normal rats treated with the serotonin 1A or 1B receptor agonists (not shown).

To ensure that the effect of the serotonin 1A and 1B receptor agonists on bile duct proliferation is specifically due to the agonist/receptor interaction in cholangiocytes, pure cells from BDL rats were incubated in vitro with 8-hydroxy-DPAT or anipirtoline in the absence or presence of the corresponding receptor antagonists [(S)-WAY 100135 dihydrochloride and SB 216641, respectively]. Similar to what was shown in vivo, both 8-hydroxy-DPAT and anipirtoline diminished PCNA protein expression of purified cholangiocytes compared with cholangiocytes treated with 0.2% BSA (Figure 2*F*). The inhibitory effect of 8-hydroxy-DPAT or anipirtoline on cholangiocyte PCNA protein expression was blocked by preincubation with the corresponding serotonin receptor antagonist (Figure 2*F*). The simultaneous administration of both receptor agonists did not result in an additive effect, either in vivo or in vitro (Figure 2*A–E*).

The Serotonin 1A and 1B Receptor Agonists Inhibit Cholangiocyte Functional Activity in the Course of Chronic Cholestasis

After 1 week of administration of 8-hydroxy-DPAT or anipirtoline to BDL rats, both secretin-stimulated bile and bicarbonate secretion were inhibited compared with BDL rats treated with saline (Figure 3*A* and *B*, respectively). 8-Hydroxy-DPAT or anipirtoline did not change basal or secretin-stimulated bile and bicarbonate secretion of normal rats (not shown). Similarly, in cholangiocytes purified from BDL rats treated with serotonin receptor agonists, both the secretin-stimulated cAMP synthesis (Figure 3*C*) and the PKA activity (Figure 3*D*) were markedly reduced compared with BDL rats treated with saline. These changes are specifically determined by the specific agonist/receptor binding in cholangiocytes. In support of this concept, both the intracellular cAMP synthesis (Figure 4*B*) and PKA activity (Figure 3*E*) of purified BDL cholangiocytes were reduced by in vitro incubation with 8-hydroxy-DPAT or anipirtoline, whereas they did not change in cells preincubated with the corresponding receptor antagonists.

The Effects of the Serotonin 1A and 1B Receptor Agonists Are Associated With Src Inactivation and Extracellular Signal-Regulated Kinase 1/2 Dephosphorylation

In cholangiocytes isolated from BDL rats treated in vivo with serotonin receptor 1A or 1B agonists, there was a reduced phosphorylation of the 139 tyrosine residue and an increased phosphorylation of the 530 tyrosine residue compared with cholangiocytes purified from BDL control rats (Figure 5*A*). This state of differential phosphorylation indicates inactivation of Src enzymatic activity.[40,41] No changes were instead observed in the expression of the total Src protein (Figure 5*A*). Similarly, the in vivo treatment with 8-hydroxy-DPAT or anipirtoline diminished ERK1/2 phosphorylation, although it did not affect the expression of the total ERK1/2 proteins (Figure 5*B*). In vitro, 8-hydroxy-DPAT and anipirtoline induced Src inactivation and ERK1/2 dephosphorylation as well, which was prevented by preincubation with the corresponding receptor antagonist (Figure 5*C* and *D*). In support of this concept, the in vitro incubation with PP2 or PD98059 inhibited cholangiocyte proliferation (Figure 6 and 2*E*, respectively).

The Effects of the Serotonin 1A and 1B Receptor Agonists Are Associated With Enhanced IP_3 Levels

Compared with cholangiocytes isolated from BDL rats treated with saline, IP_3 levels were increased in cholangiocytes purified from BDL rats treated in vivo with 8-hydroxy-DPAT or anipirtoline (Figure 7*A*). Furthermore, the increase of IP_3 levels induced by the in vitro incubation of cholangiocytes with 8-hydroxy-DPAT or anipirtoline was prevented by the presence of the corresponding receptor antagonist (Figure 7*B*).

The Inhibitory Effect of the Serotonin 1A and 1B Receptor Agonists on Cholangiocyte Proliferation Is Dependent on the Enhancement of IP_3 Signaling and the Consequent Inhibition of the cAMP/PKA/Src/ERK1/2 Cascade

The blockage of the IP_3 signaling by BAPTA/AM (an intracellular Ca^{2+} chelator) and G06976 (a Ca^{2+}-dependent PKC inhibitor) abolished the inhibitory effect of 8-hydroxy-DPAT and anipirtoline on cholangiocyte PCNA protein expression (Figure 4*A*). In the same fashion, the blockage of Ca^{2+} and Ca^{2+}-dependent PKC prevented the inhibitory effect of 8-hydroxy-DPAT and anipirtoline on intracellular cAMP synthesis (Figure 4*B*).

When cholangiocytes were preincubated in vitro with the PKA stimulator dybutyryl-cAMP, the inhibition of the PCNA protein expression induced by 8-hydroxy-DPAT and anipirtoline was no longer evident (Figure 6). In contrast, the presence of the Src inhibitor PP2 in the same experimental condition abolished the effect of the PKA stimulator (Figure 6), and the PCNA protein expression decreased to levels not statistically different from cells incubated with the serotonin 1A and 1B agonists alone.

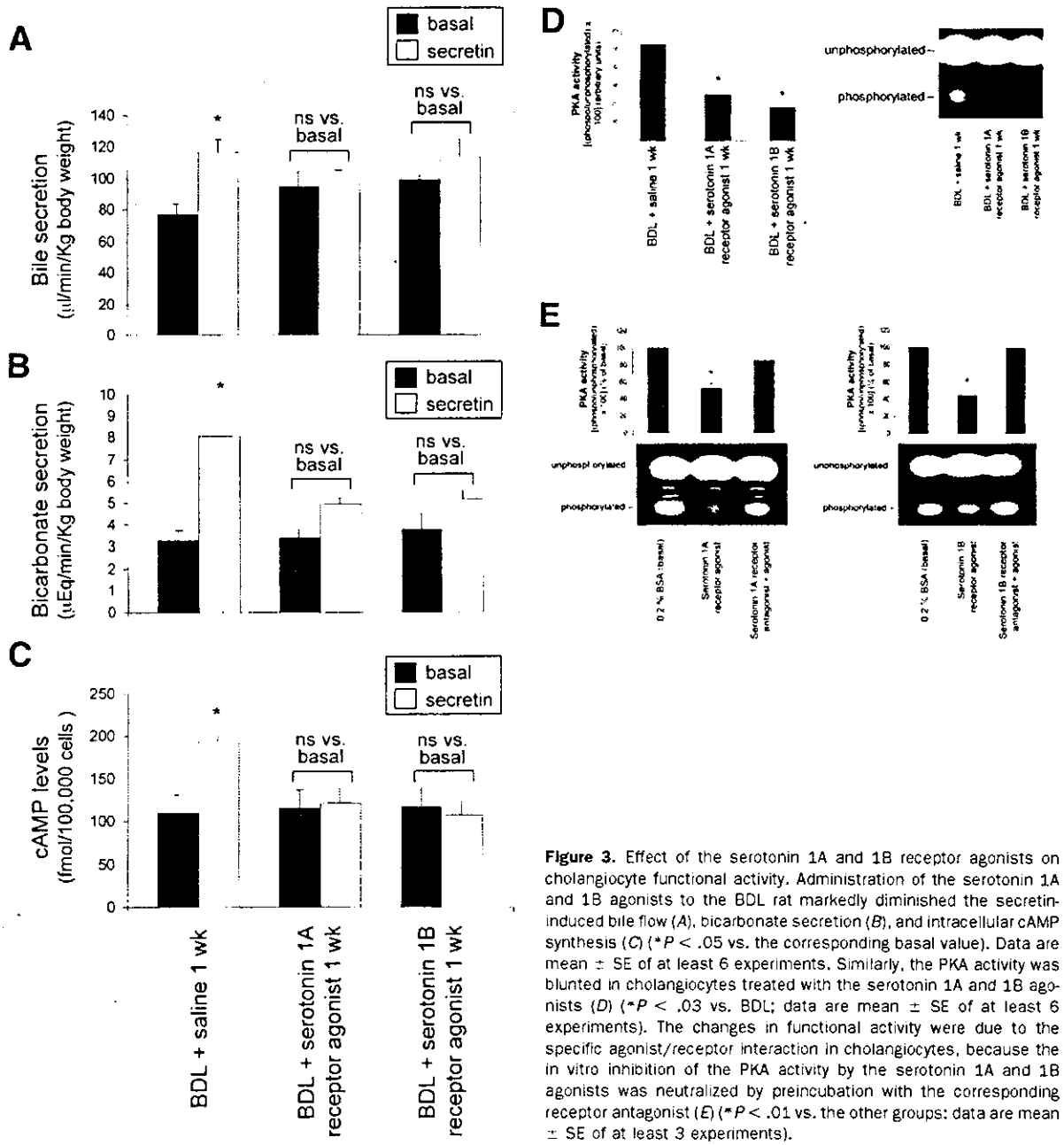


Figure 3. Effect of the serotonin 1A and 1B receptor agonists on cholangiocyte functional activity. Administration of the serotonin 1A and 1B agonists to the BDL rat markedly diminished the secretin-induced bile flow (*A*), bicarbonate secretion (*B*), and intracellular cAMP synthesis (*C*) (*$P < .05$ vs. the corresponding basal value). Data are mean ± SE of at least 6 experiments. Similarly, the PKA activity was blunted in cholangiocytes treated with the serotonin 1A and 1B agonists (*D*) (*$P < .03$ vs. BDL; data are mean ± SE of at least 6 experiments). The changes in functional activity were due to the specific agonist/receptor interaction in cholangiocytes, because the in vitro inhibition of the PKA activity by the serotonin 1A and 1B agonists was neutralized by preincubation with the corresponding receptor antagonist (*E*) (*$P < .01$ vs. the other groups; data are mean ± SE of at least 3 experiments).

Proliferating Cholangiocytes Overexpress and Oversecrete Serotonin

Pure cholangiocytes from normal rats weakly expressed the protein for serotonin. Serotonin protein expression markedly increased in cholangiocytes from BDL rats (Figure 8*A*). In a similar fashion, pure cholangiocytes from BDL rats secreted serotonin in culture medium 3-fold more than cholangiocytes from normal rats (Figure 8*B*).

The Blockage of Endogenous, Cholangiocyte-Secreted Serotonin Further Enhances the Growth of the Biliary Tree in the Course of Chronic Cholestasis

Chronic in vivo administration of the serotonin-neutralizing antibody to BDL rats significantly increased the number of PCNA-positive (Figure 9*A*, top) and CK-19–positive (Figure 9*A*, middle) cholan-

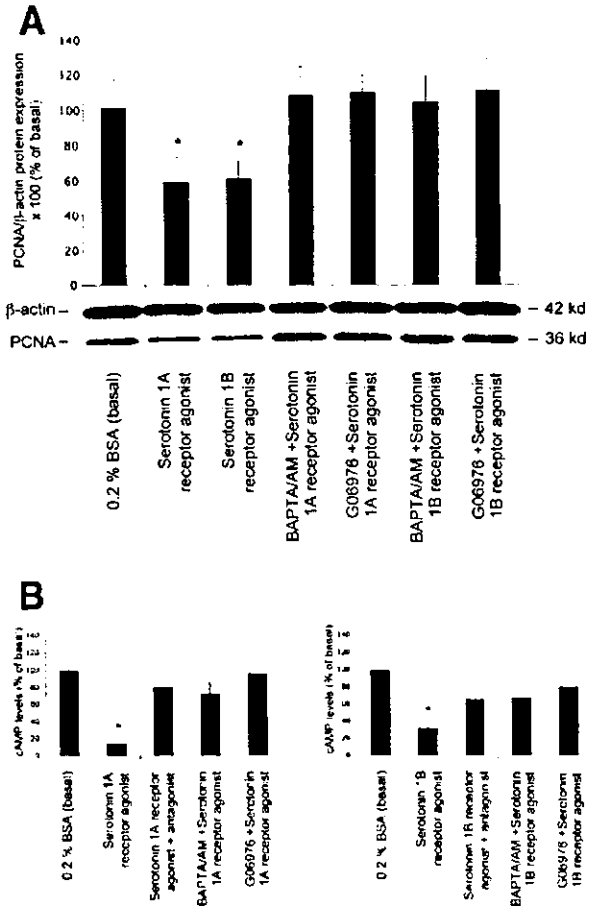


Figure 4. (*A*) In vitro, Ca^{2+} and PKC blockers abolish the inhibitory effect of the serotonin 1A and 1B receptor agonists on cholangiocyte proliferation as evaluated by immunoblots for the PCNA protein in cholangiocyte whole-cell lysates (*$P < .03$ vs. the other groups). In the same fashion (*B*), the blockage of Ca^{2+} and PKC prevented the inhibitory effect of the serotonin 1A and 1B agonists on intracellular cAMP synthesis (*$P < .03$ vs. the other groups). Data are mean ± SE of at least 6 experiments.

giocytes and the bile duct mass (Figure 9*A*, bottom) in liver sections compared with BDL rats treated with nonimmune serum. However, administration of the serotonin-neutralizing antibody to normal rats did not alter the growth of the intrahepatic biliary epithelium (Figure 9*A*).

Similarly, when cholangiocytes purified from BDL rats were incubated in vitro with the serotonin-neutralizing antibody, the PCNA protein expression was clearly enhanced as compared with cholangiocytes treated with BSA (Figure 9*B*). In contrast, the in vitro blockage of the cholangiocyte-secreted serotonin did not modify the PCNA protein expression of normal cholangiocytes (Figure 9*B*).

Discussion

The results of this study show that (1) cholangiocytes express the serotonin 1A and 1B receptor subtypes; (2) the selective activation of these receptors (by specific serotonin receptor agonists) inhibits the growth and functional activity of the intrahepatic biliary tree in the course of chronic cholestasis; (3) the enhanced intracellular IP_3 signaling and the consequent inhibition of the cAMP/PKA/Src/ERK1/2 pathway is responsible for the effects of the activation of the serotonin 1A or 1B receptors; (4) proliferating cholangiocytes overexpress and oversecrete serotonin; and (5) the neutralization of the endogenous cholangiocyte-secreted serotonin further enhances cholangiocyte proliferation in the course of chronic cholestasis.

The intrahepatic biliary epithelium is the target of both congenital and acquired chronic cholestatic liver diseases, which are collectively termed cholangiopathies.[1,2] Despite the different etiopathogenesis, cholangiopathies are commonly characterized by the dysregulation of the balance between proliferation and death of cholangiocytes that leads to progressive cholestasis, vanishing of bile ducts, and liver failure.[2] Currently, no definitive medical therapy for cholangiopathies is available, and liver transplantation is the only mean to end the progression toward liver failure.[1,42] Therefore, understanding the factors that modulate cholangiocyte survival and proliferation is an important step to further define the pathogenesis and find new effective therapeutic tools for cholangiopathies.

Our study shows that the growth of the intrahepatic biliary epithelium is also under the control of the neuroendocrine hormone serotonin. First, we observed that cholangiocytes express, mostly on the basolateral domain, both subtype 1A and 1B of the serotonin receptors, which belong to the transmembrane G-protein superfamily.[25,43] These receptors have been characterized and described on several epithelia, such as the epididymal,[44] kidney,[25] or pancreas,[45] where their activation commonly regulates different cellular functions. It is interesting to note that the activation of the serotonin 1A or 1B receptors is known to induce changes in cAMP synthesis[43] and Ca^{2+}/PKC activation,[46] molecules also known to be key regulators of cholangiocyte biology.[9,10,12,47–50]

To discover whether activation of the serotonin 1A and 1B receptors in cholangiocytes leads to changes in the development of chronic cholestasis, we administered to BDL rats the specific receptor agonists 8-hydroxy-DPAT[25] or anpirtoline,[31] respectively. After BDL, the intrahepatic biliary tree undergoes an intense process of growth because of the strong induction of cholangiocyte proliferation.[9] The more cholangiocytes proliferate, the more their functional activity is enhanced.[9] Thus, in

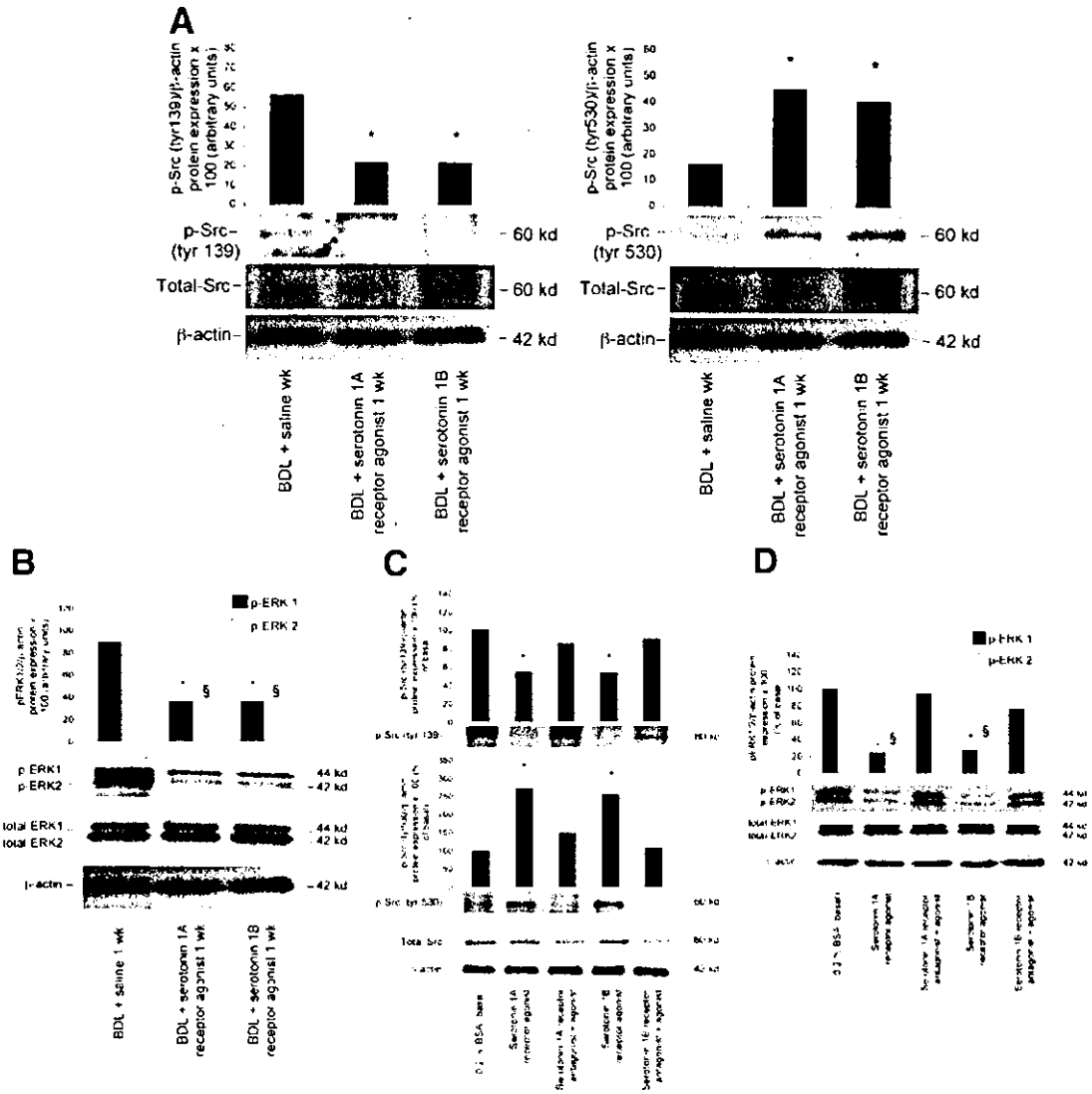


Figure 5. The serotonin 1A and 1B receptor agonists inactivate Src and dephosphorylate ERK1/2. Administration of the serotonin 1A and 1B agonists reduced Src phosphorylation at the Tyr 139 residue and enhanced phosphorylation of the Tyr 530 residue (*A*) (*$P < .05$ vs. BDL). In the same fashion, in vivo administration of the serotonin 1A and 1B agonists markedly diminished ERK1/2 phosphorylation (*B*) (*$P < .01$ vs. phosphorERK1 of BDL; §$P < .05$ vs. phosphorERK2 of BDL). In vitro, these changes in Src activation (*C*) (*$P < .02$ vs. the other groups) and ERK1/2 phosphorylation (*D*) (*$P < .01$ vs. phosphorERK1 of the other groups; §$P < .01$ vs. phosphorERK2 of the other groups) were prevented by preincubation with the corresponding receptor antagonist. Data are mean ± SE of at least 3 experiments.

proliferating cholangiocytes, secretin strongly stimulates the intracellular cAMP synthesis and PKA activity, resulting in enhanced bicarbonate secretion in bile, which is absent in vivo in normal rats.[9,12,16] The chronic administration of the serotonin 1A or 1B receptor agonists strongly blunted the proliferative response of rat cholangiocytes to BDL. In parallel, we found, in animals treated with the serotonin receptor agonists, a strong reduction of the functional activity, eg, diminished secretin-induced bile flow, bicarbonate secretion, and cAMP synthesis, associated also with reduced PKA activity. Such effects were due to the specific interaction of the serotonin agonists with their receptors, because they were reproducible in vitro in pure cholangiocytes and because the effects were prevented by the preincubation with the specific receptor antagonists. These results show that serotonin plays a relevant role in limiting the excessive cholangiocyte proliferation and choleresis in the course of chronic cholestasis, in the same fashion as other neuroendocrine hormones. In fact, in accordance with the current

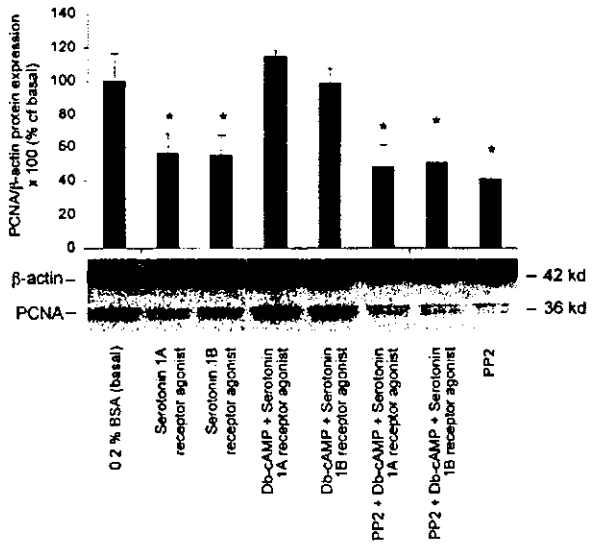


Figure 6. Changes in cholangiocyte in vitro proliferation evaluated by immunoblots for the PCNA protein expression. Overloading the cell with the PKA stimulator dybutyryl-cAMP (Db-cAMP) abolishes the inhibitory effect of the serotonin 1A and 1B agonists. However, the presence of the Src inhibitor PP2 prevented the antagonizing effect of Db-cAMP. $*P < .03$ vs. the other groups. Data are mean ± SE of at least 4 experiments.

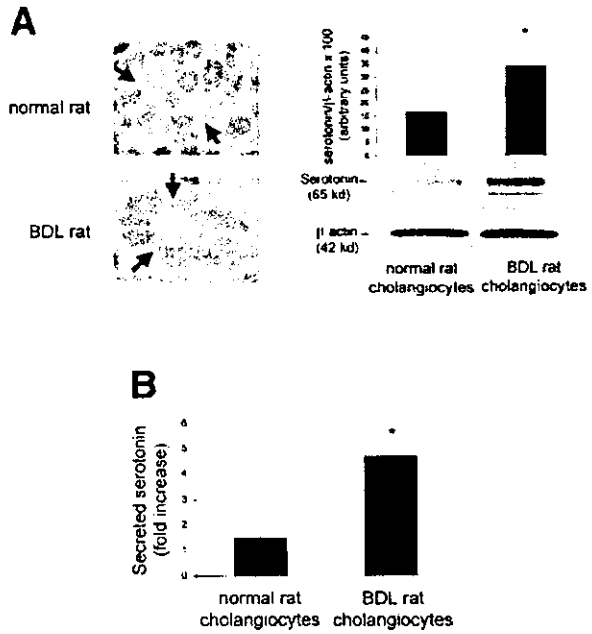


Figure 8. Proliferating cholangiocytes overexpress and oversecrete serotonin. (*A*) Serotonin expression evaluated by immunohistochemistry in liver sections (*left*, *arrows*) (original magnification, 625×) and by immunoblots in purified cholangiocytes (*right*) ($*P < .05$ vs. normal rat cholangiocytes) was markedly enhanced in proliferating BDL rat cholangiocytes compared with cells from normal rats. (*B*) BDL rat cholangiocyte secretion of serotonin in the culture medium was markedly higher than in the normal rat cholangiocyte one; data are expressed as -fold increase after in vitro incubation. $*P < .05$ vs. normal rat cholangiocytes; data are mean ± SE of at least 3 experiments.

data, previous studies showed that gastrin inhibits both the growth and functional activity of the biliary tree in the BDL rat.[10,12] Similarly, both somatostatin and insulin decrease the response to secretin of cholangiocytes from BDL rats.[16,51] Our observation that serotonin in-

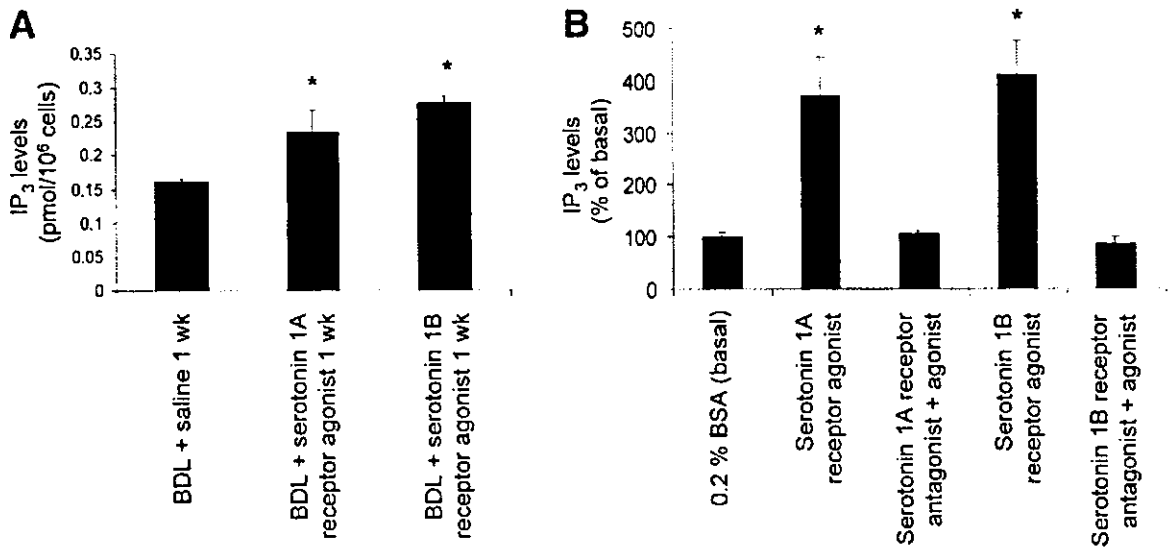


Figure 7. The serotonin 1A and 1B receptor agonists increase IP_3. In vivo, administration of the serotonin 1A and 1B agonists enhanced the intracellular IP_3 levels (*A*) ($*P < .01$ vs. BDL). These changes were abolished by incubation with the corresponding receptor antagonist (*B*) ($*P < .01$ vs. the other groups). Data are mean ± SE of at least 6 experiments.

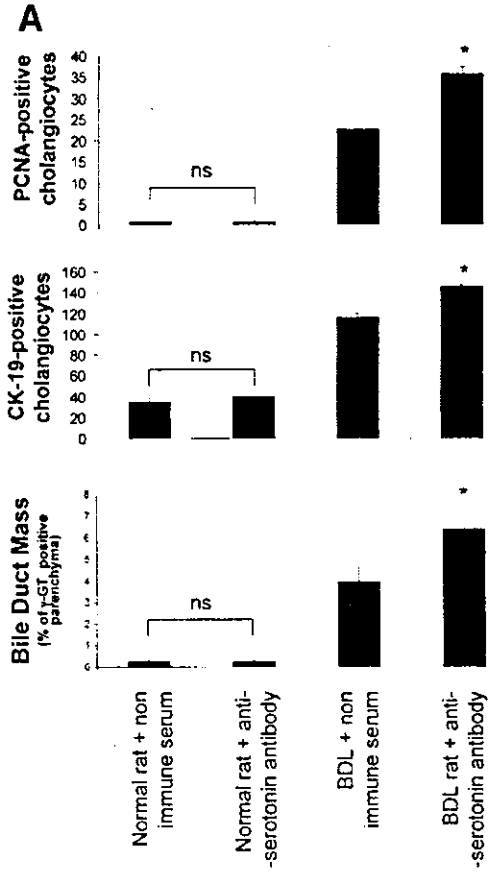
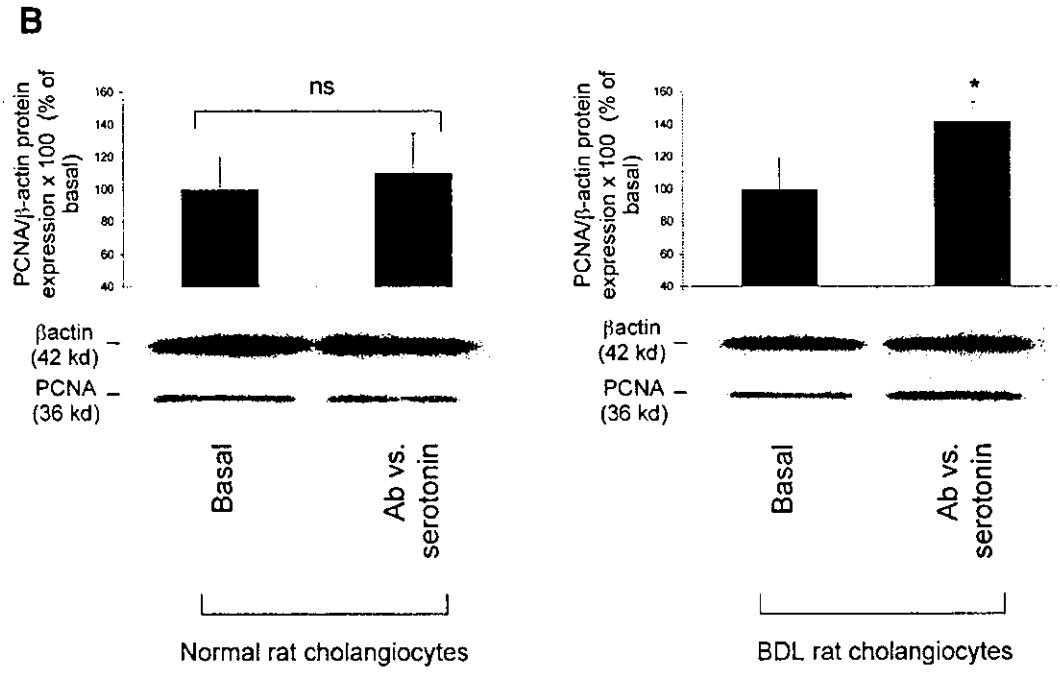


Figure 9. Effect of the serotonin-neutralizing antibody on cholangiocyte proliferation. Quantitative immunohistochemistry for PCNA (*A*) (*top*; $*P < .01$ vs. BDL plus nonimmune serum) and CK-19 (*A*, *middle*; $*P < .01$ vs. BDL plus nonimmune serum) and measurement of the bile duct mass (*A*, *botton*); $*P < .03$ vs. BDL plus nonimmune serum) showed that administration of the serotonin-neutralizing antibody further enhances the growth of the biliary tree in the BDL but not in the normal rat. Similarly, immunoblots for PCNA protein (*B*) showed that in vitro exposure to the serotonin-neutralizing antibody stimulates the proliferation of cholangiocytes isolated from BDL rats but not the proliferation of those purified from normal rats ($*P < .05$ vs. basal; data are mean ± SE of at least 3 experiments).



hibits the growth of the biliary tree is supported by other studies showing that serotonin inhibits the proliferation of other cells.[23-25] To date, only 1 study has dealt with the role of serotonin on the pathophysiology of the biliary tree. In 1985, Kortz et al[52] showed that serotonin abolished the fasting-associated bile flow without affecting canalicular events and, thus, presumably acting directly on the biliary epithelium. Therefore, our current results not only are in agreement with what was previously observed, but also provide the novel information that serotonin strongly limits cholangiocyte proliferation in the course of chronic cholestasis through specific interaction with the 1A and 1B receptor subtypes.

The detailed intracellular regulation of cholangiocyte proliferation is still largely unknown. A recent study has shown that a major role might be played, as much as in other cell types, by the activation of the Src/ERK1/2 pathway.[13] This signaling cascade is typically activated by factors involved in cellular growth[13] and plays a pivotal role in transducing the signal to cytosolic and nuclear targets.[53] Therefore, we aimed to study whether the inhibitory effects of serotoninergic agonists on cholangiocyte proliferation were associated with parallel changes in the activation of the Src/ERK1/2 pathway. It is interesting to note that we found that both in vivo and in vitro Src was inactivated by the serotonin 1A and 1B agonists. Indeed, the presence of either one or the other agonist induced dephosphorylation at Tyr 139 (one of the sites the phosphorylation of which have been recognized as an activator of Src enzyme activity)[40] and phosphorylation at Tyr 530 (an event that blocks Src enzyme activity).[41] In the same fashion, ERK1/2 phosphorylation was markedly reduced by the serotonin 1A and 1B receptor agonists. These changes were prevented, in vitro, by preincubation with the specific 1A and 1B receptor antagonists, once more showing the dependency on the agonist/receptor interaction of the observed modifications. As a confirmation of the leading role played by the Src/ERK1/2 pathway in governing the growth of the biliary epithelium, in vitro incubation with the Src inhibitor PP2[13] or the MEK inhibitor PD98059[36] inhibited cholangiocyte proliferation. In parallel with proliferative changes, serotoninergic agonists also reduced the activation of the cAMP/PKA pathway, as shown in Figures 3*C–E* and 4*B*. The correlation between the degree of activity of such pathways and cholangiocyte proliferation has been often reported.[6,7,9,12,16] However, because neither a direct mechanistic evidence nor the downstream signaling that allows the cAMP/PKA cascade to affect cholangiocyte proliferation has ever been shown, we performed a series of in vitro studies to address this issue. We found that by overloading the cell with the PKA stimulator dybutyryl-cAMP,[35] the inhibitory effect of the serotoninergic agonists was abolished. This means that neutralizing the serotonin-induced decrease of cAMP synthesis prevents the inhibition of cell proliferation. These data indicate that activation of the serotonin 1A and 1B receptors reduces cholangiocyte proliferation in a cAMP/PKA-dependent manner. Conversely, the neutralizing effect of the PKA stimulator against serotoninergic agonists was eliminated in the presence of the Src inhibitor PP2, thus indicating that Src activity is required for the modulation of cell proliferation by the cAMP/PKA cascade. Together, these data show that the serotoninergic signal is mediated by the cAMP/PKA cascade and, downstream, by the Src/ERK1/2 pathway. Interestingly, it has been previously reported that the cAMP/PKA/Src/ERK1/2 cascade allows neuroendocrine hormones or neurotransmitters to modulate cell growth.[54,55]

In cholangiocytes, the activation of the Ca^{2+}/PKC pathway is commonly associated with reduced proliferation.[10,47] The IP_3/Ca^{2+}/PKC pathway has been described among the second messengers modulated by the serotonin 1A and 1B receptors.[46] Therefore, we tested the hypothesis that the effects of the serotoninergic agonists on cholangiocyte proliferation were also mediated by such a pathway. We found that the activation of the serotonin 1A or 1B receptors enhanced the IP_3 levels. The activation of such a molecule seems to be particularly abundant not only in vitro but also in vivo. Indeed, the increase we observed in the in vivo experiments might be underestimated because of the duration of the cholangiocyte purification procedure, in light of the fact that IP_3 levels are rapidly degraded. Nevertheless, IP_3 is still measurable at the end of procedures lasting several hours, as shown in neurophysiological and neuropathologic studies.[56,57] Thus, even if clearly evident, the increase in IP_3 levels we observed after the in vivo treatment should represent just the undegraded (and thus still detectable) fraction. The blockage of the downstream components of the IP_3 signaling, Ca^{2+} and Ca^{2+}-dependent PKC, abolished the serotonin-induced inhibition of cholangiocyte proliferation. Ca^{2+} and Ca^{2+}-dependent PKC inhibitors also prevented the decrease of the intracellular cAMP synthesis due to the serotonin 1A and 1B agonists (Figure 4*B*), thus suggesting a cross talk between the IP_3/Ca^{2+}/PKC and the cAMP/PKA/Src/ERK1/2 pathways, and the latter is likely the common final path for the modulation of cell growth (Figure 10). The triggering of the same pathways by both serotonin 1A and 1B agonists also explains why an additive effect is not observed when the 2 agonists are administered simultaneously.

The biliary epithelium from human livers affected by cholangiopathies shows neuroendocrine markers not observed in unaffected livers.[17] Because, together with chromogranin A, serotonin is a typical feature of neuroendocrine cells,[58] we evaluated whether cholangiocytes

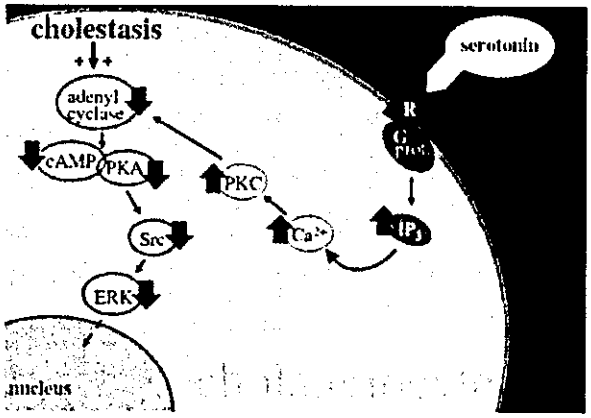


Figure 10. Schematic representation of the intracellular signal mediating the serotoninergic modulation of cholangiocyte growth. Receptor activation enhances IP_3 levels. As a consequence, the Ca^{2+}-dependent activation of PKC inhibits the cAMP/PKA pathway (leading to diminished functional activity), and this in turn is responsible for reduced activation of the Src/ERK1/2 cascade (directly affecting the proliferative processes).

express and, eventually, secrete serotonin. Surprisingly, cholangiocytes isolated from normal rat livers weakly expressed and secreted serotonin, whereas these phenomena were strongly enhanced in cholangiocytes purified from BDL rats. Similar findings were reported in the pancreas, where serotonin-containing cells have been described in the ductal epithelium.[18] Because serotonin reduces the pancreatic secretin- and acerylcholine-induced secretion, it has been postulated that certain stimuli (mechanical obstruction, increased intralumenal pressure, and so on) induce serotonin-containing cells to release this hormone, which then acts in an autocrine/paracrine way on the neighboring cells to diminish intralumenal fluids and pressure.[18] To understand the significance of the serotonin secretion by cholangiocytes, we neutralized, both in vivo and in vitro, the cholangiocyte-secreted serotonin with a specific antibody. If this did not determine major changes in normal rats, the blockage of the endogenous, cholangiocyte-secreted serotonin further enhanced the proliferation of cholangiocytes from BDL rats. Our findings thus indicate that in the course of chronic cholestasis, the immunoneutralization of serotonin is a further stimulus for cholangiocyte proliferation. These data suggest the novel concept that during chronic cholestasis, proliferating cholangiocytes oversecrete serotonin, which acts in an autocrine fashion to limit the excessive growth of the biliary tree. In addition, such observations are consistent with the lack of effects of the activation of the serotonin 1A and 1B receptors on cholangiocytes from normal rats, thus confirming the role of serotonin in antagonizing the excessive proliferation of the biliary epithelium after BDL. The role played in vivo by serotonin secreted in other organs, such as the central nervous system, pancreas, or intestine, cannot be ruled out. However, the neutralization of endogenous serotonin affects cholangiocyte growth only in BDL but not in normal rats (eg, only when cholangiocyte serotonin expression and secretion are enhanced), and those results are reproducible in vitro (eg, when there is no other source of serotonin but cholangiocytes). It can therefore be inferred that the major part of the modulation of the growth of the biliary tree should be ascribed to the serotonin secreted by cholangiocytes themselves.

In summary, we found that cholangiocytes express the serotonin 1A and 1B receptor subtypes, the activation of which markedly limits the growth of the biliary tree and, in parallel, its functional activity in the course of chronic cholestasis. In the cell, these effects are mediated by the enhanced IP_3/Ca^{2+}/PKC signaling that, inhibiting the cAMP/PKA/Src/ERK1/2 cascade, is responsible for the reduced proliferation (Figure 10). We also observed the presence of an autocrine loop that limits the excessive growth of the biliary tree because of cholestasis, a loop based on the release of serotonin by cholangiocytes and the action of serotonin on cholangiocytes themselves.

These findings open novel perspectives for new therapeutic approaches to cholangiopathies. The last stages of cholangiopathies, particularly primary biliary cirrhosis,[2] may indeed benefit from the blockage of serotonin action on cholangiocytes, thus promoting cholangiocyte proliferation and limiting the vanishing of bile ducts. This could be achieved by using drugs such as pindolol, methiothepin, ketanserin, or ritanserin,[59,60] which are known to act as serotonin 1A or 1B receptor antagonists and are already available for therapy for neuropsychiatric disorders. In contrast, enhancing the activation of such receptors with molecules (such as buspirone) acting as receptor agonists[61] or with selective serotonin reuptake inhibitors (which increase the levels of endogenous serotonin)[32] could help to prevent the development of biliary malignancies.

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Translational Regulation of XIAP Expression and Cell Survival During Hypoxia in Human Cholangiocarcinoma

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Background & Aims: Tumor progression is promoted by the ability of tumor cells to resist adverse environmental conditions such as hypoxia. We have shown that translational dysregulation contributes to transformed cell growth in malignant cholangiocytes. Translational regulation of gene expression can contribute to an immediate and rapid response to environmental changes such as hypoxia. Thus, our aims were to assess translational mechanisms involved in cell survival during hypoxia and to identify specific translationally regulated proteins involved in the cellular response to hypoxia. *Methods:* Cell viability and apoptosis in response to hypoxia were assessed in human cholangiocarcinoma cells. Translational processes were deregulated by cycloheximide or rapamycin or by targeted deletion of eukaryotic initiation factor (eIF)-4E, a rate-limiting translational initiation factor using small interfering RNA (siRNA). A protein antibody microarray was used to screen for eIF-4E-dependent proteins expressed during hypoxia. Expression of the X-linked inhibitor of apoptosis (XIAP) was decreased using siRNA. *Results:* Malignant cholangiocytes are resistant to hypoxia-induced apoptosis. Furthermore, cell survival during hypoxia required protein translation. eIF-4E was over expressed in malignant cholangiocytes. Reduction in eIF-4E expression by siRNA decreased tumor cell resistance to hypoxia, increased caspase-3 activation and apoptosis, and decreased cell survival compared with controls. XIAP was identified as a translationally regulated protein expressed during hypoxia. Modulation of XIAP expression by siRNA decreases cell death during hypoxia in vitro and in vivo. *Conclusions:* Human cholangiocarcinoma cells are highly resistant to hypoxia. Translational regulation of survival proteins such as XIAP is a mechanism mediating cholangiocarcinoma survival during hypoxia.

Tumor cells are characterized by aberrant cellular growth responses to environmental changes that normally serve to maintain tissue homeostasis. Tumor progression is promoted by the ability of tumor cells to resist adverse environmental conditions such as hypoxia or nutrient deprivation that cause cell death. Hypoxia commonly occurs in human tumors, and resistance to hypoxia-induced cell death is a characteristic feature of tumor cells.[1,2] Indeed, the ability to survive under hypoxic conditions and to maintain an adequate vascular supply are critical factors for tumor growth.[3] Although chronic hypoxia results in stimulation of angiogenesis and neovascularization, this process is often disorganized and may take several days before adequate tumor tissue oxygenation can be restored.[3] Thus, the ability of tumor cells to survive during transient or acute hypoxia is critical. Hypoxia also has a profound effect on the response to therapy and influences tumor development.[1,4,5] However, the cellular mechanisms by which tumor cells acquire resistance to hypoxia are poorly understood.

We have recently begun to study the cellular response of biliary tract epithelia to hypoxia. The epithelial lining of the biliary tract is highly sensitive to apoptosis under conditions of reduced oxygenation.[6] In contrast, malignant biliary epithelial cells (cholangiocytes) are resistant to hypoxia-induced cell death and therefore suitable for the study of intracellular survival mechanisms during hypoxia. Biliary tract malignancies (cholangiocarcinomas) are typically hypovascular tumors that are associated with a poor prognosis and are highly refractory to conventional therapies.[7,8] Cell survival during hypoxia may involve activation of cell survival pathways and/or aberrant expression of endogenous apoptosis inhibitory proteins.[9–11] Targeted interventions based on an understanding of the mechanisms of response to hypoxia may improve therapeutic approaches for cholangiocarcinoma and other tumors.

We have recently shown that translational dysregulation contributes to transformed cell growth in malignant

Abbreviations used in this paper: AMC, 7-amino-4-methylcoumarin; eIF, eukaryotic initiation factor; IRES, internal ribosome entry sequence; MAPK, mitogen-activated protein kinase; siRNA, small interfering double-stranded RNA; XIAP, X-linked inhibitor of apoptosis.

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cholangiocytes.[12] Translation is the final step in a series of several processes involved in gene expression.[13,14] Thus, regulation of gene expression at the translational level allows for an immediate and rapid response to environmental changes such as hypoxia.[15] Survival of malignant cholangiocytes during hypoxia can be mediated by a rapamycin sensitive pathway.[16] Rapamycin is a selective inhibitor of the mammalian target of rapamycin protein that regulates translation by phosphorylation and activation of several translational regulatory proteins such as the eukaryotic initiation factor (eIF)-4E-binding protein 1 and p70 S6 kinase.[17] These observations suggest that dysregulation of translationally regulated genes may contribute to malignant cholangiocyte survival during hypoxia. Therefore, our aims were to assess the role of translational mechanisms in cell survival during hypoxia and to identify specific translationally regulated proteins involved in the cellular response to hypoxia. Our studies identify a critical role for translational regulation of an endogenous antiapoptotic protein, the X-linked inhibitor of apoptosis protein (XIAP), as a mechanism mediating survival of malignant cholangiocytes during hypoxia.

Materials and Methods

Materials

Fetal bovine serum and Bradford reagent were obtained from Sigma Chemical Co. (St. Louis, MO). CMRL 1066 media, L-glutamine, and antibiotic-antimycotic mix were from Gibco BRL (Grand Island, NY). Monoclonal antibodies to eIF-4E and phospho-specific antibodies to eIF-4E (Ser 209) were obtained from Cell Signaling (Beverly, MA). Antibodies to XIAP, c-IAP-1, c-IAP-2, tubulin, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The protease inhibitor cocktail tablets were obtained from Roche Molecular Biochemicals (Indianapolis, IN). All other reagents were of analytic grade from the usual commercial sources.

Cell Culture

KMCH human malignant cholangiocytes were obtained as previously described and cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum.[18] KMp38dn cells were generated from KMCH cells stably transfected with pRc/RSV-Flag MKK3 (Ala) encoding a dominant interfering upstream activator of p38 mitogen-activated protein kinase (MAPK) with double-point mutations in Ser 189 and Thr 193 replaced by Ala.[12] H69 cells, immortalized human nonmalignant cholangiocytes, were obtained and cultured as previously described.[19] Mz-ChA-1 cells derived from metastatic gall bladder cancer (kindly provided by Dr. J.G. Fitz, University of Colorado, Denver, CO) and TFK-1 cells derived from extrahepatic cholangiocarcinoma were cultured in CMRL 1066 media with 10% fetal bovine serum, 1% L-glutamine, and 1% antimycotic antibiotic mix. Cells were cultured in 35-mm or 96-well culture plates at 37°C in a hypoxia chamber (Billups-Rothenburg, Del Mar, CA) left open in a humidified incubator in 21% O_2, 5% CO_2, balance N_2, (for normoxia studies) or gassed with a preanalyzed gas mixture containing 5% CO_2/95% N_2 and sealed (for hypoxia studies).[16] The partial pressure of oxygen in the culture media under these conditions measured with a gas analyzer (278 System; Ciba-Corning, Medfield, MA) was 140 and 40 mm Hg, respectively, at 24 hours, and there was no significant change in pH.

Viability Assay

Cells were seeded into 96-well plates (10,000 cells/well) and incubated in a final volume of 200 μL medium. Cell viability was assessed using a commercially available tetrazolium bioreduction assay for viable cells (CellTiter 96 Aqueous; Promega, Madison, WI).

Apoptosis Assay

Morphologic changes indicative of cell death by apoptosis were identified and quantitated by fluorescence microscopy and the use of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) as previously described.[18] Fluorescence was visualized using an Olympus BX40 upright fluorescence microscope (Olympus America, Inc., Melville, NY). Apoptotic nuclei were identified by condensed chromatin as well as nuclear fragmentation. At least 300 nuclei in 4 high-power fields were counted.

Caspase-3 Assays

Caspase-3 activity was quantitated in cytosolic extracts as previously described.[20] Briefly, cytosolic extracts were obtained by cell lysis in a hypotonic buffer containing protease inhibitors, followed by homogenization. Caspase activity was then determined fluorometrically using DEVD-7-amino-4-methylcoumarin (AMC), and quantitated using standard curves generated with AMC. Activation of caspase-3 in transfected cells was assessed by an immunocytochemical assay. Cells were harvested and permeabilized and fixed using the Cytofix/Cytoperm kit (BD Biosciences, Palo Alto, CA). The cells were stained with anti-caspase-3 monoclonal antibody (BD Biosciences), washed, and stained with Cy5-anti-IgG (Jackson Immunoresearch Labs). The cells were also stained with the nucleic acid dye SYTO-16 (Molecular Probes, Eugene, OR). Stained cells were suspended in an isobuoyant cell buffer at 2×10^6 cells/mL. Ten microliters of the cell suspension were applied to sample wells of the cell assay chip and assayed on the Agilent 2100 Bioanalyzer microfluidic system (Agilent, Palo Alto, CA). Five hundred to 1000 cell events were collected per sample. Fluorescence emission from the cells was detected with photodiodes at 510–540 nm and 674–696 nm. Cell events in the SYTO-16-positive population were cross gated onto the caspase-3 histogram to determine the percentage of cells undergoing apoptosis.

Immunoblot Analysis

Confluent cells in culture were washed with 1× PBS then lyzed with 0.5 mL lysis buffer containing 62.5 mmol/L Tris base, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 50 mmol/L DTT. Protein samples were separated on 4%–12% gradient polyacrylamide gels (Novex, San Diego, CA) under reducing conditions and electroblotted to positively charged 0.45 μmol/L nitrocellulose membrane (Millipore, Bedford, CA). The membranes were soaked for 5 minutes in transfer buffer (13.4 mmol/L Tris, pH 8.3, 20% methanol, 108 mmol/L glycine). Blots were preblocked in 20 mmol/L Tris, 150 mmol/L NaCl, 0.1% Tween 20, and 5% nonfat dry milk for 3–4 hours or overnight at 4°C. Membranes were incubated overnight at 4°C with the respective primary antibody, used at a 1:1000 dilution. The primary antibodies were diluted in a solution containing 20 mmol/L Tris, 150 mmol/L NaCl, 0.1% Tween 20, and 5% bovine serum albumin. The membrane was washed 3 times for 10 minutes with 20 mmol/L Tris, 150 mmol/L NaCl, and 0.1% Tween 20 (TTBS) and then incubated with the secondary antibody, a polyclonal goat anti-rabbit immunoglobulin-peroxidase conjugate (Zymed, San Francisco, CA), at a 1:2000 dilution for 60 minutes at 4°C. The secondary antibody was diluted in TTBS buffer. For all immunoblots, membranes were washed 3 times for 10 minutes with TTBS then visualized using an enhanced chemiluminescence kit (LumiGLO, Cell Signaling, Beverly, MA) following the manufacturer's directions. The relative protein expression was determined by densitometry using a CCD camera-based image analyzer (ChemiImager 4000; Alpha Innotech, San Leandro, CA).

Small Interfering RNA Design, Synthesis, and Transfection

RNA interference for gene silencing was performed in KMCH cells using small interfering 21-nucleotide dsRNA (siRNA) molecules as previously described.[12] siRNA were designed and synthesized using the Silencer siRNA construction kit (Ambion, Austin, TX). Transfection efficiency was 35%–40% in KMCH cells using Trans-IT TKO (Mirus, Madison, WI) as previously described.[21] Inhibition of target protein expression after transfection of cells was verified by immunoblot analysis. The messenger RNA (mRNA) target sequence of siRNA to eIF-4E was 5′-, AAGGATGGTATTGAGCCTATG, and the mRNA target of the scrambled nucleotide control was 5′-AAGTGCTAGATTGAGTGCTAG. The mRNA target sequence of siRNA to XIAP was 5′-AACTTGCTAACTCTCTTGGGG, and the mRNA target of the corresponding control siRNA was 5′-AATGCGTACTTCGCATCGTTG.

Cell Cycle Analysis

Cell cycle analysis was performed as previously described.[22] Cells were collected and suspended in 1× PBS at a concentration of 2×10^6 cells/mL. The samples were centrifuged at 400*g* for 3 minutes and then resuspended in a propidium iodide solution containing 0.1 mol/L propidium iodide, 0.1% vol/vol Triton X-100, and 20% RNAse A, in 1X PBS. After incubation on ice in the dark for 2 hours, the samples were analyzed by flow cytometry. Ten thousand events were recorded, and the proportion of cells in various phases of the cell cycle were analyzed using the ModFitLT DNA analysis program (Becton Dickinson, San Jose, CA).

Protein Antibody Microarray

Cells were transfected with siRNA to eIF-4E or a scrambled nucleotide control as previously described.[12] After 48 hours, the media was replaced with serum-free media for 6 hours. Cells were then incubated under hypoxic or normoxic conditions. After 24 hours, cellular protein was extracted, and the relative differences in protein expression were assessed using the antibody microarray (BD Biosciences Clontech, Palo Alto, CA) as per the manufacturer's instructions. Image and data acquisition from the antibody microarray slides was performed using an Axon GenePix 4000A laser scanner and the GenePix 4.1 software package (Axon Instruments, Foster City, CA). Internal normalization was performed following the manufacturer's protocol. A greater than 1.5-fold difference in relative protein expression was considered significant.

RT-PCR Analysis

Total cellular RNA was extracted from cells using the Ultraspec RNA isolation reagent (Biotecx Laboratories, Inc., Houston, TX) and semiquantitative RT-PCR performed as previously described.[22] In brief, cDNA was prepared from 2 to 10 μg total RNA using Moloney murine leukemia virus (MMLV) reverse transcriptase and random oligonucleotide primers. PCR was then performed using a DNA thermal cycler and a reaction mixture containing 2 μL cDNA and using the *taq* PCR core kit (Qiagen Inc., Valencia, CA). The reaction mixture was incubated at 95°C for 2 minutes, followed by 37, 3-step cycles (94°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute) and a final step at 72°C for 10 minutes. For GAPDH, 25 cycles were used. The primers used were as follows: human XIAP 5′-GGCCATCTGAGACACATGCAG-3′ (sense) and 5′-GCATTCACTAGATCTGCAACC-3′ (antisense); GAPDH 5′-TGCCAGTGAGCTTCC-3′ (sense) and 5′-CACCATGGAGAAGGC-3′ (antisense). The products were analyzed using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA), and gene expression of XIAP was normalized against GAPDH.

Cholangiocarcinoma Xenograft Model

Male athymic nu/nu mice, 8 weeks of age, were obtained from Charles River Laboratories (Wilmington, MA) and fed food and water ad libitum. The mice were housed 4 per cage, and fluorescent light was controlled to provide alternate light and dark cycles of 12 hours each. The animals received a subcutaneous injection of Mz-ChA-1 cells (3×10^6 viable cells suspended on 0.5 mL extracellular matrix gel) on their right flank. After 12 weeks, mice ($n = 2$ each) were randomly assigned to receive 20 μg per gram body weight of siRNA to

XIAP or scrambled control sequence siRNA in 50 μL PBS intratumorally, which was repeated after 3 days. Three days later, 60 mg/kg Hypoxyprobe-1 (Pimonidazole Hydrochloride; Chemicon, Temecula, CA) was injected intravenously, and tumors were excised for histologic examination after 2 hours. Sections were obtained, and immunohistochemistry was performed using monoclonal antibody to XIAP to identify XIAP expression or using Hypoxyprobe-1 monoclonal antibody to identify pimonidazole adducts as a marker of hypoxia. TUNEL staining was performed in adjacent sections to identify apoptotic cells using a commercially available kit (Wako Chemicals, Tokyo, Japan). The number of TUNEL positive cells were quantitated and expressed as average ± standard deviation of the number of positive cells in 5 high-power fields. The extent of hypoxia was determined by immunostaining for pimonidazole adducts,[23] and the amount of chromogen quantitated by digital image analysis using Adobe Photoshop.[24]

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD) from at least 3 separate experiments, unless otherwise noted. The differences between groups was analyzed using a double-sided Student *t* test when only 2 groups were present. Statistical significance was considered as $P < .05$. Statistical analyses were performed with the GB-STAT statistical software program (Dynamic Microsystems Inc., Silver Spring, MD).

Results

Survival of Malignant Cholangiocytes During Hypoxia Is Translationally Regulated

Initially, we examined the effect of hypoxia on cell viability in KMCH, TFK-1, and MzChA-1 malignant human cholangiocytes or on H69 nonmalignant human cholangiocytes. Similar to results observed in other tumor cell types, cell viability assessed using a tetrazolium bioreduction assay was not decreased in any of the malignant cells during hypoxia (Figure 1*A*). Next, we assessed the effect of hypoxia on apoptosis. All 3 malignant cholangiocytes were resistant to hypoxia-induced apoptosis (Figure 1*B*). In contrast, hypoxia decreased cell viability and increased apoptosis in H69 nonmalignant human cholangiocytes. These data suggest that survival of malignant cholangiocytes during hypoxia results from effects on apoptosis. Next, we assessed the effect of inhibitors of RNA and protein synthesis on cell survival during hypoxia (Figure 2*A*). Preincubation with the RNA synthesis inhibitor actinomycin D (10 μmol/L) did not alter resistance to hypoxia. However, preincubation with the protein synthesis inhibitors cycloheximide (10 μg/mL) or rapamycin (2 μg/mL) increased cell death during hypoxia. Cell viability was decreased during hypoxia to 73% of normoxia controls with rapamycin and to 76% of normoxia controls with cycloheximide. Furthermore, activity of caspase-3, an effector molecule in cellular apoptosis, was increased in cells preincubated with cycloheximide or rapamycin, but not with actinomycin D (Figure 2*B*). In combination, these studies indicate that tumor cell resistance to apoptosis and survival during hypoxia involve a translationally regulated response to hypoxia.

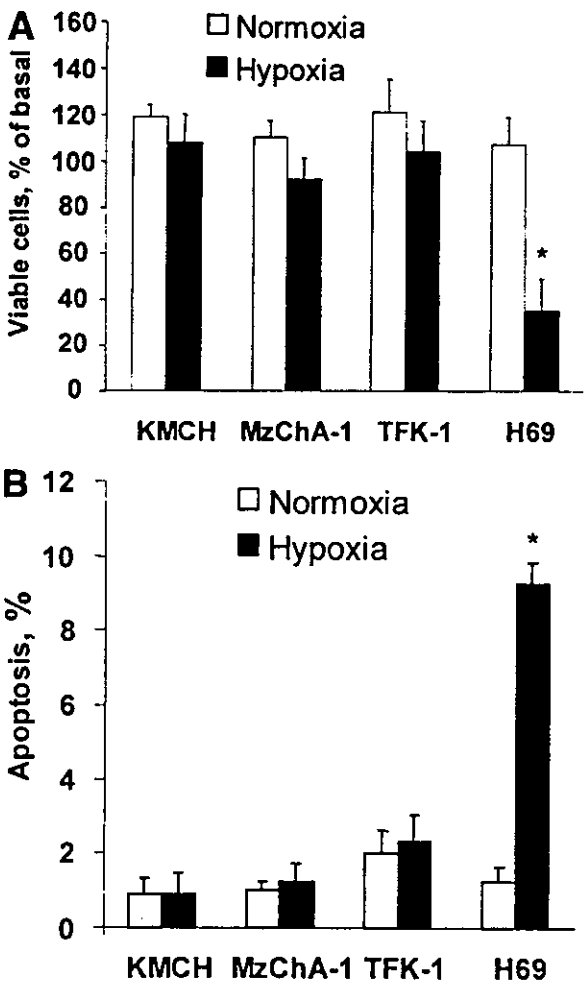

Figure 1. Malignant human cholangiocytes survive during hypoxia. Malignant cholangiocytes (KMCH, Mz-ChA-1 or TFK-1) or nonmalignant cholangiocytes (H69) were cultured under normoxic (21% O_2, 5% CO_2, balance N_2) or hypoxic conditions (95% N_2, 5% CO_2) for 24 hours at 37°C. (*A*) Cell viability was assessed using a tetrazolium bioreduction viable cell assay. In contrast to the nonmalignant H69 cells, the malignant cholangiocyte cell lines were resistant to hypoxia. Results represent the mean ± standard error of 3 studies. (*B*) Apoptosis was quantitated by fluorescence microscopy of cells as described in the Materials and Methods section. Malignant cholangiocytes were resistant to hypoxia-induced apoptosis. Results represent the mean ± standard error of 4 studies. *$P < .05$ compared with normoxia.

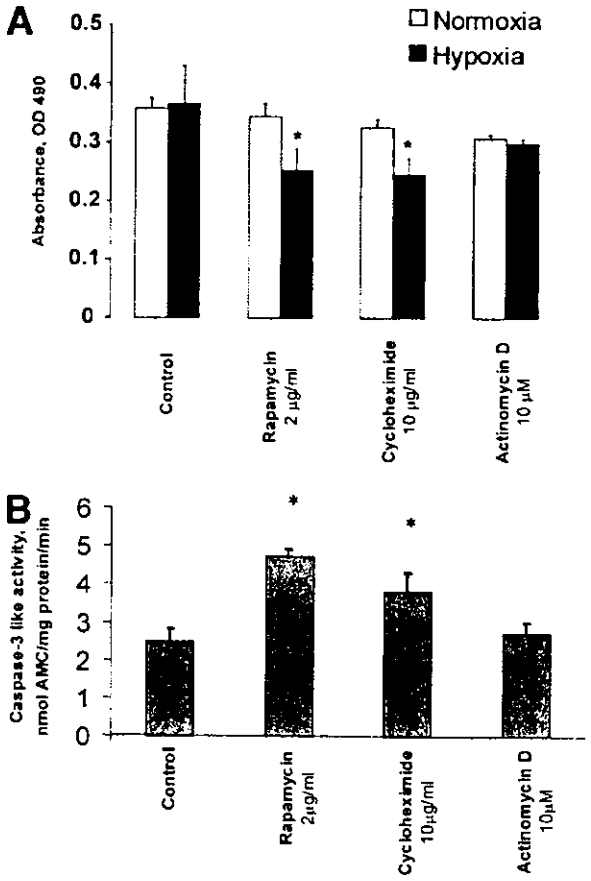

Figure 2. Cell survival during hypoxia requires protein translation. KMCH cells (10^5/mL) were plated in 6-well plates. Cells were pretreated with rapamycin 2 μg/mL, cycloheximide 10 μg/mL, or actinomycin D, 10 μmol/L for 30 minutes prior to culture under normoxic (21% O_2, 5% CO_2, balance N_2) or hypoxic conditions (95% N_2, 5% CO_2) at 37°C. (*A*) Cell viability was assessed after 24 hours. Results are expressed as absorbance readings at 490 nm as a direct measurement of metabolically active, viable cells and represent mean ± standard error of 3 studies. Preincubation with the protein synthesis inhibitors decreased cell viability during hypoxia. *$P < .05$ compared with normoxia controls. (*B*) Caspase-3–like activity in cells incubated under hypoxic conditions for 24 hours. There was no significant difference in caspase-3-like activity between the groups in cells incubated under normoxic conditions. However, an increase in caspase-3-like activity is observed during hypoxia in cells preincubated with protein synthesis inhibitors. *$P < .05$ compared with untreated controls.

Expression of eIF-4E Is Increased in Malignant Cholangiocytes

Having shown a requirement for new protein synthesis for cell survival during hypoxia, we examined the effect of hypoxia on the regulation of initiation of protein translation. The translation factor eIF-4E is rate limiting for the initiation of protein translation and is an important regulator of mRNA translation and protein synthesis. eIF-4E binds to the cap structure at the 5′-end of mRNA of eukaryotic mRNA as a component of the cap-binding complex eIF-4F. The eIF-4F complex mediates the recruitment of ribosomes to mRNA, a rate-limiting step for translation to occur. Indeed, eIF-4E is up-regulated in human cholangiocarcinoma and in the malignant cholangiocyte cell lines used in our study (Figure 3).[25] Furthermore, eIF-4E expression was not altered, but phosphorylation was increased during hypoxia (data not shown). We have shown that p38 MAPK signaling contributes to translational regulation of growth in malignant cholangiocytes.[12] Because p38 MAPK-signaling pathways can phosphorylate eIF-4E, we investigated the role of this pathway on eIF-4E phosphorylation during hypoxia. However, eIF-4E phosphorylation during hypoxia was not altered in KM-p38dn cells, which are derived from KMCH cells and have a functional defect in p38 MAPK activation. Thus, hypoxia increases eIF-4E phosphorylation by a p38 MAPK-independent pathway.

Resistance to Apoptosis During Hypoxia Is eIF-4E Dependent

To assess directly the role of eIF-4E and translationally regulated mechanisms in the cellular response to hypoxia, we used siRNA to functionally decrease eIF-4E expression as described.[12] siRNA to eIF-4E significantly decreased tumor cell resistance to hypoxia-induced cell death (Figure 4). Furthermore, siRNA to eIF-4E increased caspase-3 activation and apoptosis during hypoxia compared with scrambled

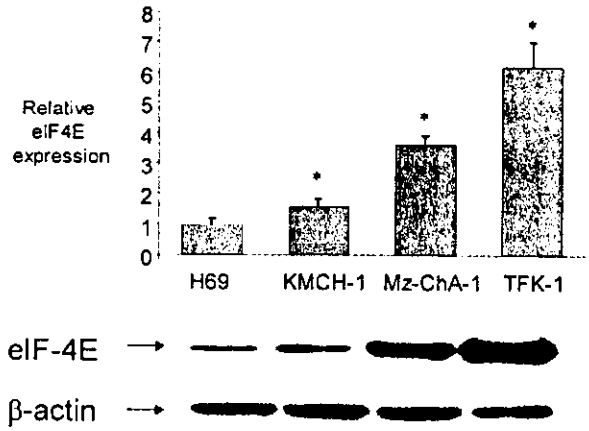

Figure 3. Immunoblot analysis of eIF4E expression in human cholangiocytes. eIF-4E expression was assessed in H69; nonmalignant human cholangiocytes; or in KMCH, Mz-ChA-1, or TFK-1 malignant human cholangiocytes. A representative *immunoblot* is illustrated below, and the mean ± standard deviation of expression relative to H69 nonmalignant cholangiocytes of 3 separate experiments is presented in the *graph* above. *$P < .05$ compared with H69 cells.

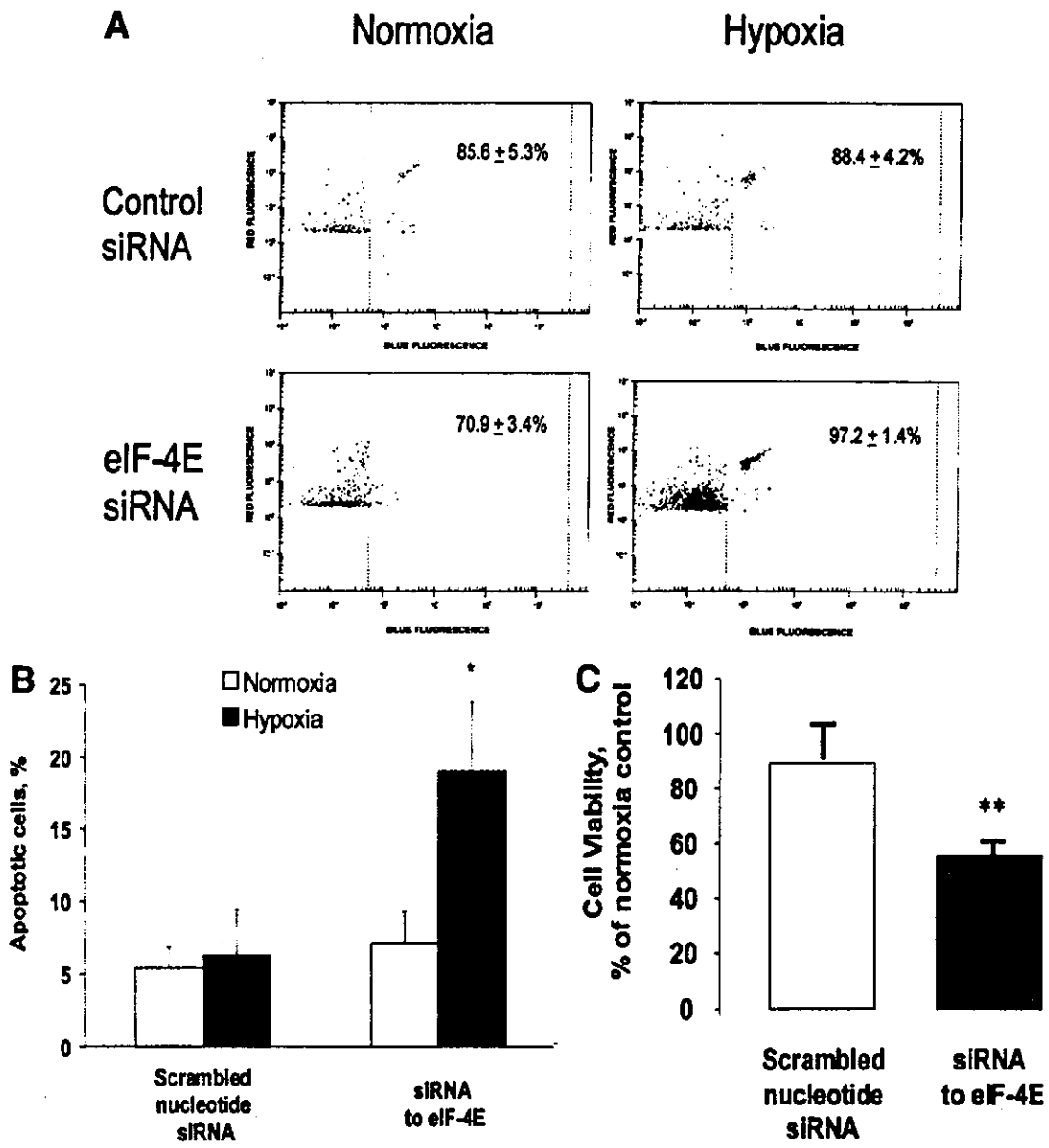

Figure 4. Caspase-3 activation and cell survival during hypoxia are eIF-4E dependent. KMCH cells were transiently transfected with siRNA to eIF-4E or a scrambled nucleotide siRNA for 48 hours. The media was changed, and cells were cultured under hypoxic or normoxic conditions for 24 hours. (*A*) Cells were fixed and permeabilized and then stained with antiactive caspase 3 monoclonal antibody/Cy5-anti-IgG and with the nucleic acid dye SYTO-16. Cells were assayed for activated caspase-3 using the Agilent 2100 Bioanalyzer microfluidic system. Cell events in the SYTO-16-positive population (*blue channel*) were cross gated onto the caspase-3 histogram (*red channel*) to determine the percentage of apoptotic cells. Cells transfected with siRNA to eIF-4E showed increased caspase-3 activation during hypoxia compared with controls. The mean and standard deviation of 4 separate determinations are shown. (*B*) Apoptosis was assessed by fluorescence microscopy after staining cells with DAPI. The number of cells showing morphologic features of apoptosis was quantitated in at least 300 cells in 4 or more high-power fields. The results represent mean ± standard error of 4 studies. $*P < .05$ compared with normoxia control. (*C*) Cell viability was assessed using a viable cell assay. Incubation with siRNA to eIF-4E decreased viability during hypoxia compared with controls. Results are expressed as cell viability during hypoxia as a percentage of viability in normoxia controls and represent mean ± standard error of 3 studies. $**P < .05$ compared with scrambled nucleotide siRNA control.

Table 1. Hypoxia Delays Cell Cycle Progression

		G0–G1	S	G2-M
Untransfected cells	Normoxia	72.2 ± 5.6	17.6 ± 2.9	10.2 ± 3.2
	Hypoxia	86.1 ± 0.7	11.5 ± 0.5	2.4 ± 0.2
Control siRNA	Normoxia	74.4 ± 1.3	19.0 ± 1.2	6.7 ± 0.6
	Hypoxia	80.4 ± 0.3	18.4 ± 1.0	1.2 ± 1.1
SIRNA to eIF-4E	Normoxia	73.1 ± 0.5	20.4 ± 0.8	6.5 ± 0.4
	Hypoxia	80.6 ± 0.3	18.7 ± 1.2	0.65 ± 0.9

NOTE. Cell cycle progression following serum stimulation was assessed in untransfected KMCH cells or cells transfected with either siRNA to eIF-4E or scrambled nucleotide siRNA control. Cells were incubated under normoxic or hypoxic conditions for 24 hours, and the cell cycle profile was determined by flow cytometry after staining with propidium iodide. Cells in G0–G1, S, and G2-M phases of the cell cycle are expressed as percentages of the total cell population. The figures represent average ± standard deviation from 3 experiments.

nucleotide controls. Thus, resistance to apoptosis in malignant human cholangiocytes is mediated by eIF-4E-dependent translation.

Cell Cycle Progression During Hypoxia

Because growth arrest is an important response to hypoxia, we next determined whether translational mechanisms were involved in mitogenic regulation of cell cycle progression during hypoxia. Hypoxia decreased cell cycle progression in KMCH cells with a failure to progress to S-phase and an increase in the proportion of cells in the G0-G1 phase. Serum-stimulation of KMCH cells increased the percentage of cells in S-phase from 10.7% ± 0.8% under basal, serum-starved conditions to 17.2% ± 0.5% after 24 hours. However, siRNA to eIF-4E did not significantly alter cell cycle progression following serum stimulation under normoxic or hypoxic conditions (Table 1). Thus, delayed cell cycle progression during hypoxia is not modulated by the translation initiation factor eIF-4E.

Hypoxia Increases XIAP Expression

To assess potential eIF-4E-regulated proteins involved in protection from cell death during hypoxia, we used a protein antibody microarray to screen for protein expression during hypoxia in KMCH cells transfected with siRNA to eIF-4E or a scrambled nucleotide siRNA. The protein antibody screen identified an increase in protein expression of XIAP during hypoxia. XIAP is an endogenous antiapoptotic protein and a member of an evolutionarily conserved family of genes involved in the regulation of apoptosis that have been linked to cancer.[26] The results of the antibody screen were confirmed by immunoblotting in cytoplasmic extracts from cells incubated under hypoxic or normoxic conditions. XIAP expression is increased in

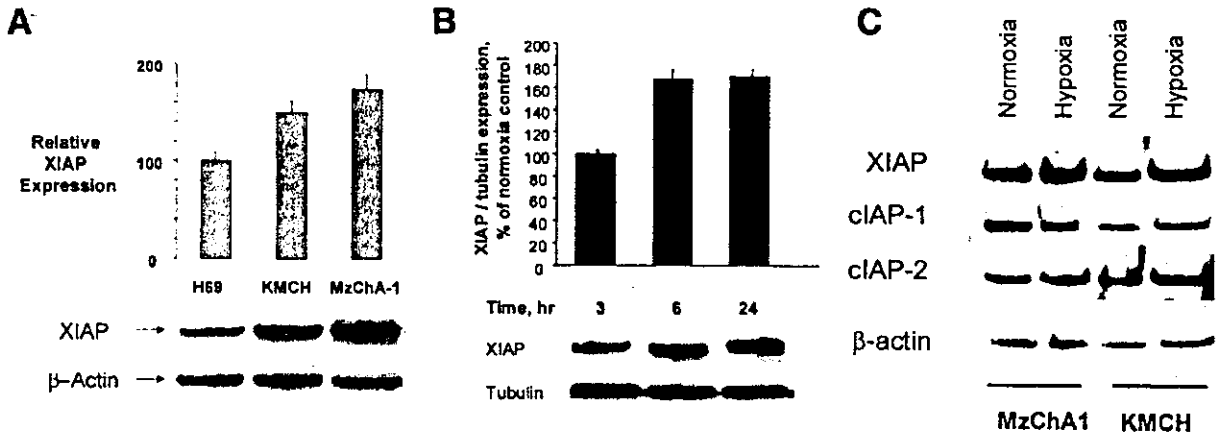

Figure 5. XIAP protein expression is increased during hypoxia. (*A*) XIAP expression was assessed in H69 (nonmalignant) and KMCH and Mz-ChA-1 (malignant) cholangiocytes. A representative *immunoblot* is illustrated, and the *graph* represents the mean ± standard deviation of relative expression from 3 separate determinations. (*B*) KMCH cells were exposed to varying periods of hypoxia. At the indicated times, samples were obtained for immunoblot analysis for XIAP. (*C*) The expression of inhibitor of apoptosis proteins XIAP, cIAP-1, and cIAP-2 was assessed in KMCH and Mz-ChA-1 human malignant cholangiocytes incubated under normoxic (N) and hypoxic conditions (H) for 24 hours. A representative immunoblot of 3 separate studies is shown. Hypoxia increased the expression of XIAP but did not alter expression of either cIAP-1 or cIAP2.

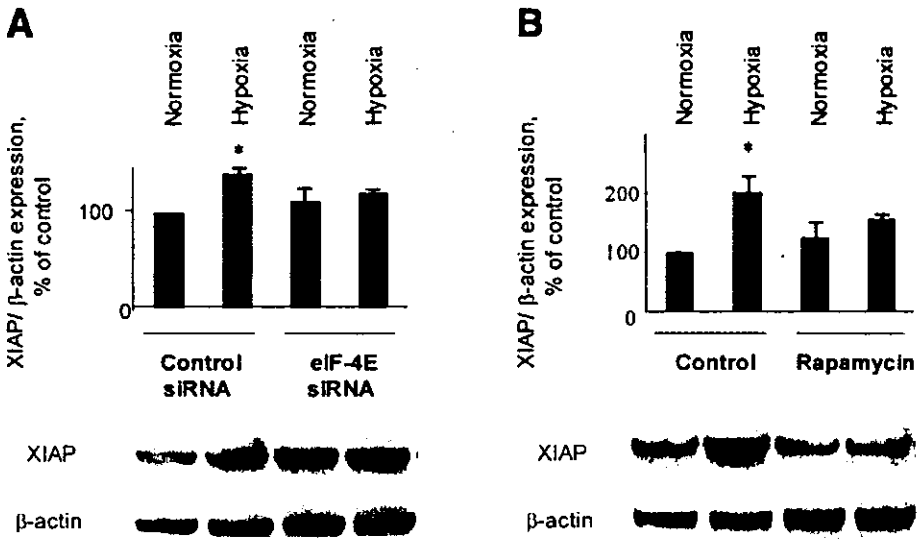

Figure 6. Translational dysregulation decreases XIAP expression during hypoxia. The expression of XIAP was assessed in KMCH malignant cholangiocytes incubated under normoxic or hypoxic conditions for 24 hours. (*A*) XIAP expression is inhibited in cells transfected with siRNA to eIF-4E compared with controls transfected with a scrambled nucleotide control. (*B*) Pretreatment with the translational inhibitor rapamycin inhibited the increase in XIAP compared with diluent (DMSO) controls. A representative *Immunoblot* is shown, and quantitative data from 3 separate studies are depicted in the graph. $^*P < .01$ compared with control.

malignant human cholangiocytes (Figure 5*A*). During hypoxia, an increase in XIAP expression occurred by 6 hours (Figure 5*B*). XIAP protein expression was increased by 252% ± 27% of normoxia controls in KMCH cells and to 192% ± 33% of normoxia controls in Mz-ChA-1 cells after 24 hours of hypoxia. Protein expression of other apoptosis inhibitor proteins cIAP-1 or cIAP-2 was not increased by hypoxia in either KMCH or Mz-ChA-1 malignant human cholangiocytes (Figure 5*C*). We also assessed XIAP mRNA expression by RT-PCR. In contrast to the increased XIAP protein expression, XIAP mRNA expression was not significantly altered during hypoxia and was 114% ± 7% and 74% ± 4% of normoxia controls after 6 and 24 hours of hypoxia, respectively. Furthermore, XIAP expression during hypoxia was decreased in cells transfected with siRNA to eIF-4E or in cells preincubated with rapamycin compared with the relevant controls (Figure 6). XIAP expression can be translationally regulated, and our studies collectively demonstrate translational regulation of XIAP during hypoxia.

XIAP Mediates Cell Survival During Hypoxia

To assess the role of XIAP in mediating resistance to hypoxia, we used siRNA to decrease cellular XIAP expression. siRNA to XIAP decreased cell viability during hypoxia compared with a scrambled nucleotide control (Figure 7). XIAP has been previously shown to mediate resistance to radiation induced cell death.[27] The reduction in cell viability was similar to that observed during incubation with the translational inhibitors cycloheximide or rapamycin but lower that that observed with siRNA to eIF-4E. It is unlikely that XIAP is the only protective factor, given that its inhibition leads to a rather modest sensitization to death, and these data support the presence of additional eIF-4E-dependent factors that are involved in mediating resistance to hypoxia.

The effect of XIAP on tumor cell survival during hypoxia in vivo was assessed in a tumor cell xenograft model in nude mice. Tumors were excised for histologic examination of XIAP, apoptosis, and tissue hypoxia after intratumoral injections of siRNA to XIAP or scrambled nucleotide control siRNA. Illustrative sections are shown in Figure 8. In regions at which the extent of tissue hypoxia was similar, as evidenced by quantitative pimonidazole adduct binding, there was an increase in the number of TUNEL-positive apoptotic cells and a decrease in XIAP expression in tumors that had received siRNA to XIAP compared with those receiving control siRNA (Figure 8). In combination, these results confirm a role for XIAP as an effector of cellular resistance to hypoxia in malignant cholangiocytes in vivo and in vitro.

Discussion

Hypoxia is a critical stress for mammalian cells that can result in cell death. Hypoxia commonly occurs in solid human tumors and is associated with a poor prognosis. Indeed, the ability of tumor cells to adapt to the hypoxic microenvironment is a critical stage in malignant progression. Tumor cell adaptations to hypoxia include alterations in cellular metabolism, neovascularization, and ability for the cells to survive in hypoxia. Thus, resistance to hypoxia-induced cell death is a characteristic feature of tumor cells. Understanding tumor cell responses to hypoxia is therefore highly germane to cancer biology. In these studies, we have identified a cellular mechanism contributing to the survival of human malignant cholangiocytes to hypoxia. The principal findings are that human malignant cholangiocytes respond to hypoxia by altered translation of the endogenous apoptosis inhibitor XIAP. Dysregulation of protein synthesis and translation by the inhibitors cycloheximide and rapamycin or by targeted deletion of the rate-limiting translational initiation factor eIF-4E decreases XIAP expression and tumor cell resistance to apoptosis during hypoxia. These observations define a mechanism of cell survival involving translational regulation of proteins such as XIAP during otherwise detrimental hypoxic conditions.

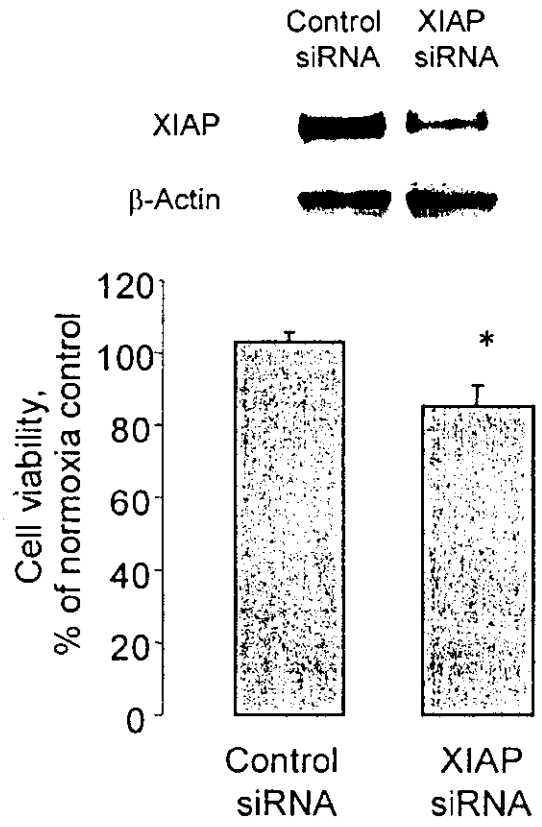

Figure 7. siRNA to XIAP increases cell death during hypoxia. KMCH cells were transfected with siRNA to XIAP or a scrambled nucleotide (control) siRNA for 48 hours. XIAP expression following transfection was assessed by immunoblot analysis. The media was changed, and cells were then cultured under hypoxic or normoxic conditions for 24 hours. Cell viability was then assessed using a viable cell assay. Incubation with siRNA to XIAP decreased viability during hypoxia compared with controls. Results represent mean ± standard error of 3 separate studies. *$P < .05$ compared with scrambled nucleotide control.

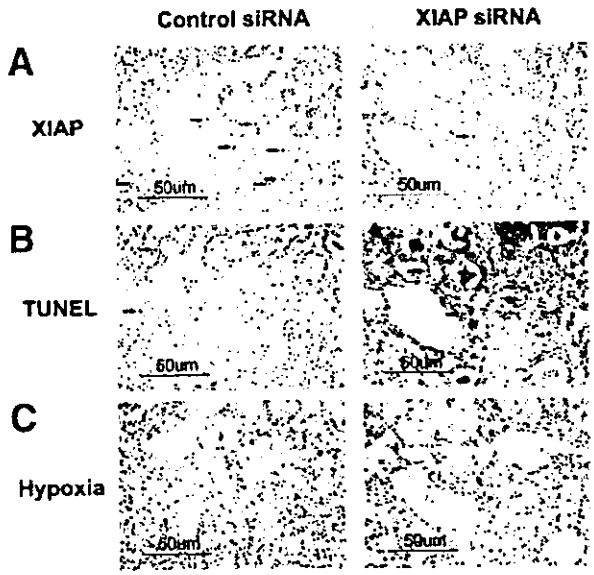

Figure 8. siRNA to XIAP decreases apoptosis in vivo. siRNA to XIAP or a scrambled nucleotide control siRNA were injected into human malignant cholangiocyte xenografts in nude mice as described in the Materials and Methods section, and sections were obtained for analysis of XIAP expression, apoptosis, and hypoxia. Representative adjacent sections are shown following (*A*) immunohistochemistry for XIAP expression, (*B*) TUNEL staining for apoptotic cells, and (*C*) pimonidazole adduct immunostaining for hypoxia. The *arrows* show XIAP or TUNEL positive staining cells, and the percentage of total area with pimonidazole binding is reported for the *lowermost* panels. In tumors that had received siRNA to XIAP, there were 20.6 ± 3.2 TUNEL-positive cells per high-power field compared with 7.6 ± 3.6 TUNEL-positive cells per high-power field in tumors that had received control siRNA (n = 5 fields). *Bars* represent 50 μm.

The cellular response to hypoxia can involve transcriptional or posttranscriptional mechanisms. Although transcriptional regulation of gene expression during hypoxia has been well characterized, the role of translational mechanisms remains poorly understood. Increasing evidence implicates the translational initiation factor eIF-4E in promoting tumor growth.[28–33] Expression of eIF-4E is increased several-fold in biliary tract as well as several other malignancies, and modulation of eIF-4E decreases tumor cell growth.[25,34] Increased eIF-4E ex-

pression may result in increased efficiency of translation of genes such as XIAP that have a long 5′ untranslated region, which makes cap-dependent translation inefficient. Our observations demonstrating a role for translational regulation of XIAP in the survival of human malignant cholangiocytes during hypoxia are thus consistent with a central role of altered eIF-4E expression in promoting tumor progression through diverse mechanisms.

These studies provide a rationale for additional studies to evaluate the use of translational inhibitors such as rapamycin for the treatment of human cholangiocarcinoma. Rapamycin is a selective inhibitor of FKBP/rapamycin-associated protein (FRAP) or the mammalian target of rapamycin (mTOR), which phosphorylates and activates the translational regulatory protein eIF-4E-binding protein 1 (4E-BP1) and thereby disrupts its inhibitory interaction with eIF-4E. The critical role of FRAP/mTOR in the regulation of translation suggests that specific inhibitors of FRAP/mTOR such as rapamycin, or its analogue CCI-779, which is now in clinical trials, can modulate tumor cell resistance to apoptosis and survival during hypoxia.

Cell stress leading to apoptosis can be associated with inhibition of protein synthesis. Indeed, hypoxia is often associated with a reduction in global protein synthesis in hepatic epithelia.[35] Thus, the increased translational response during hypoxia is paradoxical. XIAP has a well-characterized internal ribosome entry sequence (IRES) site for translation.[36] Although IRES-mediated translation was initially described for viral RNA as a mechanism for translation of uncapped viral RNA, IRES elements have been described in a few eukaryotic mRNA. IRES-mediated translation occurs during cellular stresses such as exposure to ionizing radiation-induced stress.[27,37] Although our studies show that the translation of XIAP during hypoxia is dependent on the cap-binding eIF-4E, these observations do not exclude the possibility that IRES-mediated translation also occurs, particularly during acute hypoxia. Further studies are warranted to elucidate the contributions of eIF-4E, and hence cap-dependent translation, and IRES-mediated cap-independent translation during hypoxia.

The present studies provide insight into a potential mechanism by which acquired resistance to hypoxia-induced injury in biliary tract epithelia may promote tumorigenesis. Biliary tract ischemia has been associated with cholangiocyte apoptosis and cell death, suggesting that cholangiocytes are uniquely sensitive to hypoxic injury. Acquired resistance to hypoxia may result from perturbations in critical signaling pathways involved in the cellular response to environmental changes, such as those involving translational factors. Conversely, dysregulation of apoptosis may facilitate the survival of cells with inheritable genetic damage that would have otherwise undergone apoptosis. Furthermore, increased levels of XIAP have been associated with chemoresistance and may contribute to the refractoriness of human cholangiocarcinoma to conventional chemotherapy or to radiation therapy.[38–42] By activation of cell survival mechanisms, exposure to hypoxia may modulate chemoresistance and tumor aggressiveness and thereby contribute to cholangiocarcinoma progression. Strategies to inhibit tumor cell adaptation to otherwise injurious microenvironmental changes such as hypoxia by manipulating XIAP or eIF-4E expression are attractive novel targets for therapeutic intervention in cholangiocarcinoma.

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