Cell proliferation and drug resistance in hepatocellular carcinoma are modulated by Rho GTPase signals

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Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and is responsible for 1,250,000 deaths per year worldwide. Resistance to chemotherapy is a major obstacle in HCC treatment. The mechanisms involved in drug resistance are complex and multifactorial and may be due to inadequate drug exposure or alterations in the cancer cell itself (2). Recent studies suggest that HCC may be produced by selection of both genomic and epigenetic alterations that compromise more than one regulatory pathway (33, 34). However, the regulatory pathways that account for the molecular pathogenesis of HCC remain only partly understood.

Rho is a member of the family of Rho small GTPases that is required for several cellular functions. Early studies (9, 20, 37) showed that Rho proteins regulate cell morphology and the actin cytoskeleton; however, it is now clear that they also affect gene expression, cell proliferation, and survival. Defects in the Rho pathway are increasingly implicated in cancer and metastasis (11, 25, 36), and a recent study demonstrated that Rho GAP protein DLC2 (a negative Rho GTPase regulator) has a growth suppressor function in human HCC (3). Recently, Rho GTPases have also been implicated in genotoxic stress induced by doxorubicin treatment in the HCC cell line (10). However, the precise role of Rho in the HCC is still poorly understood.

Guanine nucleotide exchange factors (GEFs) for Rho GTPases promote formation of the activated Rho GTP form and play a key role in the Rho pathway because they transduce signals from activated cell surface receptors to Rho members (28). GEFs for the Rho GTPase family are characterized by a Dbl homology (DH) domain that confers GEF activity, usually followed by a pleckstrin-homology (PH) domain. Several Rho GEFs were isolated as potent oncoproteins, and there are two examples of Rho GEF mutations in human cancer (8, 14).

Lbc, a GEF specific for Rho (40), was originally isolated as a transforming oncoprotein (onco-Lbc) with unregulated guanine exchange activity (35). Cloning of the normal Lbc homolog (proto-Lbc) showed the presence of an extended COOH-terminal region that is absent from the oncogenic form (see Fig. 2A) and appears to attenuate the potential malignant capacity of Lbc (29). Lbc is widely expressed in several epithelial tissues that are the target sites of human cancer (23, 29) including the gastrointestinal (GI) tract. However, little is known about the involvement of Lbc in human malignancy.

In this study, we analyzed the expression of Lbc in GI cancer cell lines and in normal liver and in HCC. Furthermore, we determined the effect of Lbc (oncogenic vs. normal proto-Lbc form) expression and Rho activation in HCC cells on parameters associated with malignancy such as growth rate and resistance to genotoxic stress.

MATERIALS AND METHODS

Cancer cell lines. Mz-ChA-1 cells (gallbladder) (13) were a gift from Dr. J. G. Fitz (University of Colorado, Denver), HuH-28 (intrahepatic bile duct) (15) and TFK-1 (extrahepatic bile duct) (26) cell lines were acquired from Cancer Cell Repository (Tohoku University, Japan). Mz-ChA-1, TFK-1, and HuH-28 cells were maintained in CRML 1066 medium containing 10% fetal bovine serum. The human HCC cell line Alex-0 (PRF/PLC/5) was a gift from Prof. R. Mazzanti (University of Florence, Italy) and was maintained in MEM containing 10% fetal bovine serum. The human colon carcinoma SW 480 cell
line (gift from Dr. E. Porfiri Polytechnic University of Marche, Italy) was cultured in Leibovitz’s L15 medium containing 10% fetal bovine serum.

Plasmids and antibodies. pSR-proto-Lbc:Flag, pSR-onco-Lbc:Flag mammalian expression vectors, and anti-Lbc antisera are previously described (29). Anti-Flag M2 antibody was commercially purchased (Sigma-Aldrich, St. Louis, MO). Cyclin D1, p-Akt 1/2/3, p-ERK, ERK2, and rhoA antibodies were purchased from Santa Cruz Biotechnology (San Diego, CA), PCNA, Bcl-2, and bromodeoxyuridine (BrdU) antibodies were from Dako (Denmark). p-BAD 7E11 antibody was a Cell Signaling Technology (Beverly, MA) product, and C219 antibody was purchased from Signet-Laboratories (Dedham, MA).

Northern blotting. Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA), and poly (A)⁺ was isolated by Message-Maker Reagent Assembly (Invitrogen). Poly (A)⁺ RNA (5 μg) was separated by electrophoresis under denaturing conditions and transferred to nitrocellulose membrane. Probe corresponding to nucleotide 663–1264 of the Lbc cDNA was labeled with [³²P]dCTP using a Random Primers DNA labeling system (Invitrogen). Membranes were hybridized with [³²P]-labeled probes in a buffer containing 50% formamide at 42°C overnight, and hybridized bands were analyzed by Molecular Imager FX (Bio-Rad, Hercules, CA).

Transfection. Cells grown in 35-mm dishes at 50–70% confluence were transfected with plasmids using LipofectAMINE Reagent (Invitrogen) according to manufacturer’s recommendations. Seventy-two hours after transfection, each dish was trypsinized and cells were seeded into three to four 100-mm dishes containing complete medium in the presence of 1 mg/ml of G418 sulfate (Geneticin Invitrogen, Carlsbad, CA). After 10–15 days, discrete colonies were harvested and expanded.

Western blotting. Protein from whole cell extracts was separated in SDS-polyacrylamide gels and transferred onto nitrocellulose membrane. Filters were blocked in 5% nonfat dry milk in TBS containing 0.05% Tween and incubated with primary antibodies. The reaction was revealed with the enhanced chemiluminescence detection method (ECL kit, Aylesbury, UK). Membrane fraction proteins (29) and protein nuclear extracts (5) were prepared as previously described. The intensity of the bands corresponding to protein was quantified by densitometric analysis evaluated by Chemi-Doc (Bio-Rad).

Biopsy data. All biopsies for this study were taken from the files of the Department of Gastroenterology, Polytechnic University of Marche. Twenty liver biopsies of patients with HCC (16 men and 4 women; range 44–75 yr old, mean 64 yr old) were examined. Twelve patients were HCV positive, and six were HbsAg positive. Five specimens of normal liver (3 men and 2 women; range 40–60 yr old, mean 55 yr old) were also used as controls. Histological grade of HCC was determined according to the criteria of the International Working Party (32). None of the patients received any treatment to HCC.

Immunohistochemistry. Five micrometer-thick sections of formalin-fixed and paraffin-embedded material were processed for immunohistochemistry using the polyclonal antibody anti-Lbc (1:50) for 2 h at room temperature. Reacting sites were detected using DAKO EnVision System. Sections were counterstained with hematoxylin and examined with a microscope (Olympus BX 40, Olympus Optical, Tokyo, Japan). The intensity of the staining was semiquantified into four degrees (− negative; + light; ++ medium; +++ strong) in a random, blinded fashion in three different fields containing 100 cells for each section.

Rho binding domain assay. Equal amounts of cell lysates from Alex-0 stable transfected cells were incubated with affinity gel-bound GST-Rhotekin RD fusion protein (Upstate, Lake Placid, NY), and

![Fig. 1. Immunohistochemical detection of endogenous Lbc in human specimens. A: normal liver. Lbc expression is not detectable. B: hepatocarcinoma showing a positive diffuse cytoplasmic staining. C: nontumoral surrounding tissue without an evident positive staining. Original magnification in A and B: ×150. *Bile duct.](http://ajpgi.physiology.org/)

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Fig. 2. Lbc expression in human cancer cell lines. A: schematic representation and domain organization of Onco-Lbc and Proto-Lbc. The Dbl homology (DH) and pleckstrin homology (PH) domains are shown. B: Northern blot analysis of Lbc mRNA expression in human cancer cell lines: SW480, Alex-0, Mz-Cha-1, TFK-1, and HuH-28. To normalize for differences in RNA loading, the same blot was also probed for β-actin after stripping the Lbc signal. C: immunoblot analysis of endogenous Lbc protein. 50 μg of total protein of human cancer cell line lysates were immunoblotted with anti-Lbc antiserum.

Fig. 3. Effect of Lbc overexpression on Alex-0 cells. A: Western blot analysis of onco- or proto-Lbc expression in Alex-0 stably transfected cells. Equal amounts of total cell proteins from ONCO-5, PROTO-2, and pSRv-3 cell lines representing G418 selected clones were immunoblotted with anti-Flag M2 antibody. B: GTP-Rho pull-down assay in Alex-0 stable transfectants. Total Rho and GTP-bound Rho levels were determined as described in MATERIALS AND METHODS. Representative immunoblots and densitometric analyses are shown. Columns and bars represent means ± SE of 3 experiments [ONCO-5 or PROTO-2 vs. pSRv-3 (*P < 0.002); ONCO-5 vs. ONCO-5 + C3 or PROTO-2 vs. PROTO-2 + C3 (*P < 0.002)]. C: onco- and proto-Lbc induce BrdU incorporation by Alex-0 stable transfectants (ONCO-5, PROTO-2) and control (pSRv-3). Data were expressed as %BrdU-positive cells ± SE (ONCO-5 or PROTO-2 vs. pSRv-3, *P < 0.002).
Rho pull-down assay was performed according to manufacturer’s recommendations. Active Rho bound to beads was detected by blotting with anti-RhoA. Thirty micrograms of total cell lysate were used for determination of total Rho.

Cell proliferation assay. Cell proliferation was determined by indirect immunoperoxidase staining of nuclei, which have incorporated BrdU, as previously described (30). Data were expressed as the percentage of BrdU-positive nuclei.

C3 exoenzyme treatment. Cells were incubated with C. botulinum C3 exoenzyme (2 μg/ml; Biomol Research Laboratories, Plymouth Meeting, PA) (38), a Rho-specific inhibitor (1, 16). Cell viability after C3 incubation (6 h) was evaluated by trypan blue exclusion.

Cytotoxicity studies. Alex-0 cells stably transfected with onco-Lbc:Flag, proto-Lbc:Flag, or vector (1.5 × 10^5 per 35-mm dish) were incubated with medium containing different concentrations of doxorubicin (Sigma-Aldrich) or the vehicle (DMSO). In a different experiment, transfected cells preincubated for 6 h with C. botulinum C3 exoenzyme (2 μg/ml) were incubated with different concentrations of doxorubicin or the vehicle. After 24 h, medium was replaced with fresh medium and cells were kept in culture for a further 24 h. Cell viability was measured by trypan blue exclusion and expressed as a percentage of respective controls.

Assay for detection of caspase-3/7 activity. Alex-0 cells stably transfected with onco-Lbc:Flag, proto-Lbc:Flag, or vector (1.5 × 10^5 in a volume of 50 μl) pretreated or not with C3 were incubated with medium containing 6–9 μM doxorubicin (Sigma-Aldrich) or the vehicle (DMSO). Caspase-3/7 activity was evaluated according to the vendor instruction using “Apo-ONE Homogeneous Caspase-3/7 Assay” (Promega). Caspase-3/7 activity was expressed as increasing fold of respective controls.

Statistical analysis. All data were expressed as means ± SE. For statistical analysis of differences between two groups, a Student’s t-test was used.

RESULTS

Lbc protein expression in specimens of human hepatocarcinoma and normal tissues. The number of patients with well-, moderately, and poorly differentiated HCCs was 5, 12, and 3 respectively. After incubation with anti-LBC antibody normal human liver sections showed absence of specific staining (Fig. 1A). In all HCC samples, a strong positive immunohistochemical staining (Fig. 1B) with a diffuse cytoplasmic pattern was evident independently of HCC grading. In 11 samples, clearly evident nontumoral surrounding tissue did not show specific LBC staining (Fig. 1C).

The random blinded semiquantitative evaluation of LBC staining was as follows: well-differentiated HCCs: differentiated 0 ± 6.6%, ++44.6%, +++48.8% cells; in moderately differentiated HCCs: differentiated 0 ± 6.9%, ++40.5%,...
++++52.6% cells; and in poorly differentiated HCCs: differentiated –0 + 5.5%, +++44.4%, +++50.1% cells.

Lbc mRNA and protein expression in human cancer cell lines. Northern blot analysis (Fig. 2B) shows that all cell lines tested express two Lbc mRNA transcripts: a large transcript of ~10 kb and one of two smaller transcript forms, which lie between the 9.5- and the 7.5-kb size markers. Western blot analysis detected expression of the 102-kDa wild-type proto-Lbc in all the cell lines analyzed (Fig. 2C).

Characterization of Alex-0 stable Lbc transfected cell lines. To investigate the effect of chronic Rho activation in HCC, cells stably expressing Lbc forms were generated. Clones of Alex-0 cell lines stably transfected with pSR proto-Lbc:Flag, pSR onco-Lbc:Flag plasmids, or pSR vector alone were isolated by selection with Geneticin. Three cell lines for each construct were generated, and the one showing the higher expression level of transfected protein and higher phenotypic changes, respectively designated PROTO-2 and ONCO-5, was used in the study. Western blot analysis of the two stable transfected clones by anti-Flag antibody confirmed expression of the predicted sized 102-kDa proto-Lbc:Flag and 47-kDa onco-Lbc:Flag, respectively, in PROTO-2 and ONCO-5 cell line (Fig. 3A). In contrast, expression was not detected in clones transfected with the vector alone such as the pSRv-3 line.

Onco- and proto-Lbc expression induce increased levels of activated Rho in vivo. To determine whether onco- and proto-Lbc expression affect Rho activity in stable transfected cells, a GTP-Rho pull-down assay was performed to evaluate change in cellular GTP-Rho levels. The level of GTP bound to Rho was measured in cell lysates from PROTO-2, ONCO-5, and pSRv-3 clones by affinity purification with Rhotekin RBD. As shown in Fig. 3B, expression of both forms of Lbc significantly induce increased levels of GTP-Rho compared with the vector clone (P < 0.002; Fig. 3B); and the oncogenic Lbc form shows a slightly higher level than the protooncogenic form. The higher levels of cellular GTP-Rho was reversed by treatment with C3 (P < 0.002; Fig. 3B).

Proliferation rate and activation of signaling pathways in stable transfected cells. The effect of chronic Lbc expression on Alex-0 cell proliferation rate was measured in stable transfected PROTO-2, ONCO-5, and control pSRv-3 clones. The percentage of BrdU-positive (S-phase) nuclei was significantly higher in PROTO-2 and ONCO-5 clones than in the control clone (P < 0.002; Fig. 3C). Western blot analysis for expression of the proliferation marker PCNA showed that PCNA protein expression is increased in ONCO-5 and PROTO-2 clones with respect to vector control clone, as shown by densitometric analysis, which is in keeping with the BrdU data (P < 0.03; Fig. 4A).

Fig. 5. Effect of C3 on PCNA, p-ERK, and cyclin D1 expression in Alex-0 stably transfected cells. A: PCNA and cyclin D1. B: p-ERK protein levels were evaluated by Western blot analysis in cells treated for 24 h with C3. C: Botulinum exoenzyme. Same amount of nuclear extracts was probed with anti-cyclin D1 antibody and subsequently with anti-PCNA antibody. In addition, a portion of the lysates was directly immunblotted for β-actin. Equal amounts of total cell protein were analyzed for p-ERK and ERK-2 (loading control) expression. Representative immunoblots and densitometric analyses are shown. Columns and bars represent means ± SE of 3 experiments (*P < 0.02).
Next, we investigated whether Lbc chronic expression led to changes in key signaling components associated with cell growth. Western blot analysis of cyclin D1 expression in the stably transfected clones showed an increase in protein levels in ONCO-5 and PROTO-2 clones compared with the control line (P < 0.03; Fig. 4A). Furthermore, Western blot analysis of ERK1/2 phosphorylated forms (p-ERK) revealed an increased expression of both ERK isotypes in ONCO-5 and PROTO-2 lines compared with the control line (P < 0.02 Fig. 4B). These results suggest that activation of p42/44 MAPK pathway and cyclin D1 might be involved in the Lbc-dependent increase in the proliferation rate of Alex-0 cancer cells.

Inhibition of proliferation and signaling pathways by Rho inactivation in stable transfected cells. Since Lbc is a Rho GEF, the contribution of the Rho signaling pathway to modulation of HCC cell proliferation and signaling was studied. PROTO-2 and ONCO-5 clones were treated with C. Botulinum exoenzyme C3, which is known to inhibit Rho function by ADP ribosylation. Incubation with C3 dramatically reduced PCNA expression (P < 0.02; Fig. 5A) as well as the levels of Cyclin D1 (P < 0.02; Fig. 5A) and phosphorylated ERK1/2 (P < 0.02; Fig. 5B) expression in PROTO-2 and ONCO-5 clones, although cell viability was not affected. These findings support the notion that Rho signaling is able to modulate proliferation through intracellular pathways that involve ERK1/2 and Cyclin D1 activity.

In vitro doxorubicin cytotoxicity. The effect of Lbc expression on resistance of HCC cells to genotoxic stress was evaluated by incubating stable transfected Alex-0 cells with doxorubicin (0.05–9 μg/ml) or the vehicle DMSO for 24 h. Viable cells were counted by trypan blue dye exclusion by hemocytometer. After incubation with 3, 6, and 9 μg/ml doxorubicin, PROTO-2 and ONCO-5 clones showed a significantly higher number of viable cells with respect to control pSRv-3 clone (P < 0.002; Fig. 6A). The higher resistance to doxorubicin cytotoxicity was reversed by incubation with C3 (P < 0.002; Fig. 6B). Our data were also confirmed by the evaluation of caspase-3/7 activation, which plays a key effector role in apoptosis. In fact, caspase activation by doxorubicin significantly increased after C3 preincubation in both transfected cell lines (P < 0.005; Fig. 6D).

Expression of P-glycoprotein in stable Lbc transfected cells. Since increased levels of P-glycoproteins can mediate resistance to cytotoxic drugs, P-glycoprotein levels were next determined. The immunoblotting of PROTO-2, ONCO-5, and control line lysates was carried out with C219, an anti-P-glycoprotein antibody (Fig. 6C). Consistent with previous studies (18), P-glycoprotein expression was detected in Alex-0 cells and the level did not appear to differ in transfected cells. P-glycoprotein expression does not seem to be involved in the higher resistance to doxorubicin of transfected cells.

Expression of survival factors involved in doxorubicin resistance. Finally, expression of key proteins involved in cell survival such as Bcl-2, BAD, and Akt was analyzed in the Alex-0 stable transfected lines (Fig. 7). No difference was observed in the expression of the phosphorylated form of Akt (p-Akt). In contrast, higher levels of Bcl-2 and phosphorylated BAD (p-BAD) expression were observed in Lbc-transfected cells compared with the control (P < 0.05). These data suggest that Bcl2 and BAD might...
be involved in conferring higher resistance to doxorubicin of PROTO-2- and ONCO-5-expressing Alex-0 cells.

**DISCUSSION**

Our examination of Lbc Rho GEF expression in human specimens and cell lines revealed the interesting finding that whereas Lbc is absent in normal adult liver, it is abundantly expressed in the hepatocarcinoma cancer cell line and tissue samples. These results indicate that Lbc expression is up-regulated in the corresponding cancer tissues and imply that these carcinomas have increased Rho pathway signaling.

Furthermore, our study shows for the first time that Lbc expression, leading to Rho signaling in HCC cells, is able to significantly increase cell growth, which is a common feature in hepatocarcinogenesis irrespective of the environmental causative agent, and, moreover, to confer increased resistance to doxorubicin toxicity. The evidence that both increased proliferation and drug resistance are reversed by a Rho inhibitor identifies a potential new target for the development of more effective anticancer therapies in HCC.

Several studies have linked Rho proteins to the disregulation of mitogenic signals in cancer cells (25). Our data show that both forms (onco and proto) of Lbc increase Rho-GTP formation and promote cell growth in transfected Alex-0 cells, and the effect appears higher in cells overexpressing the oncogenic form. These data are in keeping with the finding that although both onco- and proto-Lbc contain a Rho GEF (DH) domain, which confers Rho exchange activity (22), onco-Lbc is constitutively active, whereas proto-Lbc is likely subject to regulation (7, 23).

The extensive heterogeneity of genomic lesions displayed by HCCs suggests that more than one regulatory pathway is compromised (33). The finding that proto- and onco-Lbc-expressing cells exhibit increased levels of ERK and cyclin D1 implies that chronic Rho activation may play a central role in stimulating these two key growth promoting components. This is supported by our finding that ERK and cyclin D1 activation are blocked by treatment with exoenzyme C3, a well-known specific Rho inhibitor. MAPK/ERK signaling transduces convergent signals into nuclei, resulting in various cellular responses including proliferation and differentiation. MAPK/ERK activation, which is significantly higher in HCCs than in their adjacent noncancerous lesions, appears to play an important role in hepatocarcinogenesis, especially in the progression of HCC, at least in part through cyclin D1 modulation (17). In fact, cyclin D1, which is required for cell-cycle progression into the G0/G1 phase and is able to induce genetic instability and genomic DNA amplification resulting in transformation of
mammalian cells, is overexpressed in human HCC (21) and could be induced by Rho (25, 39) through activation of ERK1/2 (12).

Drug resistance is a serious obstacle to cancer eradication, and HCCs are highly resistant to chemotherapeutic agents. Rational attempts to tackle drug resistance need to be based on an understanding of the mechanisms involved that are likely to be complex and multifactorial, allowing the cancer cell many escape routes to survival (2). Our findings show that both onco- and proto-Lbc overexpression confers increased resistance to genotoxic stress induced by doxorubicin, which is not mediated by P-glycoproteins and is reversed by treatment with exoenzyme C3, a well-known specific Rho inhibitor. Our data confirm the potentially important role of signal-transduction inhibitors designed to specifically target deregulated pathways driving malignant progression in cancer treatment, especially when used as chemosensitizers in combination with “traditional” drugs (2).

Doxorubicin induces cell death in cancer cells by apoptosis (4, 31), and several genes involved in programmed cell death regulation have been recently shown to modulate therapeutic resistance. Takahashi et al. (31) demonstrated that overexpression of Bcl-2 renders human HCC cells resistant to doxorubicin cytotoxicity. Although they did not detect Bcl-2 expression in Alex-0 cells, we readily found Bcl-2 expression in the same cell line, probably due to a higher quantity of protein (100 vs. 30 μg) that we analyzed by SDS-PAGE. Our results demonstrate that Bcl-2 levels are increased in onco- and proto-Lbc-transfected cells, and a recent report found that the expression of Bcl-2 in a human neuroblastoma cell line was reduced by Rho inhibitor, suggesting a role for Rho in the control of Bcl-2 expression (19).

Onco- and proto-Lbc-transfected cells showed increased levels of phosphorylated Bad. It has been demonstrated that activation of ERK1/2 (induced in Lbc-transfected cells) in neoplastic cells results in phosphorylation of the proapoptotic protein Bad and in its dissociation from Bcl-2, allowing these antiapoptotic proteins to prevent mitochondrial events required for apoptosis (24, 27). Thus it is possible that chronic Rho signaling induced by Lbc expression may modulate Bcl-2 and antiapoptotic proteins to prevent mitochondrial events required for apoptosis (2). In contrast, we found no changes in p-AKT expression in Lbc-transfected cells, suggesting that the phosphatidylinositol 3-kinase/Akt pathway, which has been shown to have a role in breast cancer cell survival and therapeutic resistance (4), is not involved in the observed resistance to doxorubicin.

Identification and functional characterization of genes that may play a critical role in human HCC development would be a significant contribution toward not only the definition of the molecular pathogenesis of HCC, but would also broaden the potential for developing effective treatment of HCC (33). Our findings detailed here, showing that expression of a Rho GEF, Lbc, affects multiple aspects of HCC tumorigenesis, support the notion that the Rho GTPase pathway components may provide novel targets for therapy.

REFERENCES


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