucosa. * $P < .001$ for inflamed CD compared with control and UC. ** $P < .01$ for inflamed CD compared with noninflamed CD, and UC compared with normal mucosa. *** $P < .004$ for noninflamed CD compared with control (groups included 11 control, 10 inflamed CD, 10 noninflamed CD, and 10 UC). (C) From top to bottom: CD3-positive lymphocytes (red) in the lamina propria and survivin-positive crypt cells (green) in normal mucosa; abundant CD3 (red) and survivin (green) co-expressing lymphocytes infiltrating colonic CD; CD3-positive lymphocytes (red) in the lamina propria and survivin-positive crypt cells (green) in UC mucosa; CD3-positive (red), survivin-negative lymphocytes infiltrating survivin-positive neoplastic cells (green) in colon adenocarcinoma. Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Representative of 10–14 samples for normal and IBD mucosa and 3 colon cancers.

Figure 2.

Levels of survivin protein in fresh, activated, and rested LPTs. (A) Increased survivin and p-survivin in freshly isolated CD compared with control and UC LPTs ($P < .001$ and $P < .04$, respectively). (B) Increased nuclear p-survivin in freshly isolated UC and CD compared with control LPTs ($P < .05$). (C) Up-regulation of total and p-survivin in control, CD, and UC LPTs upon exposure to IL-2 ($P < .02$ –.001 compared with day 0). (D) Subcellular distribution of survivin in IL-2-activated LPTs. Increased survivin in activated UC and CD LPT cells compared with control LPTs. (E) Up-regulation of survivin in control, CD, and UC LPTs upon activation with anti-CD3/CD28 or CD2/CD28 ($P < .05$ and $P < .04$ compared with day 0, respectively). (F) Spontaneous increase of survivin in fresh CD LPTs and disappearance of survivin in control and UC LPTs cultured in the absence of IL-2 ($P < .03$ and $P < .02$, respectively). GAPDH used as loading control for total and cytoplasmic survivin, and lamin B1 for the nuclear fraction. Immunoblots are representative of 12–18 samples in each control, CD, and UC group; bands represent a loading of 10 μ g protein lysate/lane regardless of the total (T), cytoplasmic (C), or nuclear (N) origin of survivin. P values were calculated using the ratio of survivin to GAPDH or lamin B1 obtained by densitometric analysis and comparing the ratio values among control CD and UC.

Figure 3.

Effect of survivin and HSP90 silencing on protein levels, proliferation, and apoptosis of LPTs. (A) Immunoblot showing the effect of survivin and scrambled siRNA on protein levels of IL-2–stimulated control, CD, and UC LPTs. Bcl-2 was used as specificity control and GAPDH was used as loading control (figure is representative of 4 experiments). (B) Upper panel: time-dependent suppression of anti-CD3/CD28 plus IL-2–induced LPT proliferation upon treatment with survivin siRNA (each column indicates 1 control, 1 CD, and 1 UC LPT isolate). Lower panel: lack of significant effect on anti-CD3/CD28 plus IL-2–induced LPT proliferation by treatment with HSP90 siRNA (each column indicates 1 control, 1 CD, and 1 UC LPT isolate). Percentage of suppression compared with that of scrambled siRNA. (C) Increased LPT apoptosis induced by survivin, but not HSP90, siRNA compared with scrambled siRNA ($*P < .012$; $n = 3$ for control, CD and UC, respectively, for both survivin and HSP90 siRNA). (D) Flow cytometric analysis of increased LPT apoptosis induced by survivin siRNA as assessed by annexin-V/7-Aminoactinomycin D. Red indicates double-negative cells and green indicates annexin-V–positive cells (representative of 3 experiments).

Figure 4.

Effect of inhibiting proteasomal activity on time-dependent expression of survivin in LPTs. Western blots of IL-2-activated LPT extracts showing results with 3 different anti-survivin antibodies recognizing distinct survivin epitopes (anti-survivin-1, -2, and -3), one antibody against Bcl-2, and reciprocal immunoprecipitation of ubiquitin and survivin probed with antibodies against survivin (anti-survivin 3) and ubiquitin, respectively. Anti-survivin 1 is a 6E4 mouse monoclonal antibody, anti-survivin 2 is a mouse monoclonal antibody clone 60.11, and anti-survivin 3 used for immunoprecipitation (IP) is a rabbit polyclonal antibody (Supplementary Materials). Antibodies directed at distinct survivin epitopes ensured that all detected bands did contain survivin. Results displayed derive from a CD LPT isolate. Dimethyl sulfoxide (DMSO) is the solvent for the proteasomal inhibitor MG132, and E64 is a protease inhibitor used as control for MG132. GAPDH was used as loading control. Figure is representative of 1 control, 2 CD, and 2 UC LPTs.

Figure 5.

Proteasomal activity of LPT and survivin sensitivity to proteolysis. (A) Immunoprecipitation of survivin in fresh control, CD, and UC LPT lysates followed by immunoblotting with an anti-ubiquitin antibody. Figure is representative of 6–8 samples in each group. (B) Differential levels of proteasomal activity in fresh control, CD, and UC LPTs. * $P < .04$ for CD compared with control LPT activity, and for degree of lactacystin-induced inhibition in CD compared with UC LPT ($n = 17$ for control, CD, and UC, respectively). (C and D) Effect of proteinase K activity on survivin levels in cytosolic and nuclear fractions of IL-2-stimulated LPTs. Immunoblots showing a dose-dependent decrease of survivin in the cytosolic fraction of control and UC, but not CD LPT lysates (* $P < .02$, ** $P < .004$, by densitometry of the survivin: lamin B1, survivin: γ -actin, and Bcl-2: γ -actin ratios, respectively), and a dose-dependent decrease of survivin in the nuclear fraction of the same lysates. Immunoblots of the same lysates show a dose-dependent decrease in the cytosolic fraction of Bcl-2, which was used as control. Figure is representative of 4 experiments.

Figure 6.

Association of survivin with HSP90 in LPTs and dissociating effect of 17-AAG. (A) Reciprocal immunoprecipitation of survivin and HSP90 in fresh control, CD, and UC LPT lysates. Immunoblots showing immunoprecipitated survivin probed with an anti-HSP90 antibody, and immunoprecipitated HSP90 probed with an anti-survivin antibody. Figure is representative of 6 experiments. (B) Effect of heat shock on expression of survivin, HSP90, and Bcl-2 by LPTs. Immunoblots showing time-dependent increase of survivin and HSP90 and a decrease of Bcl-2 in fresh control, CD, and UC LPTs. GAPDH was used as loading control. Figure is representative of 4 control, CD, and UC LPTs, respectively. (C) Effect of inhibition of HSP90 chaperone activity on LPT survivin levels. Immunoblots showing the dose-dependent effect of 17-AAG on survivin levels of IL-2-stimulated control, CD, and UC LPTs. MG132 was used as control to block survivin degradation through the proteasomal pathway, and dimethyl sulfoxide (DMSO) was used as solvent control for both 17-AAG and MG132. γ -actin was used as a loading control. Figure is representative of 3 control, CD, and UC LPTs, respectively. (D) Dose-dependent increase of control, CD, and UC LPT apoptosis induced by 17-AAG (n = 3 control, CD, and UC LPTs).

Figure 7.

Association of survivin with HSP90 in LPTs. Confocal microscopy of LPT preparations showing co-localization of survivin (green) and HSP90 (red) in fresh, heat-shocked (6 hours), IL-2-stimulated (7–10 days), and alone (absence of IL-2 for 7 days) control, CD, and UC LPTs. Co-localization of survivin and HSP90 is indicated by the orange color generated by the combination of green (survivin) and red (HSP90) colors. Micrograph panel is representative of 5–10 isolates in each group.