Lithium Sensitizes Tumor Cells in an NF-κB-independent Way to Caspase Activation and Apoptosis Induced by Tumor Necrosis Factor (TNF)

EVIDENCE FOR A ROLE OF THE TNF RECEPTOR-ASSOCIATED DEATH DOMAIN PROTEIN*

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We have previously shown that lithium salts can considerably increase the direct cytotoxic effect of tumor necrosis factor (TNF) on various tumor cells in vitro and in vivo. However, the underlying mechanism has remained largely unknown. Here we show that the TNF-sensitizing effect of lithium chloride (LiCl) is independent of the type of cell death, either necrosis or apoptosis. In the case of apoptosis, TNF/lithium synergism is associated with an enhanced activation of caspases and mitochondrial cytochrome c release. Sensitization to apoptosis is specific for TNF-induced apoptosis, whereas Fas-mediated or etoposide-induced apoptosis remains unaffected. LiCl also potentiates cell death induced by artificial oligomerization of a fusion protein between FKBP and the TNF receptor-associated death domain protein. TNF-induced activation of NF-κB-dependent gene expression is not modulated by LiCl treatment. These results indicate that LiCl enhances TNF-induced cell death in an NF-κB-independent way, and suggest that the TNF receptor-associated death domain protein plays a crucial role in the TNF-sensitizing effect of LiCl.

Tumor necrosis factor (TNF)1 is a cytokine that is mainly produced by activated macrophages; also lymphoid cells, natural killer cells, neutrophils, keratinocytes, and fibroblasts can produce this cytokine in response to various challenges (1). The first interest in TNF arose from its potential antitumor properties because of a specific cytotoxic effect of TNF on several transformed cell lines (2). Also in vivo, TNF showed considerable antitumor activity in a number of murine tumor models (3). The antitumor effect of TNF is mediated by direct cytotoxic effects on the tumor cells as well as by several effects on the host cells. Because TNF receptors (TNF-R) are ubiquitously expressed, it is not surprising that almost all cell types respond to TNF. Many cell types respond to TNF by the expression of specific proteins, including other cytokines, adhesion proteins, and enzymes involved in the production of proinflammatory mediators (1). These proinflammatory effects of TNF lead to several toxic side effects in vivo after injection of high TNF doses (4). The latter, as well as the rather low sensitivity of several tumor cells to the direct cytotoxic effect of TNF, have limited the use of TNF as an antitumor agent. However, combination treatments that allow the use of lower nontoxic TNF doses might still offer opportunities for TNF in the treatment of cancer. Combination treatments have already proven their success in the clinic, as demonstrated by isolated limb perfusion of melanoma and carcinoma patients (5, 6). However, a systemic treatment is still hampered by toxic side effects. Unexpectedly, lithium chloride was found previously to considerably increase the direct cytotoxic activity of TNF on tumor cells in vitro and in animal studies, without significant side effects (7). The potential use of lithium as a sensitizing agent for the antitumoral activities of TNF is interesting in view of the well known use of lithium in the treatment of manic depression (8). So far, the underlying mechanism for TNF/lithium synergism is still unclear.

TNF-treated cancer cells die from either necrosis or apoptosis (9). Necrosis is often referred to as accidental cell death and is induced when the plasma membrane is damaged in such a way that cells swell and finally burst, releasing their cellular content. In contrast to necrosis, apoptosis is the commonest physiological form of cell death that also occurs during embryonic development, tissue remodeling, and immune regulation. Cells undergoing apoptosis show a sequence of cardinal morphological features including membrane blebbing, cellular shrinkage, and condensation of chromatin. It is also a clean way of dying, in which cells fall apart in so-called apoptotic bodies, which are removed by phagocytosis. So far, the reason why some cells die by necrosis whereas others die by apoptosis is still unknown. Recently, a new family of cysteine proteases (caspases) has been shown to play a crucial role in apoptosis. All caspases, currently 14 in number, share the unusual property of being cysteine proteases with specificity for cleaving substrates on the carbonyl side of aspartic acid residues (10, 11). Caspases are synthesized as proenzymes with a tripartite structure consisting of an N-terminal prodomain that is variable in length, a large subunit with an approximate size of 20 kDa (p20), and a second smaller subunit of about 10 kDa (p10); the mature caspase is a (p20p10)2 heterotetramer. Processing of caspases to a mature product occurs following different apoptotic stimuli (12) and requires cleavage at specific aspartic acid residues located between the different subdomains. The...
question of how an apoptotic stimulus is linked to the activation of caspase-8 (13, 14). This protein associates through its prodomain with the Fas-associated death domain (FADD), which is a component of the p55 TNF-R (TNF-R1) and the Fas receptor complex. Stimulus-induced recruitment of caspase-8 to the receptor leads to oligomerization-induced autoprocessing and activation of caspase-8 (14, 15), which then processes and activates caspase-3 and caspase-7. Binding of the trimeric TNF ligand to the extracellular domain of TNF-R1 induces oligomerization of the receptor death domains (DD). This leads to recruitment of the adapter protein TNF-R-associated DD (TRADD), which acts as a scaffold for the assembly of a signaling complex containing different proteins that mediate several TNF activities. The DD of TRADD associates with that of FADD, which mediates apoptosis by activating caspase-8 and a downstream caspase cascade (14). Apoptosis initiated by Fas triggering follows a similar pathway, but in this case FADD is directly recruited to the Fas receptor without involvement of TRADD. Caspases also initiate a mitochondrial pathway leading to release of several mediators that further amplify the caspase cascade by acting as direct activators of specific caspases (16–18). In addition to the proapoptotic TRADD/ FADD/caspase-8 pathway initiated by TNF, TNF-R triggering also initiates an antiapoptotic pathway believed to be mediated by the transcription factor NF-κB. The expression of several proinflammatory and antiapoptotic proteins is known to be NF-κB-dependent (19, 20). Moreover, inhibition of NF-κB-dependent gene expression has recently been shown to sensitize cancer cells to TNF-induced apoptosis (21).

In the present study, we analyze the level at which lithium ions interfere with the TNF-signaling pathway leading to cell death. We demonstrate that lithium sensitizes cancer cells to TNF-induced cell death in an NF-κB-independent way. Furthermore, we present evidence for a role of TRADD as a potential target for LiCl.

MATERIALS AND METHODS

Cells and Reagents—The human rhabdomyosarcoma cell line KYM37E4 was maintained in RPMI 1640 supplemented with 10% fetal calf serum. The murine fibrosarcoma cell line L929 was grown in modified Eagle’s medium supplemented with 10% fetal calf serum. The Fas-expressing human rhabdomyosarcoma cell line RD was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The protease inhibitors benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone and benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone were purchased from Enzyme Systems Products (Dublin, CA); they were dissolved as stock solutions of 100 mM in Me2SO and stored at −70 °C until use. Acetyl-Tyr-Val-Ala-Asp-chloromethylketone and benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin in a total volume of 150 μl of reaction buffer (10 mM HEPES-NaOH, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM K2PO4, 0.5 mM EGTA, 2 mM MgCl2, 5 mM pyruvate, 0.1 mM phenylmethysulfonyl fluoride), containing 0.001% digitonin. Lysates were cleared by centrifugation, and 100 μg of cytosolic protein was subjected to SDS-polyacrylamide gel electrophoresis. To measure β-catenin accumulation, 106 cells were incubated in serum-free medium for 12 h and subsequently treated with GSK-3 inhibitors for 6 h. Cells were washed twice in PBS, and whole-cell extracts were prepared by adding 1× sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% mercaptoethanol). Lysates were subjected to SDS- polyacrylamide gel electrophoresis. Proteins were transferred to Hybond nitrocellulose membranes, blocked with 5% dry milk in phosphate-buffered saline containing 0.1% Tween 20; they were probed with anti-cytokeratin c, anti-human caspase-3 or -8, or anti-β-catenin according to the manufacturer’s instructions. Blots were developed with a chemiluminescence method (PerkinElmer Life Sciences).

Fluorometric Assay for Caspase-3-like Activity—Cellular extracts were prepared as described under “Isolation of Cell Extracts and Western Blotting.” 25 μg of protein was incubated for 1 h at 30 °C with 50 μM of acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin in a total volume of 150 μl of reaction buffer (10 mM HEPES-NaOH, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM K2PO4, 0.5 mM EGTA, 2 mM MgCl2, 5 mM pyruvate, 0.1 mM phenylmethysulfonyl fluoride, 1 mM diethiothreitol). The release of aminomethylcoumarin was determined by spectrofluorometry (CytoFluor; PerSeptive Biosystems, Cambridge, MA). Data are expressed as increase in fluorescence as a function of time (ΔF/min).

NF-κB Reporter Gene Assay—L929/Fluc cells stably expressing a luciferase reporter gene under control of the minimal chicken β-globin promoter, preceded by three NF-κB-binding sites (22), were seeded on day −1 in a 96-well plate at 2 × 103 cells/well. The next day, the cells were either untreated or pretreated with 10 mM LiCl for 1 h and subsequently stimulated with TNF. After 3 h of incubation, cells were lysed in 200 μl of lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM cyclohexanediaminetetraacetic acid, 10% glycerol, and 1% Triton X-100). The luciferase activity was determined as described previously (27).

Cytotoxicity Assays—Cells were seeded on day −1 at 2 × 104 cells/well in a 96-well plate. The next day, cells were stimulated with a serial dilution of TNF, anti-Fas, or etoposide in the absence or presence of LiCl or GSK-3 inhibitors. Transcription- or translation-independent cell death was analyzed in the presence of 1 μM/ml actinomycin D or 10 μg/ml cycloheximide, respectively. Typically, cells were incubated for 18 h, and cell viability was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as previously described (28, 29). Cell death of L929 cells stably expressing FKBP3–TRADD was induced by stimulating the cells with 62.5 nM of the dimerizing agent AP1510 (ARIAD) for 24 h. The percentage of cell survival was calculated using the equation 100% × (Auntreated - Apretreated) / Auntreated × 100% (Auntreated—untreated cells – Apretreated medium)/ (Auntreated—untreated cells – Aygrosin medium).

RESULTS

The TNF-sensitizing Effect of LiCl Is Independent of the Type of Cell Death—to investigate whether the TNF/LiCl synergy depends on the type of cell death, we analyzed the effect of LiCl on TNF-induced killing of L929 and KYM37E4 cells. L929 cells were previously shown to die by necrosis (23), whereas KYM37E4 die in an apoptotic way in response to TNF. Moreover, necrosis of L929 cells is caspase-independent, whereas apoptosis of KYM37E4 cells strictly depends on caspase activ-
Sensitization by Lithium of Tumor Cells

FIG. 1. Sensitizing effect of LiCl on TNF-induced necrosis and apoptosis of L929 and Kym37E4 cells. Different concentrations of LiCl (△, control; △, 2.5 mM; ▲, 5 mM; ■, 10 mM) were added 1 h prior to TNF treatment. LiCl treatment as such had no effect on cell viability (data not shown). Results are representative of three independent experiments (S.D. <10%).

Mitochondria are believed to contribute to TNF-induced caspase activation and apoptosis by releasing cytochrome c, which is directly involved in the activation of caspase-9, a direct activator of caspase-3 (31). To analyze whether LiCl-induced sensitization of caspase-3 activation in response to TNF was associated with an enhanced release of cytochrome c in TNF + LiCl-treated cells, we analyzed cytosolic cytochrome c levels by Western blotting. This showed that LiCl also increased the TNF-induced activation of caspase-3-like activity in Kym37E4 cell extracts (Fig. 3). This was associated with an increased proteolytic processing of caspase-3, as demonstrated by the faster disappearance of procaspase-3 in TNF/LiCl-treated cells versus cells treated with TNF alone (Fig. 4).

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The Apoptosis-sensitizing Effect of LiCl Is Specific for TNF and Localized at the Level of TRADD—In order to analyze whether the sensitizing effect of LiCl is specific for TNF, we compared the effect of LiCl on apoptosis induced by TNF and Fas agonistic antibodies. To avoid a potential contribution of cell type-specific effects, we analyzed the effect of TNF and Fas in L929.hFas cells. Treatment with TNF leads to typical necrosis, whereas treatment of the same cells with agonistic Fas antibodies results in typical apoptosis (23). Whereas LiCl was able to induce a 100-fold sensitization to TNF cytotoxicity in L929 cells, Fas-mediated killing of the same cell line was not modulated by LiCl (Fig. 5, A and B). Similarly, when Kym37E4 cells were stimulated with TNF, etoposide, or staurosporine (data not shown), only TNF-induced apoptosis was enhanced by LiCl treatment (Fig. 5, C–E). These results demonstrate that the sensitizing effect of LiCl is specific for TNF.

Fas-mediated signaling to cell death is largely overlapping TNF signaling. Fas directly binds to FADD, whereas TNF-R initiates FADD clustering via the adapter protein TRADD. The specific effect of LiCl on TNF-induced cell death suggests that LiCl interferes with a step upstream of FADD. To distinguish between TRADD or TNF-R as targets for LiCl, we generated an L929 cell line that stably expresses TRADD fused to two tandem repeated FKBP dimerization domains. This fusion protein can be oligomerized by the cell-permeable artificial ligand AP1510 (34), resulting in TNF-R-independent cell death. These data suggest that self-association of TRADD is necessary and sufficient to induce cell death. Moreover, LiCl was still able to enhance cell death induced by the oligomerizer (Fig. 6). These data strongly suggest that the potentiating effect of LiCl on TNF cytotoxicity is situated downstream of TNF-R, most likely at the level of TRADD.

LiCl-induced TNF Sensitization Does Not Involve NF-κB or GSK-3β—NF-κB becomes activated in several cell lines after stimulation with TNF and is responsible for the transcription of many proinflammatory genes. More recently, an antiapoptotic function for NF-κB has been reported (35). The signal transduction pathway leading to NF-κB activation in response to TNF has been largely elucidated (36). TRADD seems to act as a point of bifurcation, from which both proapoptotic and NF-κB activation pathways start. Interaction of TRADD with FADD initiates a signaling pathway leading to apoptosis (37, 38), whereas the interaction of TRADD with receptor-interacting protein and TNF-R-associated factor-2 is crucial for NF-κB activation (37, 39). To analyze whether LiCl potentiates TNF-induced cell death by preventing TNF-induced NF-κB activation and expression of antiapoptotic genes, we studied the effect of LiCl on the TNF-induced expression of an NF-κB-dependent reporter gene in L929 cells. As shown in Fig. 7, there was no effect of LiCl on NF-κB-dependent gene expression, indicating that LiCl sensitizes cells to TNF-induced cell death in an NF-κB-independent way. Moreover, the sensitizing effect of LiCl could still be observed in the presence of inhibitors of transcription or translation, such as actinomycin D and cycloheximide, respectively (Fig. 8). As previously demonstrated (40), transcriptional or translational inhibition by these inhibitors is associated with a strong TNF-sensitizing effect. These results show that the effect of LiCl on TNF cytotoxicity is independent of de novo transcription or protein synthesis, making the involvement of an LiCl-induced change in expression of proapoptotic or antiapoptotic proteins unlikely. Recently, an increased cytotoxic effect of TNF was shown in GSK-3β-deficient cells (41), suggesting a protective function for GSK-3β against TNF-induced cell death. In addition, GSK-3 was demonstrated to be essential for NF-κB-dependent gene expression in response to TNF. Because LiCl is known to directly inhibit GSK-3 activity (42), one could imagine a potential role for...
GSK-3 in the LiCl-induced sensitization against TNF. However, our observation that LiCl has no effect on TNF-induced NF-κB activation suggests that GSK-3 is not involved in the lithium-induced TNF sensitization of the cells studied. Nevertheless, it is conceivable that GSK-3 could still play a role in the LiCl-induced TNF sensitization through an effect independent of NF-κB. Therefore, we analyzed the effect of the GSK-3 inhibitors Ro31–8220 (43), valproic acid (44), and indirubin-3′-monoxine (45) on TNF-induced cell death. However, none of these inhibitors was able to mimic the effect of LiCl on TNF cytotoxicity (partially shown in Fig. 9A). GSK-3 inhibition was confirmed by analyzing the effect of the drugs on the accumulation of β-catenin (partially shown in Fig. 9B), which has previously been shown to be negatively regulated by GSK-3-mediated phosphorylation (46). The above findings make it rather unlikely that inhibition of GSK-3 is involved in the TNF-sensitizing effect of LiCl in the cells studied.

**DISCUSSION**

We have previously shown that treatment of cells with lithium salt considerably increases the direct cytotoxic effect of TNF on tumor cells, both in vitro and in vivo (7). However, the underlying mechanism has remained largely unknown. In this report, we present evidence that LiCl potentiates TNF cytotoxicity by acting at an early step in the TNF-signaling pathway. First, we demonstrate that the TNF-sensitizing effect of LiCl is independent of the type of cell death, more particularly necrosis in the case of L929 fibrosarcoma cells and apoptosis in the case of KYM rhabdomyosarcoma cells. Although it is still unclear which signals determine whether a cell will die by necrosis or apoptosis in response to TNF, recent experiments in our laboratory showed that TNF-induced necrosis is not associated with caspase activation, whereas apoptosis is strictly dependent on caspases (30). The fact that LiCl potentiates both types of cell death indicates that LiCl acts upstream of the point of bifurcation of signaling pathways leading to necrosis or apo-
ptosis. Second, potentiation of TNF-induced apoptosis by LiCl was associated with an increased processing of procaspase-8 and an early release of cytochrome c from the mitochondria. Caspase-8 is known as an initiator caspase, being part of the TNF-R complex (47). Third, although TNF-induced and Fas-induced signaling pathways leading to caspase activation and apoptosis converge at the level of FADD, only differing in the requirement of TRADD as an adapter protein between TNF-RI and FADD, LiCl specifically increases TNF-induced cell death. Although we cannot exclude the existence of a TNF-specific pathway (still unknown), the results mentioned above suggest that LiCl acts upstream of FADD, more specifically at the level of the TNF-R itself or of TRADD. However, cell death induced by enforced oligomerization of a stably expressed FKBP2-TRADD fusion protein with a synthetic oligomerizer (26) was still potentiated by LiCl cotreatment, excluding an effect of LiCl at the level of the TNF-R. Moreover, we were unable to demonstrate any effect of LiCl on the binding of TNF to its receptor (data not shown). By binding to receptor-interacting

**FIG. 5.** LiCl specifically potentiates the cytotoxic effect of TNF. L929.hFas cells were pretreated for 1 h with LiCl (control; 10 mM) and stimulated with a serial dilution of TNF (A) and anti-Fas antibodies (B). Similarly, KYM37E4 cells were pretreated with LiCl (control; 10 mM) and stimulated with a serial dilution of TNF (C) or etoposide (D). LiCl treatment alone had no effect on cell viability (data not shown). Results are representative of three independent experiments.

**FIG. 6.** LiCl potentiates cell death induced by TRADD oligomerization. L929 cells stably expressing an FRKBp2-TRADD fusion protein were pretreated for 1 h with LiCl and subsequently stimulated with the oligomerizer AP1510 (solid lines) or TNF (open lines). Treatment with LiCl as such had no effect on cell survival (open triangles). Results are representative of three independent experiments.

**FIG. 7.** LiCl has no effect on NF-κB-dependent gene expression in response to TNF. L929 luc cells stably expressing an NF-κB-dependent luciferase reporter gene were either untreated (open circle) or treated (open squares) for 1 h with 10 mM LiCl and subsequently stimulated for 3 h with a serial dilution of TNF. Cells were then lysed and assayed for luciferase (Luc) activity.

**FIG. 8.** LiCl enhances TNF cytotoxicity in the presence of transcriptional or translational inhibitors. L929 cells were treated with TNF (solid lines) or TNF plus 10 mM LiCl (broken lines) in the absence (open circle) or presence of 1 μg/ml actinomycin D (open squares) or 10 μg/ml cycloheximide (open triangles). Cell viability was measured after 18 h. Results are representative of at least three independent experiments (S.D. <10%).
protein and TNF-R-associated factor-2, TRADD is also involved in the initiation of NF-κB activation in response to TNF (37, 38). However, LiCl did not modulate TNF-induced NF-κB activation. These results also exclude the possibility that LiCl enhances TNF-induced apoptosis by preventing the antiapoptotic function of NF-κB. Moreover, the sensitizing effect of LiCl could still be observed in the presence of transcriptional or translational inhibitors, excluding a role for de novo protein synthesis in LiCl-induced TNF sensitization. The inability of LiCl to modulate NF-κB activation in response to TNF is in contrast with a recently published observation that LiCl inhibits TNF-induced NF-κB activation via inhibition of GSK-3 (41). Because GSK-3-deficient cells are hypersensitive to TNF, it has also been concluded that inhibition of GSK-3 and NF-κB-dependent gene expression by LiCl is directly responsible for the TNF-sensitizing effect of LiCl. The reason for this discrepancy with our results is still unclear. However, it should be noted that the inhibitory effect of LiCl on TNF-induced NF-κB activation was previously shown in the presence of 30 mM LiCl (41), which is considerably higher than the concentration needed for the potentiating effect on TNF-induced cell death, namely 2.5–10 mM. Moreover, if LiCl would inhibit NF-κB-dependent gene expression in response to TNF, we would expect that LiCl also inhibits the TNF-induced expression of NF-κB-responsive genes such as interleukin-6, which is not the case (28). In contrast, TNF-induced levels of interleukin-6 were even enhanced in the presence of LiCl. Furthermore, we also obtained evidence against an NF-κB-independent function of GSK-3 in lithium-induced TNF sensitization. First, the GSK-3 inhibitors indirubin-3′-monoxide, valproic acid, and Ro31–8220 had no effect on the cytotoxic activity of TNF. Second, treatment of the cells with insulin, which is known to inhibit GSK-3 activity (48), was unable to mimic the TNF-sensitizing effect of LiCl. Although we cannot exclude a cell type-specific involvement of GSK-3 in the modulation of TNF sensitivity, the above observations suggest at least the existence of an additional mechanism responsible for the sensitizing effect of LiCl on TNF-induced cell death.

The therapeutic application of TNF in cancer treatment is limited by the fact that many tumor cells are only weakly sensitive to its direct cytotoxic action. To overcome this problem of TNF resistance as well as to be able to use lower doses of TNF with less side effects, agents that specifically increase the sensitivity of tumor cells to TNF might be very promising. Since lithium has been used for many years in the clinic to treat manic depression, the TNF-sensitizing effect of LiCl is intriguing and merits further experiments aimed to understand the underlying mechanism. We have demonstrated in this study that LiCl sensitizes cells to TNF-induced apoptosis in an NF-κB- and GSK-3-independent way. Moreover, we obtained evidence for a crucial role of TRADD in the TNF/LiCl synergism. It would be interesting to investigate how LiCl affects the signaling potential of TRADD.

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