

Inhibition of GSK-3 β enhances reovirus-induced apoptosis in colon cancer cells

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Abstract. Reovirus functions as an oncolytic agent for many types of cancer including colon cancer. Although most studies have emphasized the role of activated Ras signaling in enhancing reoviral oncolysis in susceptible cells, we note that many colon cancers also display elevated β -catenin. Thus, it is possible that enhanced β -catenin may augment reoviral susceptibility in colon cancer cells. To explore this hypothesis, HEK293 cells were treated with the glycogen synthase kinase (GSK)-3 β inhibitor LiCl, thereby inducing β -catenin, followed by reoviral infection. Co-administration with LiCl indeed enhanced cell death compared to reovirus infection alone, but this was not associated with elevated reoviral replication. Similarly, HEK293 cells expressing the Frizzled-1 receptor in Wnt3a-conditioned medium also showed reovirus replication equivalent to that in cells in control medium, further suggesting that up-regulation of β -catenin does not enhance the replication of reovirus. Instead, we observed that inhibition of GSK-3 β with LiCl decreased reovirus-induced NF- κ B activation, leading to accelerated apoptosis via

caspase 8 activation. We further found that colon cancer HCT116 cells were sensitized to apoptosis by co-treatment with reovirus and a GSK-3 β inhibitor, AR-A014418. Finally, we identified that inhibition of NF- κ B sensitized apoptosis of HEK293 or HCT 116 cells during reovirus infection. Taken together, we propose that inhibition of GSK-3 β sensitizes reovirus-induced apoptosis of colon cancer cells by down-regulation of NF- κ B activity, offering a potentially improved therapeutic strategy for the treatment of colon cancer.

Introduction

The human reovirus is a ubiquitous, non-enveloped virus with 10 segments of double-stranded RNA (1). Virus infection is usually restricted to the upper respiratory and gastrointestinal tracts and is often asymptomatic or causing mild disease (2,3). However, reovirus also displays striking cytolytic activity in certain types of transformed cells (4,5). Evidence that Ras-transformed cells are preferentially susceptible to reovirus T3D (type 3 Dearling strain) via inactivation of the protective PKR (dsRNA-activated protein kinase) phosphorylation pathway has been reported (6-8) although our study has revealed that Ras activation alone does not enable reovirus to induce oncolysis in hepatocellular carcinoma cells expressing HBX, an oncoprotein of hepatitis B virus (9). Furthermore, the use of reovirus in the treatment of gliomas in immunocompetent hosts and the inoculation of reovirus into the brains of non-human primates have not produced significant toxicities (10), thereby supporting the current tests of reovirus in several clinical trials (11).

On the other hand, it has been reported that reoviral oncolysis is associated with the induction of apoptosis in various types of cancer (12-14). Further investigation has revealed that TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily is involved in reovirus-induced apoptosis (15). Caspase 8 and JNK MAPK molecules

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Abbreviations: GSK, glycogen synthase kinase; NF- κ B, nuclear factor- κ B; HEK, human embryonic kidney; LiCl, lithium chloride; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase

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also participate in apoptotic signaling following reoviral infection (16,17).

Glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed serine/threonine kinase that regulates glycogen synthesis (18). GSK-3 has 2 isoforms (GSK-3 α and GSK-3 β) which are highly homologous but exhibit different substrate specificities *in vitro* (19). GSK-3 α and GSK-3 β have been implicated in cell-fate determination and differentiation in a variety of organisms (20). In particular, GSK-3 β functions as a member in complexes of the adenomatous polyposis coli (APC), Axin, and β -catenin. In this model, GSK-3 β can directly phosphorylate β -catenin and target it for degradation via ubiquitin-mediated proteosomes (21). Thus, GSK-3 β participates as part of a tumor suppressor complex that regulates the levels of the oncoproteins, β -catenin (22). Consistent with this, disruption of the murine GSK-3 β gene results in embryonic lethality, and mouse embryonic fibroblasts (MEFs) derived from these animals are more sensitive to apoptosis (23). Further studies have revealed that GSK-3 β -knockout MEFs harbor an intrinsic defect in the activation of NF- κ B during TNF- α signaling (23), raising the possibility that GSK-3 β indeed contributes to cell survival at least in part through the involvement of NF- κ B signaling.

To examine whether the up-regulation of β -catenin which is frequently noted in colon cancer cells might enable the preferential replication of reovirus in a manner similar to that following Ras activation, we initiated the present study. Here, we report that inhibition of GSK-3 β can indeed enhance reovirus-induced apoptosis of the cell, by down-regulation of NF- κ B activity and irrespective of up-regulation of β -catenin. This raises the possibility that the combined administration of a GSK inhibitor together with reovirus could be an efficient regimen for the treatment of colon cancer cells.

Materials and methods

Cell cultures and virus amplification. Human embryonic kidney (HEK)293, HEK293 stably expressing Frizzles-1 (HEK293-Fz), murine L929, and colon cancer HCT116 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. The Dearing strain of reovirus serotype 3 purchased from the ATCC (Manassas, VA) was propagated in L929 cells. The virus was purified as described elsewhere (24,25), and titer of virus was measured as plaque forming units (PFU).

Reagents and antibodies. To inhibit GSK-3 β activity, LiCl and AR-A014418 were purchased from Calbiochem (San Diego, CA), and Bay 11-7082 was obtained from Calbiochem to inhibit NF- κ B activity. For immunoblotting, anti-caspase 3, caspase 8, PARP, and β -tubulin antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA). Anti-phospho-GSK-3 β (Ser9) and GSK-3 β antibodies were purchased from Cell Signaling Technology (Danvers, MA). Polyclonal anti-reovirus antibodies described previously (1) were used for the detection of λ , μ and σ reoviral proteins.

Preparation of nuclear extract. Nuclear extracts were prepared according to a modified method described elsewhere (26). Cells were washed and harvested with ice-cold PBS, and the

cell pellets were lysed with 500 μ l of buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 10% glycerol, 0.5 mM PMSF and 1X cocktail protease inhibitor] on ice for 20 min, followed by centrifugation at 10,000 \times g for 15 min. The supernatant was taken as a cytosol fraction, and the pellets were washed with 500 μ l of buffer A, and the remaining pellets were subsequently solubilized in 500 μ l of buffer C [20 mM HEPES (pH 7.9), 450 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.5 mM PMSF and 1X cocktail protease inhibitor] on ice for 30 min. Extracts were centrifuged at 12,000 \times g for 15 min, and the supernatant was taken as a nuclear fraction.

Western blot assay. Cells were harvested and lysed with lysis buffer [150 mM NaCl, 1% NP-40, 50 mM Tris-HCl (pH 7.5)] containing 0.1 mM Na₂VO₃, 1 mM NaF and protease inhibitors (Sigma, St. Louis, MO). For immunoblotting, proteins from whole cell lysates were resolved by 10 or 12% SDS-PAGE and then transferred to nitrocellulose membranes. Primary antibodies were used at 1:1,000 or 1:2,000 dilutions, and secondary antibodies conjugated with horseradish peroxidase were used at 1:2,000 dilutions in 5% nonfat dry milk. After a final washing, nitrocellulose membranes were exposed for an enhanced chemiluminescence assay using the LAS 3000 (Fuji, Japan).

Nuclear NF- κ B pull-down assay. Using oligonucleotide conjugated with agarose, NF- κ B transcription factor was isolated as described elsewhere (27). Nuclear extracts from cells treated with LiCl, reovirus, or reovirus and LiCl were prepared as described above. In accordance with the manufacturer's instructions, NF- κ B consensus oligonucleotide-agarose conjugate (100 μ g; Santa Cruz) was incubated with 500 μ g of nuclear extracts in binding buffer [10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 50 μ g/ml poly(dI-dC), 1 μ g/ml aprotinin, leupeptin, and pepstatin, and 2 mM Na₃VO₄] at room temperature for 2 h with gentle rotation. Samples were centrifuged (15,000 \times g) for 3 min and washed three times with binding buffer at 4°C. After washing, 25 μ l of 2X sample buffer was added and boiled for 5 min. The result was analyzed by SDS-PAGE and immunoblotting using anti-p65 NF- κ B antibody (Santa Cruz).

Results

Inhibition of GSK-3 β with LiCl enhances reovirus-induced cell death. To begin testing whether the elevated expression of β -catenin noted in colon cancer cells might enhance the replication of reovirus, we treated HEK293 cells with LiCl (a known GSK-3 β inhibitor) for 24 h to achieve up-regulation of β -catenin. As expected, LiCl induced elevated expression of β -catenin as revealed by immunoblotting (Fig. 1A) in a dose-dependent manner. The level of β -catenin was maximized (up to 10-fold) at 20 mM of LiCl, and since we observed a significant inhibition of cell proliferation at 30 mM of LiCl (data not shown), we used 20 mM of LiCl thereafter. Live HEK293 cells treated with LiCl, reovirus alone, or reovirus and LiCl were counted at 36 h post-treatment using trypan

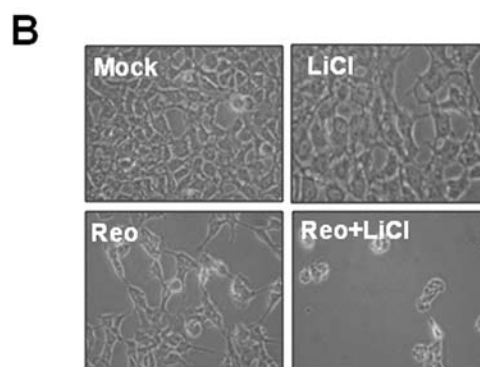
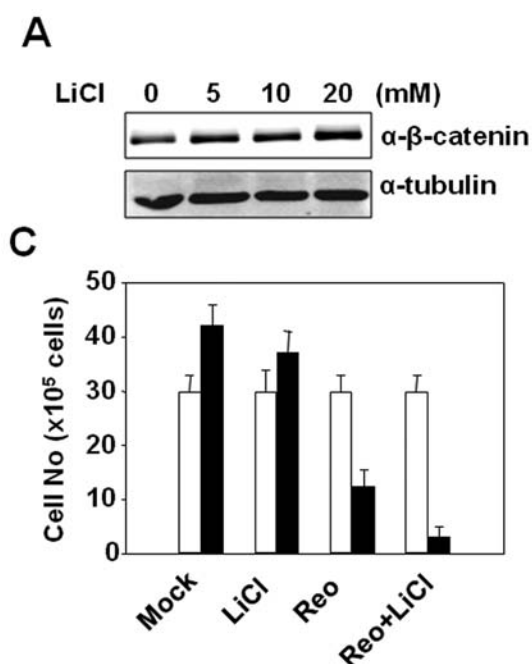


Figure 1. GSK-3 β inhibition enhances reovirus-induced cell death. (A) HEK293 cells were treated with LiCl at 5, 10 or 20 mM and harvested at 24 h post-treatment. Expression of β -catenin was examined by immunoblotting with anti- β -catenin antibody. (B and C) HEK293 cells were treated with LiCl (20 mM), reovirus (Reo) (MOI=1) alone, or reovirus (MOI=1) and LiCl (20 mM), and the cells were photographed (x200) and counted using trypan blue after 36 h. White bars indicate the cell number of inoculation at 0 h while black bars indicate the cell number treated with indicated agents at 36 h post-treatment.

blue exclusion. As noted in Fig. 1B, LiCl treatment alone slightly inhibited cell proliferation to a certain extent compared to mock treatment. Infection of HEK293 cells with reovirus (MOI=1) caused partial cell lysis at 36 h post-infection (Fig. 1B). However, co-treatment with LiCl and reovirus efficiently destroyed HEK293 cells compared to infection of HEK293 cells with reovirus alone (Fig. 1B and C). Only 10% of HEK293 cells survived at 36 h after co-treatment with reovirus and LiCl, whereas 35% of HEK293 cells survived 36 h after treatment with reovirus alone, consistent with the speculation that up-regulation of β -catenin by inhibition of GSK-3 β promotes reovirus-induced cell death.

Enhancement of reovirus-induced cell death by LiCl is not linked to reovirus propagation. If this is true, then elevated expression of β -catenin in the presence of LiCl might be predicted to enhance reoviral replication and consequently reovirus-induced cell death. We therefore tested this possibility by determining reoviral protein levels in cells treated with reovirus or reovirus and LiCl using polyclonal anti-reovirus antibodies. However, the levels of reoviral protein detected in cell lysates treated with reovirus alone were actually greater than those in the cell lysates co-treated with reovirus and LiCl (Fig. 2). Thus, elevated reovirus replication is not likely a direct cause of enhanced cell death in the cells treated with reovirus and LiCl. The up-regulation of β -catenin by the inhibition of GSK-3 β is not linked to reovirus replication in this experimental model.

Elevated expression of β -catenin by Wnt signaling does not confer preferential replication of reovirus. To further show that enhanced β -catenin does not increase the replication of reovirus, we employed HEK293 cells expressing human Frizzled-1 (HEK293-Fz) as a receptor of Wnt-3a. HEK293-Fz cells were cultured in Wnt3a-conditioned medium (CM) for the indicated time intervals and amounts (Fig. 3A and B), resulting in maximal induction of β -catenin by 5-fold. Under

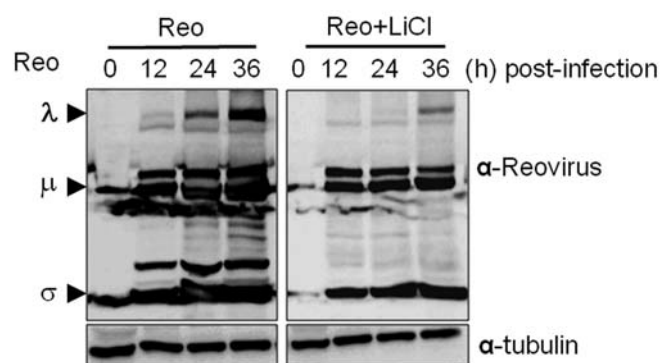


Figure 2. Enhancement of reovirus-induced cell death under LiCl is not linked to elevated reoviral protein synthesis. HEK293 cells were treated with reovirus (Reo) (MOI=1) or reovirus (MOI=1) and LiCl (20 mM) and harvested at 12, 24 and 36 h post-infection. Reoviral replication was determined by measuring protein levels using polyclonal antibodies against the reovirus. Polyclonal anti-reovirus antibodies were used for the detection of λ , μ and σ reoviral proteins.

conditions of continual Wnt3a signaling and β -catenin induction, HEK293-Fz cells were infected with reovirus, and reoviral protein levels were analyzed with immunoblotting over 36 h. As shown in Fig. 3C, reoviral protein levels from HEK293-Fz cells treated with Wnt3a-CM were similar to those from HEK293-Fz treated with control-CM. This result further demonstrated that the elevated expression of β -catenin by Wnt-3a signaling did not enhance reovirus replication in the HEK293-Fz cells.

Inhibition of GSK-3 β suppresses reovirus-induced NF- κ B activity, leading to the promotion of reovirus-induced apoptosis. First, we attempted to confirm that LiCl treatment inhibits GSK-3 β activity as described elsewhere (28) using an anti-phospho-GSK-3 β (Ser9) antibody. As shown in Fig. 4A, LiCl treatment enhanced GSK-3 β phosphorylation,

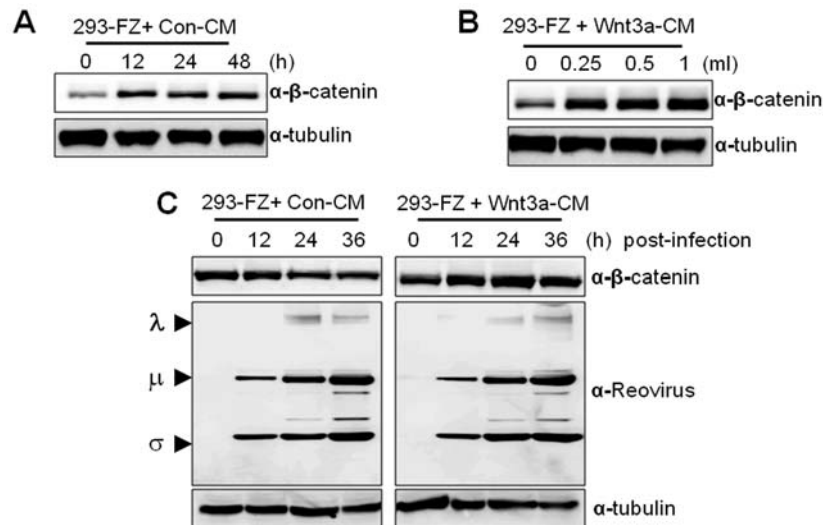


Figure 3. The enhanced level of β -catenin by Wnt3a does not confer a preferential replication of reovirus. (A and B) HEK293-Fz (293-FZ) cells were treated with Wnt3a-conditioned medium (CM) for 12, 24 and 48 h or with different amounts (0.25, 0.5 and 1 ml) for 24 h and harvested for the measurement of the β -catenin level. (C) HEK293-Fz cells were treated with reovirus in the presence of control conditioned medium (Con-CM) or reovirus in the presence of conditioned medium containing Wnt3a (Wnt3a-CM; 0.5 ml) and harvested at 12, 24, and 36 h post-treatment. The increased level of β -catenin in the presence of Wnt3a-CM was examined with anti- β -catenin antibody. Reoviral replication was examined by protein level of reovirus using polyclonal antibody.

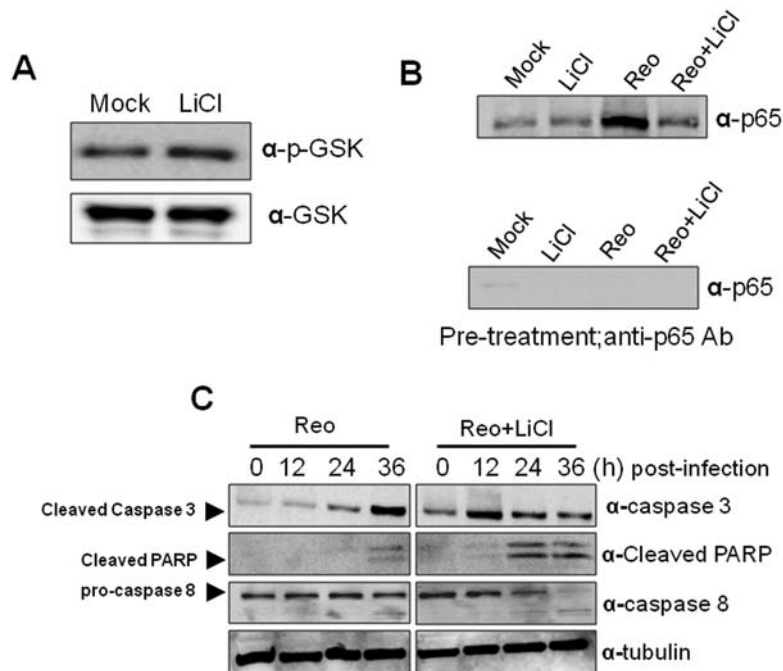


Figure 4. Inhibition of GSK-3 β promotes reovirus-induced apoptosis by inhibition of NF- κ B. (A) HEK293 cells were treated with LiCl (20 mM) for 24 h and harvested for the detection of phosphorylation of GSK-3 β (Ser9). Phosphorylated GSK-3 β was examined with anti-phospho-GSK-3 β (Ser9) antibody. (B) HEK293 cells were treated with LiCl (20 mM), reovirus (Reo) (MOI=1), or reovirus (MOI=1) and LiCl (20 mM), and the nuclear extracts from the samples were prepared after 12 h. NF- κ B target oligonucleotides conjugated with agarose (100 μ g) were added to the nuclear extracts, and the protein bound to the oligonucleotides was harvested and confirmed by immunoblotting using anti-p65 antibody since p65 is a subunit of the NF- κ B complex. To confirm specific binding between the NF- κ B oligonucleotide and p65, p65 antibody was added to the nuclear extract followed by treatment with Protein A/G to remove p65 protein. (C) HEK293 cells were treated with reovirus (MOI=1) alone or reovirus (MOI=1) and LiCl (20 mM) and harvested at 12, 24 and 36 h post-treatment. Protein levels of pro-caspase 8, cleaved caspase 3, and cleaved PARP were examined by immunoblotting with anti-caspase 8, 3 and anti-PARP antibodies, respectively.

indicating that LiCl treatment reduces GSK-3 β activity, leading to up-regulation of β -catenin. We therefore wished to test other possible roles of GSK inhibition in the enhance-

ment of reovirus-induced cell death. A role for GSK-3 β in NF- κ B activation has previously been reported (23,29), and we therefore wondered whether NF- κ B modulation mediated

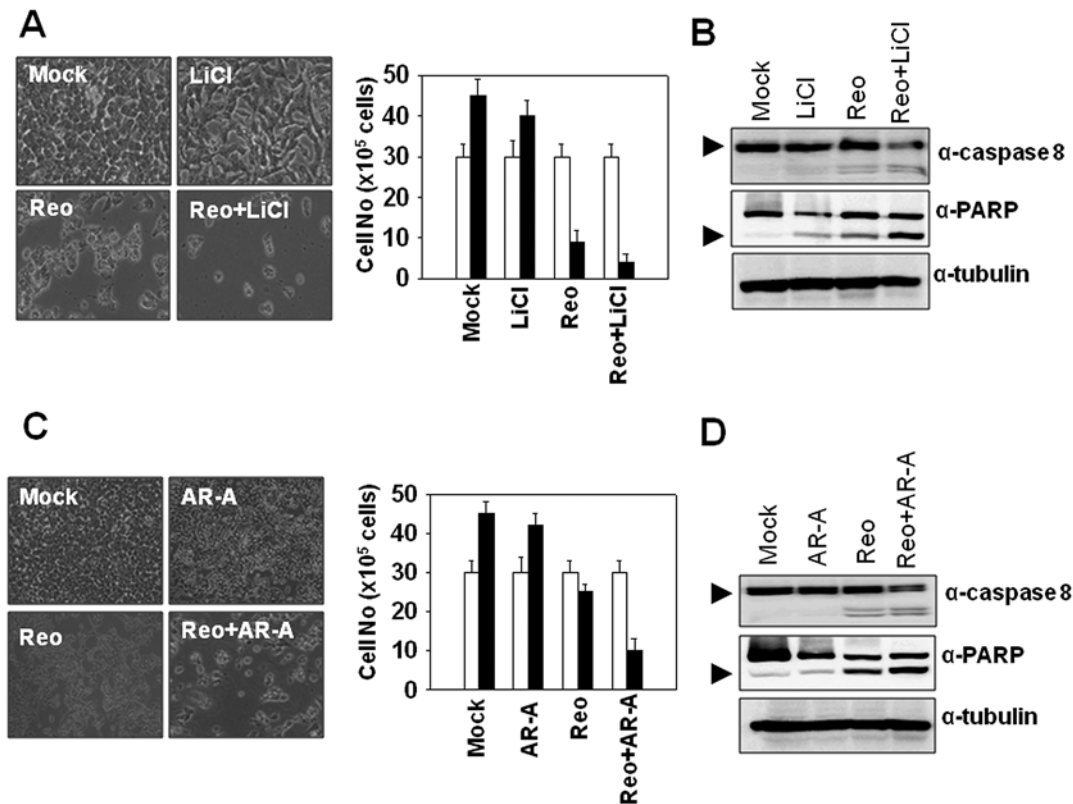


Figure 5. GSK-3 β inhibition accelerates reovirus-induced apoptosis in colon cancer HCT116 cells. (A and B) HCT116 cells were treated with LiCl (20 mM), reovirus (Reo) (MOI=1), or reovirus (MOI=1) and LiCl (20 mM), and the cell viability was recorded under the microscope (x200) at 36 h post-treatment. The cells were counted using trypan blue after 36 h. White bars indicate the cell number of inoculation at 0 h while black bars indicate the cell number treated with indicated agents at 36 h post-treatment. The cells were harvested at 36 h post-treatment, and cell lysates were separated on 10% SDS-PAGE for immunoblotting with anti-caspase 8 antibody and anti-PARP antibody. Arrowheads indicate pro-caspase 8 and cleaved PARP, respectively. (C and D) HCT116 cells were treated with AR-A014418 (AR-A; 10 μ M), reovirus (MOI=1), or reovirus (MOI=1) and AR-A014418 (10 μ M), and cells were photographed, counted, and proteins were analyzed as described in A and B.

by GSK inhibition might promote reovirus-induced cell death. After we prepared nuclear extracts from HEK293 cells treated with reovirus alone or reovirus and LiCl, we analyzed the binding ability of p53 (the major subunit of the NF- κ B dimer) to NF- κ B oligonucleotide target. As shown in Fig. 4B, treatment with reovirus strongly induced the binding ability of p53 protein to the NF- κ B oligonucleotide at 12 h post-infection. However, co-administration of reovirus with LiCl prevented the enhanced binding of p53 to the NF- κ B oligonucleotide. As a control, we added anti-p53 antibody to the nuclear extract fraction, and then removed p53 in the nuclear extract with protein A/G-conjugated agarose, preventing binding to the NF- κ B oligonucleotide (Fig. 4B). Our result suggests that GSK-3 β positively regulates reovirus-induced NF- κ B activation and that LiCl can block this activation.

Furthermore, to examine the consequences of NF- κ B blockade by inhibition of GSK-3 β , we investigated apoptosis in the cells treated with reovirus alone or reovirus and LiCl. Treatment with LiCl alone did not induce apoptosis in HEK293 cells, whereas cells treated with reovirus alone showed activation of caspase 3 and partial cleavage of PARP (a caspase 3 substrate) and caspase 8 by 36 h. In contrast, HEK293 cells treated with reovirus and LiCl exhibited a clear acceleration of apoptotic events with extensive cleavage of PARP by 12 h and activation of caspase 3 and caspase 8 at

24 h post-infection (Fig. 4C). The results suggest that GSK-3 β inhibition by LiCl accelerates the kinetics of reovirus-induced apoptosis of the cell.

GSK-3 β inhibition enhances reovirus-induced apoptosis of colon cancer cells. As we observed that GSK-3 β inhibition by LiCl enhances reovirus-induced apoptosis in HEK293 cells (Figs. 1 and 4), we wondered whether this effect of GSK-3 β inhibition might also be detected in colon cancer cells. To address this issue, we employed the HCT116 colon cancer cell line which shows an up-regulated level of β -catenin due to a mutated β -catenin gene. When HCT116 cells were treated with LiCl alone, cell proliferation was very slightly inhibited compared to mock treatment (Fig. 5A). When the cells were treated with reovirus and LiCl, cell viability was significantly decreased compared to that of cells treated with reovirus (Fig. 5A). Only 15% of HCT116 cells survived at 36 h after co-treatment with reovirus and LiCl, whereas ~35% of HCT116 cells survived 36 h after treatment with reovirus alone (Fig. 5A). We also tested a small molecule GSK-3 β inhibitor AR-A014418 and found that treatment with this agent enhanced reovirus-induced cell death similarly to LiCl treatment (Fig. 5C). The reovirus-induced cell death in both cases was attributed to apoptosis through caspase 8 cascades in the HCT116 cells (Fig. 5B and D). These results suggest that co-treatment with reovirus and GSK inhibitors

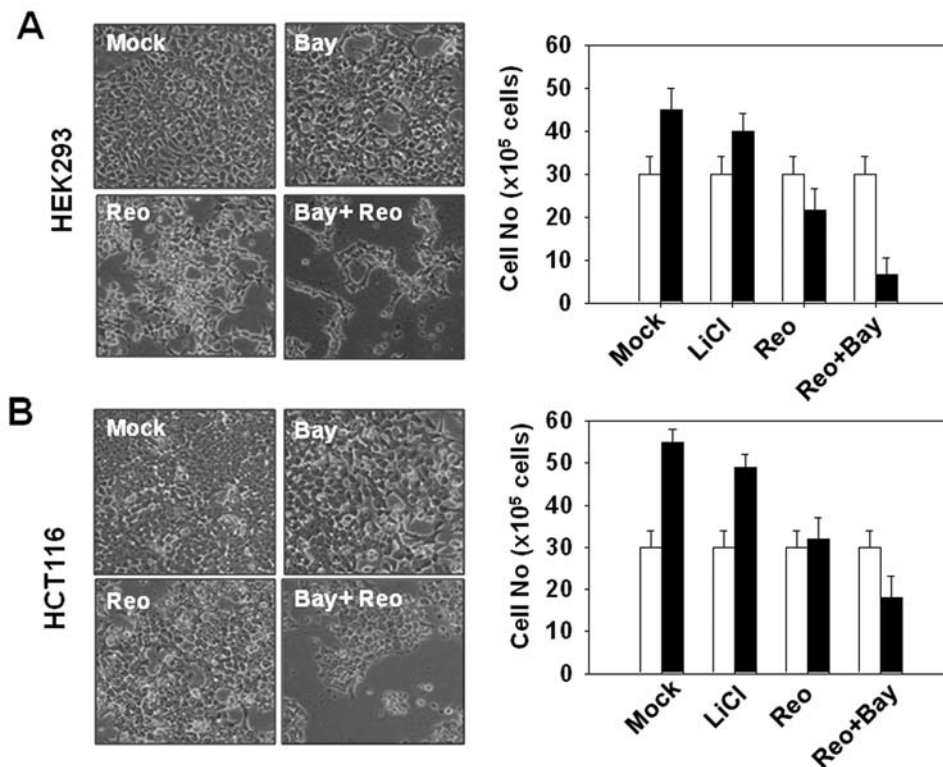


Figure 6. NF- κ B inhibition promotes reovirus-induced apoptosis of HEK 293 cells or HCT colon cancer cells. (A and B) HEK293 and HCT116 cells were treated with Bay 11-7082 (Bay; 10 μ M), reovirus (Reo) (MOI=1), or reovirus (MOI=1) and Bay 11-7082 (10 μ M), and the cell viability was recorded under the microscope (x200) at 24 and 48 h post-treatment, respectively. The cells were counted using trypan blue at 24 or 48 h post-treatment. White bars indicate the cell number of inoculation at 0 h while black bars indicate the cell number treated with indicated agents at 24 or 48 h post-treatment.

may efficiently kill colon cancer cells more rapidly and to a greater extent than with reovirus alone.

Inhibition of NF- κ B sensitizes apoptosis of HEK293 or HCT116 cells during reovirus infection. To prove whether inhibition of NF- κ B enhances reovirus-mediated apoptosis in HEK293 and HCT116 cells, we treated the cells with Bay 11-7082 during reovirus infection. As shown in Fig. 6A, Bay 11-7082 treatment (10 μ M) slightly inhibited cell proliferation in HEK 293 cells compared to mock treatment. When the cells were treated with reovirus and Bay 11-7082, cell viability was significantly decreased compared to that of cells treated with reovirus alone. Only 10% of HEK293 cells survived at 24 h after co-treatment with reovirus and Bay 11-7082, whereas 45% of HEK293 cells survived after treatment with reovirus infection alone (Fig. 6A). We also observed that the NF- κ B inhibitor enhanced reovirus-mediated apoptosis of HCT116 cells as seen in HEK293 cells. Co-treatment with this NF- κ B inhibitor and reovirus induced up to approximately 80% cell death at 48 h post-treatment while reovirus infection alone induced 45% cell death at 48 h post-treatment (Fig. 6B). Accordingly, the results suggest that inhibition of NF- κ B sensitizes apoptosis of HEK293 cells or HCT116 colon cancer cells during reovirus infection.

Discussion

Colorectal cancer is the third most common cancer diagnosed in the US and is increasing in incidence as well in Asian

countries (30). Mutations not only in the K-ras proto-oncogene but also in components of the Wnt/ β -catenin signaling pathway are frequently detected in colorectal cancers (31). Although Ras signaling plays a critical role in dictating host cell permissiveness to reovirus (6), other signaling pathways may also contribute susceptibility to reoviral replication and oncolysis (9). Since it has been reported that reovirus efficiently kills many colon cancer cell lines (12) *in vitro* and in a rodent model *in vivo* (12,14,25), we wondered whether up-regulation of β -catenin might in some way enhance reovirus replication. However, we found that overexpression of β -catenin as induced by exposure to the GSK-3 β inhibitor LiCl, did not enhance reoviral replication in HEK293 and HCT116 cells. Thus, at present it appears that β -catenin does not play a role similar to that of the activated Ras oncoprotein to facilitate reovirus replication in the cell. Consistent with our results, treatment of pancreatic cancer cells with GSK-3 β inhibitor also decreases cell survival and proliferation (29).

GSK-3 β is active in resting cells, leading to the constitutive degradation of β -catenin via ubiquitin-proteasomes (21). During Wnt/ β -catenin or PI-3 kinase signaling, GSK-3 β becomes phosphorylated and inactivated, and subsequently β -catenin is up-regulated (32,33). Considering that inhibition of GSK-3 β results in the up-regulation of various proto-oncogenes such as β -catenin, cyclin-D1, and c-Myc, the inhibition of GSK-3 β may increase the risk of carcinogenesis (34). On the other hand, GSK-3 β -knockout MEFs have been used to show that GSK-3 β participates in cell survival and proliferation (23), and GSK-3 β inhibition also suppressed the

development of cancer in a xenograft of a tumor model (35).

Consistent with this, GSK-3 β also provides a pro-survival signal in a Wnt3/ β -catenin-independent manner (36,37). GSK-3 β physically interacts with and activates MEKK1, leading to stimulation of JNK, p42/p44 MAPK, and NF- κ B pathways (36). In addition, GSK-3 β depletion abolishes the TNF-induced phosphorylation of p65, a subunit of the NF- κ B complex (37). Herein, a role for GSK-3 β as a pro-survival signal molecule is consistent with our results showing the reduction of reovirus-induced NF- κ B activation and the enhancement of reovirus-induced apoptosis through GSK-3 β inhibition by LiCl or the small molecule inhibitor AR-A014418 (Figs. 4 and 5). Furthermore, we demonstrated that inhibition of NF- κ B enhances reovirus-mediated cell death in HEK293 cells or HCT116 colon cancer cells during reovirus infection, confirming that blockage of NF- κ B with the GSK-3 β inhibitor promotes reovirus-induced apoptosis (Figs. 4 and 5). Therefore, inhibitors of GSK-3 β may have therapeutic potential in cancers and inflammation as mediated by NF- κ B.

Taken together, our results raise the possibility that combined treatment with reovirus and GSK-3 β inhibitors may provide an enhanced therapeutic approach for the treatment of colon cancer compared to reovirus treatment alone.

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