**α2β1 Integrin Promotes Chemoresistance against Doxorubicin in Cancer Cells through Extracellular Signal-regulated Kinase (ERK)**

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Dallal Naci1,1, 1Mohammed-Amine El Azreq1, 2Nizar Chetoui1, 1Laura Lauden1,2, 3François Sigaux1, 4Dominique Charron1, 4Reem Al-Daccak1, and Fawzi Aoudjit1,3

From the 1Centre de Recherche en Rhumatologie/Immunologie, Centre Hospitalier Universitaire de Québec, Pavillon CHUL, and Faculté de Médecine, Université Laval, 2705 Boulevard Laurier, Local T1-49, Québec G1V4G2, Canada, 2Institut National de la Santé et de la Recherche Médicale (INSERM) U940, Institut Universitaire d’Hématologie Université Paris Denis Diderot, Hôpital Saint Louis, 75010 Paris, France, and 3INSERM U944, Institut Universitaire d’Hématologie Université Paris Denis Diderot, Hôpital Saint Louis, 75010 Paris, France

**Background:** Mechanisms by which β1 integrins regulate resistance of cancer cells to chemotherapy.

**Results:** α2β1 integrin but not fibronectin-binding integrins inhibits doxorubicin-induced apoptosis of leukemic T cells via JNK inhibition and Mcl-1 up-regulation in an MAPK/ERK-dependent pathway.

**Conclusion:** α2β1 integrin/ERK pathway promotes chemoresistance of cancer cells.

**Significance:** Activation of this pathway can contribute to the appearance of the drug resistance phenotype.

The role and the mechanisms by which β1 integrins regulate the survival and chemoresistance of T cell acute lymphoblastic leukemia (T-ALL) still are poorly addressed. In this study, we demonstrate in T-ALL cell lines and primary blasts, that engagement of α2β1 integrin with its ligand collagen I (ColI), reduces doxorubicin-induced apoptosis, whereas fibronectin (Fn) had no effect. ColI but not Fn inhibited doxorubicin-induced mitochondrial depolarization, cytochrome c release, and activation of caspase-9 and -3. ColI but not Fn also prevented doxorubicin-induced cell death in T lymphocytes (5), and ligation of death receptors in dendritic cells (6). Integrin-mediated cell survival has been associated with the activation of the two major cell survival pathways, the phosphatidylinositol 3-kinase (PI3K)/serine/threonine kinase (AKT) and the MAPK/ERK pathways (1). Integrin-mediated cell survival is essential for the maintenance of Mcl-1 levels and for ColI-mediated survival of T-ALL cell survival. Furthermore, activation of MAPK/ERK, but not PI3K/AKT, is required for ColI-mediated inhibition of doxorubicin-induced JNK activation and apoptosis and for ColI-mediated maintenance of Mcl-1 levels.

Integrins are α/β heterodimeric membrane proteins that mediate cell adhesion to the surrounding extracellular matrix (ECM).4 In addition to their anchoring function, integrins induce several intracellular signals that modulate cell behavior (1). Normal epithelial and endothelial cells depend on integrin signals for cell cycle progression and disruption of matrix attachment induces their apoptosis, a process termed anoikis (2). We and others (3, 4) have shown that integrins can also protect the cells against cytokine withdrawal, activation-induced cell death in T lymphocytes (5), and ligation of death receptors in endothelial cells (6). Integrin-mediated cell survival has been associated with the activation of the two major cell survival pathways, the phosphatidylinositol 3-kinase (PI3K)/serine/threonine kinase (AKT) and the MAPK/ERK pathways (1).

Growing evidence suggests that tumor cell interactions with ECM of their microenvironment are important for tumor resistance against chemotherapy-induced apoptosis. However, the mechanisms by which integrins promote chemoresistance in cancer cells are still not fully understood. The α4β1 integrin, which binds fibronectin (Fn) protects hematopoietic malignancies, including myeloma (7) B cell leukemia (8), and myeloid leukemia cell lines (9) from the apoptotic effects of melphalan and Ara-c, and from radiation-induced apoptosis (10).

Malignant T cells such as T cell acute lymphoblastic leukemia (T-ALL) cell lines express several β1 integrins, which serve as receptors for collagens, fibronectin and laminins (11). T-ALL is a hematopoietic malignancy, which also grows in the bone marrow that is rich in ECM (12). We have previously demonstrated that collagen type I (ColI) inhibited Fas-induced apoptosis of the Jurkat T-ALL cell line (13). ColI was also shown to protect Jurkat cells from serum starvation-induced apoptosis (14). In contrast, Fn has been shown to induce apoptosis of some leukemic T cell lines (15). Whether ECM proteins regu-

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4 The abbreviations used are: ECM, extracellular matrix; ColI, collagen I; Fn, fibronectin; T-ALL, T cell acute lymphoblastic leukemia; MSC, mesenchymal stem cell; DN, dominant-negative.
late the response of T-ALL cells to chemotherapy is currently unclear. Resistance of cancer cells to apoptosis is a major hurdle in anti-cancer therapies, and understanding how these cells escape apoptosis is likely to lead to new therapeutic avenues.

Chemotherapy-induced apoptosis involves the activation of the mitochondrial death pathway (16), which is regulated tightly by the balance between pro- and antiapoptotic Bcl-2 family proteins (17, 18). Activation of Bcl-2 proapoptotic proteins leads to the permeabilization of the mitochondria, and to the release into the cytosol of apoptogenic factors such as cytochrome c. Cytochrome c then participates in the activation of caspase-9, which, in turn, activates executioner caspases.

In this study, we investigated the regulation of doxorubicin-induced apoptosis of T-ALL cells by β1 integrin signaling. Doxorubicin is a drug widely used in anti-cancer therapy, including in T cell malignancies. We show that engagement of α2β1 integrin by its ligand Coll, inhibited doxorubicin-induced apoptosis of T-ALL cells by inhibiting activation of the c-Jun N-terminal kinase (JNK). This resulted in the maintenance of the prosurvival McI-1 levels. The protective effect of Coll is mediated through the activation of the MAPK/ERK survival pathway. In contrast to Coll, Fn, previously shown to be a weak inducer of MAPK/ERK in Jurkat cells (13, 19), had no effect on doxorubicin-induced JNK activation, did not maintain McI-1 levels and thereby did not protect T-ALL cells from doxorubicin-induced apoptosis. Our study demonstrates an important survival role for α2β1 integrin and its ligand Coll in drug-induced apoptosis of T-ALL cells and suggests that its activation can contribute to the generation of drug resistance.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—Collagen type I and doxorubicin were from Sigma. Human fibronectin was purchased from Millipore (Billerica, MA). The inhibitors of PI3K/AKT (LY294002), JNK (SP600125), and MEK-1 (U0126) were from Calbiochem (San Diego, CA). Antibodies were obtained as follows: anti-phospho-p44/42 MAPK (E-4), anti-ERK2 (C-14), anti-caspase 3, which detects the native and the active fragments of caspase-3, anti-Mcl-1, anti-Bcl-2, and anti-β-actin (C-2) were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-AKT (Ser-473), anti-AKT, anti-caspase-9, which detects the native and active fragments of caspase-9, anti-Bcl-xL, anti-phospho-JNK1/2 (G9), and anti-JNK-2 (9252) were from Cell Signaling Technologies (Beverly, MA). Phycocerythrin-conjugated anti-human CD49b (α2 integrin) and allopheocyanin-conjugated anti-CD29 (β1 integrin) and isotypic control antibodies were from BD Pharmingen. The anti-β1 (4B4) and anti-α2 (P1E6) integrin blocking antibodies were purchased from Beckman Coulter (Brea, CA) and Millipore, respectively.

**Cell Culture and Primary T-ALL Blasts**—The human T-ALL cell lines Jurkat (E6.1) and HSB-2 were obtained from ATCC (Manassas, VA) and were maintained in RPMI 1640 medium supplemented with 10% of fetal bovine serum (FBS), 2 mmol/liter glutamine, and 100 units/ml penicillin and streptomycin (complete medium). T-ALL patients were diagnosed and treated at Hôpital Saint-Louis (Paris, France). Informed consent was obtained from the patients or relatives in accordance with the Declaration of Helsinki, and the study was approved by the Hôpital Saint-Louis and Institut Universitaire d'Hematologie Institutional Review Board. The study was carried out with cryopreserved leukemic cells from the bone marrow of patients at diagnosis. Two patients were diagnosed as stage III (cortical immature T-ALL) and one patient as stage IV (mature T-ALL). The expression of α2 and β1 integrin chains on these T-ALL blasts was determined by the use of phycocerythrin-conjugated anti-α2 (CD49b) and allopheocyanin-conjugated anti-β1 (CD29) specific antibodies. Samples were analyzed by Canto II flow cytometer (BD Biosciences).

**Determination of Apoptosis and Clonogenic Survival**—T-ALL cells were resuspended at 1×10⁶/ml in RPMI 1640 medium containing 2.5% serum. The cells were then seeded in 24-well plates (5×10⁵/well) and activated or not for 4 h with 100 μg/ml of ECM (Coll, Fn). The cell cultures were then treated with doxorubicin at 250 ng/ml for Jurkat and HSB-2 T cell lines, and at 600 ng/ml for T-ALL primary blasts. After 16 to 24 h of drug treatment, apoptosis was determined by annexin V staining and flow cytometry analysis using the FACSCalibur cytometer (BD Biosciences) as we described previously (13). Apoptosis also was determined by a cell death detection ELISA kit measuring DNA fragmentation (Roche Applied Science) as we described previously (13).

For clonogenic survival assays, the cells were activated or not with ECM for 4 h and then treated with doxorubicin for 24 h. The cells were then washed and seeded in complete RPMI medium containing 1% methylcellulose (StemCell Technologies, Vancouver, BC) at 1×10⁴ cells/ml. After 21 days, colonies with >40 cells were counted.

**Co-culture of Jurkat Cells with Bone Marrow Stromal Cells**—Human bone marrow-derived mesenchymal stem cells (MSCs) were a generous gift from Dr. Nicholas Pineault (Hema-Québec, Québec, Canada) and were described previously (20). The co-culture of Jurkat cells with MSCs and drug treatment was carried out as described previously (21, 22). MSCs at passage 2 or 3 were seeded in 24-well plates for 24 h to form a monolayer. Jurkat cells (5×10⁵ in 500 μl of RPMI medium) were kept in suspension or co-cultured with the monolayer of MSCs for 24 h. The cultures were then treated with doxorubicin (250 ng/ml) for 24 h. Jurkat cells were separated from MSCs by pipetting with ice-cold PBS. This treatment did not affect the MSC monolayer nor did it result in the detachment of MSCs. Apoptosis of Jurkat cells in suspension or cocultured with MSCs was then evaluated as described above.

**Caspase Activation, Expression of Bcl-2 Proteins, Activation of ERK, AKT, and JNK**—Activation of caspase-9 and caspase-3, expression of Mcl-1, Bcl-2, and Bcl-xL, and phosphorylation of ERK1/2, JNK1/2, and AKT were determined by immunoblot analysis using specific antibodies as we described previously (23).

**Mitochondrial Depolarization and Cytochrome c Release**—Loss of the mitochondrial membrane potential (ΔΨm) was measured by staining the cells with DioC₆(3) (Molecular Probes, Eugene, OR) and flow cytometry analysis as described previously (24). Cytochrome c release was determined by immunoblot analysis of cytosolic fractions with an anti-cytochrome c antibody (clone 7H8.2C12; BD Pharmingen) as we described previously (25). The purity of the cytosolic fractions was verified by immunoblot analysis using an antibody against...
the mitochondrial cytochrome c oxidase (antibody 20E8-C12, Molecular Probes) as we described previously (25). We found that only the unbroken cell fraction, which contains the mitochondria and not the cytosolic fraction, contains cytochrome c oxidase (data not shown).

Plasmids and Cell Transfection—The plasmids encoding the dominant-negative forms of MEK-1 (DN-MEK-1) and AKT (DN-AKT) were used in our previous studies (13, 23, 25). Jurkat cells were transfected by electroporation as we described previously (13).

RNA Interference—Silencer-validated siRNA-specific for Mcl-1 (siRNA ID 120644; sense, 5′-GGACUUUUUAUACCUGUUAUtt-3′; antisense, 5′-AUAAACAGGUAAAAGUGC-Ctg-3′) and Silencer negative control siRNA were from Ambion and were used in our previous study (23). A second Silencer-validated siRNA sequence targeting Mcl-1 (siRNA ID 4170; sense, 5′-CCAGUAAAAUCUUCAGAAAtt-3′; antisense, 5′-UUUCAAAGAAGUAUCUGGa-3′) and its Silencer negative control sequence were also from Ambion. 5 × 10^6 Jurkat cells were transfected with 200 nmol/liter of Mcl-1 specific siRNA or negative control siRNA using the nucleofector method from Amaxa according to the manufacturer’s instructions. Jurkat cells were mixed with siRNA in the T cell nucleofector solution (solution 5) and transfected using the C016 program. The cells were then cultured in RPMI supplemented with 10% of fetal bovine serum for 6 h. Viable cells were recovered by Ficoll-hypaque density gradient centrifugation and used in subsequent apoptosis experiments. The inhibition of Mcl-1 protein expression was assessed by immunoblot analysis using Mcl-1-specific antibody.

Statistical Analysis—Statistical analysis was performed by the Student’s t test. Results with \( p < 0.05 \) were considered significant.

RESULTS

Collagen Type I-αβ1 Integrin Inhibits Doxorubicin-induced Apoptosis—The effects of Coll and Fn, two major matrix proteins of the bone marrow microenvironment, on doxorubicin-induced apoptosis were examined in two established T-ALL cell lines, Jurkat and HSB-2. We found that preactivation of Jurkat and HSB-2 cells for 3–4 h with Coll but not with Fn inhibited doxorubicin-induced apoptosis; the percentage of annexin V-positive cells was decreased by 30% (Fig. 1a), and DNA fragmentation was inhibited by ~38% (Fig. 1b). Preactivation of the cells for >4 h with Coll but not with Fn also inhibited doxorubicin-induced apoptosis, whereas treatment of the cells with poly-L-lysine; a non-integrin binding ligand, did not modulate doxorubicin-induced apoptosis (data not shown).

We then tested whether the combination of both matrix proteins would lead to a synergistic cell survival. Preactivation of the cells simultaneously with Fn and Coll only slightly decreased doxorubicin-induced apoptosis in comparison with cells preactivated with Coll alone; however, this did not reach statistical significance (Fig. 1c). Changing the concentrations of Coll or Fn or varying the time of cellular preactivation did not lead to a further decrease in doxorubicin-induced apoptosis of cells preactivated with both Coll and Fn in comparison with cells preactivated only with Coll (data not shown).

Several studies have previously shown that αβ1 integrin is the major Coll receptor expressed on T cells, including T cell lines (5, 26, 27). Herein, we found that blocking anti-α and anti-β integrin antibodies but not control isotypic antibody significantly block the ability of Coll to reduce doxorubicin-induced apoptosis (Fig. 1d). The blocking antibodies had no effect on doxorubicin-induced apoptosis (data not shown). Together these results indicate that the protective effect of Coll involves αβ1 integrin.

Bone marrow-derived MSCs, which are the producers of ECM in the hematopoietic microenvironment, previously have been shown to protect B cell and myeloid leukemia from chemotherapy (21, 22). Therefore, we evaluated the role of MSCs and of αβ1 integrin in the protection of Jurkat cells from doxorubicin-induced apoptosis. In agreement (28), we found that co-culture of Jurkat cells with MSCs resulted in a reduction of doxorubicin-induced apoptosis compared with cells grown in suspension (Fig. 1e). In addition, we found that anti-α2 and anti-β1 integrin blocking antibodies but not control antibodies also abolished the protective effect of MSCs on Jurkat cells (Fig. 1e). We then carried out clonogenic survival assays to assess whether Coll promoted long term survival. As shown in Fig. 1f, treatment with doxorubicin led to the formation of very few colonies in both T cell lines. However, treatment with doxorubicin in the presence of Coll but not of Fn led to a significantly higher number of colonies in Jurkat and HSB-2 T cell lines. Taken together, these results indicate that Coll via αβ1 integrin could constitute a major pathway contributing to T-ALL drug resistance.

Coll Inhibits Doxorubicin-induced Mitochondria Depolarization, Cytochrome c Release, and Caspase Activation and Maintains Mcl-1 Protein Levels—The mitochondrial death pathway plays a critical role in drug-induced apoptosis including in doxorubicin-induced apoptosis of T-ALL cell lines (29, 30). Thus, we examined whether Coll modulates this apoptotic pathway. Treatment of Jurkat cells with doxorubicin induces the loss of mitochondrial membrane potential, which is reduced by Coll but not by Fn (Fig. 2a). Similarly, Coll but not Fn also reduces doxorubicin-induced cytochrome c release (Fig. 2b). We then examined the regulation of caspase activation. Doxorubicin induces the activation of both caspase-9 and caspase-3 as determined by the reduction in the levels of pro-caspase forms and in the appearance of caspase-9 and caspase-3 active fragments (Fig. 2c). The presence of Coll reduces the capacity of doxorubicin to activate both caspases, whereas Fn, which had no effect on doxorubicin-induced apoptosis, did not modulate doxorubicin-induced caspase activation (Fig. 2c). Coll but not Fn also reduced doxorubicin-induced caspase activation in HSB-2 cells (supplemental Fig. S1). These results indicate that the prosurvival effect of Coll occurs at the level of the mitochondria.

The balance between pro- and anti-apoptotic Bcl-2 proteins regulates the mitochondrial death pathway and some studies have suggested that integrins can regulate Bcl-2 prosurvival proteins (31, 32). Accordingly, we studied the regulation of the three major Bcl-2 prosurvival proteins. Treatment of Jurkat cells with doxorubicin in the presence or absence of Coll had no
effect on the protein levels of Bcl-2 and Bcl-xL (Fig. 2d). However, doxorubicin treatment dramatically decreased the levels of Mcl-1, and Coll but not Fn, restored those levels (Fig. 2d). Coll restored Mcl-1 levels up to 80% in doxorubicin-treated cells (average of three independent experiments). The maintenance of Mcl-1 protein levels by Coll/α2β1 integrin signaling
also occurs in HSB-2 cells (supplemental Fig. S2). Together, these results suggest that Coll can inhibit doxorubicin-induced mitochondrial signaling and apoptosis by maintaining the levels of Mcl-1.

To test this possibility, we performed an Mcl-1 knockdown by RNA interference and tested the ability of Coll to protect against doxorubicin-induced apoptosis. Transfection of Jurkat cells with Mcl-1-specific siRNA (ID 120644) but not with control siRNA drastically reduced Mcl-1 protein levels (reduction of 85 to 90%; average of three experiments) (Fig. 2e; left panel). As expected, Coll restored Mcl-1 levels upon doxorubicin treatment in control siRNA-transfected cells but not in Mcl-1 siRNA-transfected cells. In addition, Mcl-1 siRNA enhances doxorubicin-induced mitochondrial membrane depolarization, and more importantly, the protective effect of Coll observed in control siRNA-transfected cells is abrogated in Mcl-1 siRNA-transfected cells (Fig. 2e, right panel). For more specificity, we used a second siRNA sequence targeting Mcl-1 (ID 4170). We found that this sequence also drastically reduces the levels of Mcl-1 and abolishes the protective effect of Coll (supplemental Fig. S3). Similar findings were obtained when apoptosis was measured by annexin V binding (data not shown). Together, these results demonstrate that the protective effect of Coll on doxorubicin-induced mitochondrial membrane depolarization and apoptosis occurs through the maintenance of Mcl-1 protein levels. It is noteworthy that knockdown of Mcl-1 by itself led to an increase in mitochondrial depolarization, which is in line with Mcl-1 as a major regulator of mitochondrial integrity and survival of leukemic cells.

Coll Inhibits Doxorubicin-induced JNK Activation—Previous studies have shown that JNK is involved in doxorubicin-induced apoptosis of T-ALL cells (29, 33) and that Mcl-1 stability and levels can be regulated by JNK (34). Thus, we considered the possibility that doxorubicin reduces Mcl-1 levels through a mechanism involving JNK, and that Coll could restore Mcl-1 levels by blocking JNK activation. In agreement, we found that treatment of T-ALL cell lines with doxorubicin increases the phosphorylation of JNK (Fig. 3a, and the JNK inhibitor SP600125 reduces doxorubicin-induced apoptosis by ~50–60% and also reduces doxorubicin-induced caspase-9 activation (supplemental Fig. S4). We then examined the role of JNK in the regulation of Mcl-1 protein levels in doxorubicin-treated cells. The results show that the JNK inhibitor SP600125 inhibits the ability of doxorubicin to down-regulate Mcl-1 protein levels in Jurkat and HSB-2 cells (Fig. 3b, left and right panel, respectively) indicating that JNK activation is essential for the down-modulating effect of doxorubicin on Mcl-1 levels. Finally, we found that Coll but not Fn inhibited the ability of doxorubicin to activate JNK in T-ALL cell lines (Fig. 3c). Together, these results indicate that doxorubicin reduces Mcl-1 protein levels through JNK and Coll could restore these levels by preventing activation of JNK.

Coll-mediated Cell Survival Is MAPK/ERK-dependent—Integrin-mediated signaling results in many cell types into the activation of the PI3K/AKT and MAPK/ERK survival pathways. We have previously shown in T cell lines that engagement of α2β1 integrin with Coll activated the MAPK/ERK but not the PI3K/AKT pathway (13, 19). Herein, we found that treatment of Jurkat cells with doxorubicin had no effect on ERK phosphorylation, and Coll induced a significant increase in ERK phosphorylation both in the absence and in the presence of doxorubicin (Fig. 4a). In contrast, doxorubicin or Coll did not regulate AKT, which is constitutively phosphorylated in Jurkat cells (Fig. 4b). These results suggest that the protective effect of Coll could be mediated via the activation of the MAPK/ERK survival pathway.

To test this possibility, we examined the effect of the MEK-1/ERK inhibitor U0126 on Coll-mediated cell survival. Treatment of T-ALL cell lines with U0126 but not with LY294002 (PI3 kinase/AKT inhibitor) abolished the ability of Coll to protect the cells from doxorubicin-induced apoptosis (Fig. 4c). As a control, treatment of the cells with U0126 abolished Coll-induced ERK phosphorylation, and treatment of the cells with LY294002 abolished AKT phosphorylation (data not shown). Because chemical inhibitors could possess off-target effects, we studied the implication of ERK and AKT using a genetic approach. Expression in Jurkat cells of a dominant-negative form of MEK-1 (DN-MEK-1) but not of AKT (DN-AKT) partially reduced the protective effect of Coll. We found that Coll reduced doxorubicin-induced apoptosis by ~40% in control transfected cells and only by 15% in DN-MEK-1-transfected cells (Fig. 4d). As we found previously (13, 25, 35), expression of DN-AKT and DN-MEK-1 also respectively diminished phosphorylations of AKT and ERK (data not shown). Together, these data confirm the implication of MAPK/ERK in Coll-mediated cell survival.
We then assessed whether the effects of Coll on the maintenance of Mcl-1 levels and on the inhibition of JNK activation (see Figs. 2d and 3c) also were dependent on MAPK/ERK. The results show that the MEK-1 inhibitor but not the PI3K/AKT inhibitor reduced the ability of Coll to restore Mcl-1 levels in doxorubicin-treated cells (Fig. 4e) and abolished the ability of Coll to inhibit doxorubicin-induced JNK activation (Fig. 4f). Together, these results indicate that α2β1 integrin promotes resistance to doxorubicin by activating the MAPK/ERK signaling pathway.

Coll Protects T-ALL Blasts from Doxorubicin-induced Apoptosis—To assess whether our findings could have a clinical significance, we examined the expression and the function of α2β1 integrin in primary T-ALL blasts isolated from the bone marrow. Three different patient samples were obtained and analyzed in our study. The three samples expressed significant levels of α2 integrin chain. Between 49 to 58% of the total cells expressed α2 integrin; although with different MFI (high levels of α2 integrin were detected on samples 1 and 2, whereas sample 3 expresses lower levels). However, all samples expressed

**α2β1 Integrin in T-ALL Survival**

**Figure a**

- Low A$_{570}$ (% of total cells)

|          | medium | Doxorubicin |
|----------|--------|-------------|
|          |        |             |
| Coll I   |        |             |
| Fbn      |        |             |

**Figure b**

- cyt c
- β-actin

**Figure c**

- Caspase-9
- p47
- p37
- p35
- active

- Caspase-3
- p35
- p17
- p11
- active

- β-actin

**Figure d**

- Mcl-1
- Bcl-2
- β-actin

- Bcl-xL

**Figure e**

- Mcl-1
- β-actin

- Control siRNA
- Mcl-1 siRNA

**Figure f**

- Low A$_{570}$ (% of total cells)

|          | medium | Doxorubicin |
|----------|--------|-------------|
|          |        |             |
| Coll I   |        |             |
| Fbn      |        |             |

**Figure g**

- Coll Protects T-ALL Blasts from Doxorubicin-induced Apoptosis—To assess whether our findings could have a clinical significance, we examined the expression and the function of α2β1 integrin in primary T-ALL blasts isolated from the bone marrow. Three different patient samples were obtained and analyzed in our study. The three samples expressed significant levels of α2 integrin chain. Between 49 to 58% of the total cells expressed α2 integrin; although with different MFI (high levels of α2 integrin were detected on samples 1 and 2, whereas sample 3 expresses lower levels). However, all samples expressed
high levels of the β1 integrin chain (Fig. 5a). Having established the expression of α2β1 integrin, we tested whether Coll would protect these primary T cell blasts from doxorubicin-induced apoptosis. Preactivation of all three primary T-ALL blasts samples with Coll reduced doxorubicin-induced apoptosis, whereas Fn had no significant effect (Fig. 5b). Doxorubicin-induced apoptosis was reduced by 27% in sample 1, 25% in sample 2, and by 15% in sample 3. Finally, the MEK-1 inhibitor induced apoptosis was reduced by 27% in sample 1, 25% in sample 2, and by 15% in sample 3. The MEK-1 inhibitor U0126 but not the PI3K/AKT inhibitor LY294002 also abolished Coll-mediated cell survival in primary T-ALL blasts (Fig. 5c). Together, these results demonstrate that the protective effect of Coll is not restricted only to T-ALL cell lines and that the Coll−α2β1 interaction could be an important cell survival pathway in the resistance of T-ALL toward doxorubicin-induced apoptosis.

DISCUSSION
The tumor microenvironment is recognized as a critical factor promoting tumor progression and survival. The bone marrow, which is rich in ECM proteins, is the growth site for the development of hematological malignancies (12). However, the role of β1 integrin signaling in the resistance of malignant T cells to apoptosis is still poorly addressed. In this study, we demonstrate that Coll through its receptor α2β1 integrin inhibits apoptosis of T-ALL cells that is induced by doxorubicin, a chemotherapeutic drug widely used in anti-cancer therapy.

FIGURE 2. Coll inhibits doxorubicin-induced mitochondrial apoptosis by maintaining the levels of Mcl-1. a, Coll (Coll I) inhibits doxorubicin-induced loss of mitochondrial membrane potential. Jurkat cells were preactivated for 4 h with Coll or Fn (Fn), after which they were treated for 16 h with doxorubicin (Dox). The loss of the mitochondrial membrane potential (low ΔΨ\textsubscript{m}) was measured by DIOC\textsubscript{3} staining and flow cytometry analysis. The results represent mean values of three independent experiments with S.D. as indicated. *, p < 0.05 between doxorubicin + Coll and doxorubicin samples. b, Coll inhibits doxorubicin-induced cytochrome c release. After activation, the cytosolic fractions were prepared and analyzed by immunoblotting using an anti-cytochrome c (cyc c) Ab. The blot was stripped and reprobed with anti-β-actin Ab to confirm equal loadings. c, Coll inhibits doxorubicin-induced caspase activation. The cells were activated as described in a, and after cell lysis, activation of caspase-9 and -3 was determined by immunoblot analysis using specific anti-caspase-9 and anti-caspase-3 antibodies that recognize the native forms and the active fragments of caspases. The membrane was stripped and reprobed with anti-β-actin antibody to confirm equal loading. The results in b and c are representative of three independent experiments. d, Coll maintains Mcl-1 levels in doxorubicin-treated cells. The cells were preactivated with Coll or Fn, after which they were treated for 12 h with doxorubicin (Dox). After treatment, the cells were lysed, and the expression of Mcl-1, Bcl-2, and Bcl-x\textsubscript{L} was determined by immunoblot analysis using specific antibodies. The blots were stripped and reprobed with anti-β-actin antibody to ensure equal loading. The results are representative of three independent experiments. e, Mcl-1 siRNA abolishes the protective effect of Coll. Jurkat cells were transfected with control siRNA or with Mcl-1 siRNA. After transfection, the cells were activated with Coll and treated or not with doxorubicin (Dox). Expression of Mcl-1 protein in control and Mcl-1 siRNA-transfected cells was assessed by immunoblot analysis, and β-actin levels were determined as control to ensure equal loading (left panel). Apoptosis of cells transfected with control and Mcl-1 siRNA was determined after 16 h of treatment with doxorubicin by measuring the loss of the mitochondrial membrane potential (right panel). The results represent mean values of three independent experiments with S.D. as indicated. *, p < 0.05 between doxorubicin + Coll and doxorubicin samples in control siRNA-transfected cells.

FIGURE 3. Coll inhibits doxorubicin-induced JNK activation. a, doxorubicin (Dox) activates JNK in Jurkat cells. The cells were treated with doxorubicin for the indicated periods of time, and JNK activation was determined by immunoblot analysis using a specific antibody recognizing the phosphorylated form of JNK1. The blot was stripped and reprobed with anti-JNK-2 antibody to ensure equal loading. b, activation of JNK is necessary for doxorubicin-induced Mcl-1 down-regulation. The cells were treated for 12 h with doxorubicin (Dox) in absence or in the presence of 10 μM of the JNK inhibitor SP600125. The cells were lysed, and expression of Mcl-1 protein was determined by immunoblot analysis. The blot was stripped and reprobed with anti-β-actin antibody to ensure equal loading. c, Coll (Coll I) but not Fn (Fn) blocks the ability of doxorubicin to activate JNK. The cells were preactivated or not with Coll or Fn and then treated for 12 h with doxorubicin. JNK activation was determined by immunoblot analysis as described above. The results shown in the three panels are representative of three independent experiments. Nonspecific bands are indicated as NS (a and c).
In contrast to Coll, Fn did not protect T-ALL cells from doxorubicin-induced apoptosis despite the fact that malignant T cells express Fn-binding integrins α4β1 and α5β1 (11). However, Fn, through α4β1 integrin, has been shown to reduce drug-induced apoptosis in other hematological malignancies such as myeloma and myeloid leukemia (7, 9). This suggests that hematological tumors could respond differently to their tissue microenvironment depending on the integrin expression profile and on the signaling events active in the cells. The fact that Fn does not protect T-ALL cells from doxorubicin-induced apoptosis is reminiscent to previous studies. We and others (13, 14, 19, 27, 37) have reported that in Jurkat cells, soluble Coll binds to α2β1 integrin, reduces chemotherapy-induced apoptosis in breast cancer cells (25), and soluble Coll and ECM ligands also induce intracellular signaling and modulate cell behavior in solid tumors. Soluble Coll ligands also induce intracellular signaling and modulate cell behavior in solid tumors. Soluble Coll has been shown to influence TGFβ receptor signaling and gene expression in breast cancer cells (38, 39), and soluble integrin ligands such as Coll also rescue neuroblastoma cells from apoptosis under nonadherent conditions (40). ECM remodeling and degradation by metalloproteinases, which is associated with cancer growth and invasion, is likely to lead to the release of ECM components that can act as “soluble” pep-

Coll/α2β1 integrin interaction not only inhibited doxorubicin-induced apoptosis but also promoted clonogenic growth of T-ALL cells. Taken together with our previous study that has shown that α2β1 integrin reduces chemotherapy-induced apoptosis in breast cancer cells (25), our results strongly suggest that α2β1 integrin can be an important pathway contributing to the development of drug resistance in T-ALL and other cancer cells.

In our study, we used soluble Coll to ligate α2β1 integrin because T-ALL cell lines attach poorly to immobilized ECM. We and others (13, 14, 19, 27, 37) have reported that in Jurkat cells, soluble Coll binds to α2β1 integrin and induces intracellular signaling. Soluble ECM ligands also induce intracellular signaling and modulate cell behavior in solid tumors. Soluble Coll has been shown to influence TGFβ receptor signaling and gene expression in breast cancer cells (38, 39), and soluble integrin ligands such as Coll also rescue neuroblastoma cells from apoptosis under nonadherent conditions (40). ECM remodeling and degradation by metalloproteinases, which is associated with cancer growth and invasion, is likely to lead to the release of ECM components that can act as “soluble” pep-

Coll/α2β1 integrin interaction not only inhibited doxorubicin-induced apoptosis but also promoted clonogenic growth of T-ALL cells. Taken together with our previous study that has shown that α2β1 integrin reduces chemotherapy-induced apoptosis in breast cancer cells (25), our results strongly suggest that α2β1 integrin can be an important pathway contributing to the development of drug resistance in T-ALL and other cancer cells.

FIGURE 4. The protective effect of Coll is dependent on MAPK/ERK but not on PI3K/AKT pathway. Jurkat cells were activated for 4 h with Coll (Coll I) in the presence of absence of doxorubicin (Dox) and activation of ERK (a) and AKT (b) was determined by immunoblot analysis using specific antibodies recognizing the phosphorylated forms of ERK1/2 and AKT. The results represent mean values from three independent experiments with S.D. as indicated. *, p < 0.05 between doxorubicin + Coll or doxorubicin + Coll/LY294002 and doxorubicin samples. Similar results were found with Jurkat cells (not shown). c, Jurkat cells were transfected with control plasmid or with plasmids encoding dominant-negative forms of MEK-1 (DN-MEK-1) and AKT (DN-AKT). After transfection, the cells were activated with Coll for 4 h and then treated with doxorubicin (Dox). Apoptosis was then determined by annexin V staining and flow cytometry analysis. The results represent mean values from three independent experiments with S.D. as indicated. *, p < 0.05 between doxorubicin + Coll and doxorubicin samples in control and in DN-AKT-transfected cells. d, Jurkat cells were pretreated with U0126 or with LY294002 inhibitors prior to their activation with Coll. The cells were then treated for 12 h with doxorubicin (Dox), and the expression of Mcl-1 was determined by immunoblot analysis. The results are representative of three independent experiments. e, the cells were treated and activated as described in d, and activation of JNK was determined by immunoblot analysis as described above. The results are representative of three independent experiments. Nonspecific bands are indicated as NS.
**FIGURE 5. ColI/α2β1 integrin inhibits doxorubicin-induced apoptosis in primary T-ALL blasts.**

*a*, expression of α2 and β1 integrin chains on primary T-ALL blasts was determined by immunostaining and flow cytometry analysis. The number of positive cells (%) and the mean fluorescence intensity values are indicated. 

*b*, ColI (Coll I) reduces doxorubicin-induced apoptosis of primary T-ALL blasts. The cells were preactivated or not for 4 h with ColI or Fn (Fbn), after which they were treated for 24 h with doxorubicin. Apoptosis was then determined by annexin V staining and flow cytometry analysis. The results represent mean values of triplicates with S.D. as indicated. *, p < 0.05 between doxorubicin + Coll and doxorubicin samples.

*c*, ColI-mediated inhibition of doxorubicin-induced apoptosis in primary T-ALL blasts (patient 2) is MAPK/ERK-dependent. The cells were pretreated or not with 10 μM of the MEK-1 inhibitor (U0126) or with 10 μM of the PI3K/AKT inhibitor (LY294002) for 1 h before being activated with Coll for 4 h. The cells were then treated or not for 24 h with doxorubicin, and cell apoptosis was evaluated by DNA fragmentation ELISA assay. The results represent mean values of triplicates with S.D. as indicated. *, p < 0.05 between doxorubicin + Coll and doxorubicin samples.
tides within the tumor microenvironment. T cell malignancies are also associated with metalloproteinases and ECM remodeling as well as dissemination (41–44). Thus, our study suggests that the Coll released from tissue ECM can bind to α2β1 integrin and provides T-ALL cells with a survival advantage against the cytotoxic effect of doxorubicin. In addition to soluble Coll, we found that co-culture of Jurkat cells with MSCs protected them from doxorubicin-induced apoptosis, which is in agreement with a previous report (28). Moreover, our results showed that the protective effect of MSCs also involves α2β1 integrin. Interestingly, the study of Guo et al. (28) reported that the effect of MSCs on Jurkat cell resistance to doxorubicin involves Notch1 signaling. Together, these studies suggest that MSCs are likely to activate several signaling pathways to support T-ALL resistance to chemotheraphy. Although it remains to be tested, it is also possible that a cross-talk exists between Notch1 and α2β1 integrin pathways in T-ALL chemoresistance. Because MSCs are known to produce and to express at their cell surface various ECM proteins, including type I collagen (α2β1 integrin ligand) (45), our study indicates that interactions of T-ALL cells with MSCs via α2β1 integrin can constitute one important signaling pathway in their resistance against doxorubicin-induced apoptosis.

The mitochondrial death pathway plays a critical role in drug-induced apoptosis including in doxorubicin-induced apoptosis (29, 30). Our results demonstrate that the protective effect of Coll occurs at the level of the mitochondria. We found that Coll reduces mitochondrial membrane depolarization, cytochrome c release, activation of caspase-9 and caspase-3, and maintained the levels of prosurvival Mcl-1 levels in doxorubicin-treated cells. Coll signaling had no effect on the basal levels of Bcl-xL, Bcl-2, and Mcl-1, but it prevented doxorubicin from downregulating Mcl-1 protein levels. In agreement with our results, doxorubicin-induced apoptosis in leukemic cell lines has been associated recently with the down-modulation of Mcl-1 levels and the subsequent activation of the mitochondrial death pathway (30). Knockdown experiments showed that the maintenance of Mcl-1 levels by Coll is necessary for the inhibition of doxorubicin-induced mitochondrial membrane depolarization and apoptosis. Together, these results indicate that Coll/α2β1 integrin signaling regulates doxorubicin-induced apoptosis of T-ALL cells by maintaining Mcl-1 levels, which contributes to the protection of mitochondria. Interestingly, Mcl-1 knockdown in T-ALL cells led to an increase in basal cell apoptosis. This effect has also been observed in different tumor cells, including melanoma (23) and myeloma (46), further emphasizing the importance of Mcl-1 in regulating mitochondrial integrity and cell survival.

We also demonstrated that the maintenance of Mcl-1 levels by Coll signaling in doxorubicin-treated cells could be attributed to the inhibition of doxorubicin-induced JNK activation. Indeed, we found that doxorubicin-induced down-regulation of Mcl-1 levels is mediated via JNK and that Coll inhibited doxorubicin-induced JNK activation. Our results showed that JNK is important for doxorubicin-induced caspase activation and apoptosis in T-ALL cells, which is in line with previous studies (29, 33). In addition, JNK has also been involved in the degradation of Mcl-1 through its phosphorylation and ubiquitination, a pathway that has recently been shown to play a key role in the sensitization of breast cancer cells to TRAIL by antimicrotubules agents (47) and in the synergy between the Bcl-2 pan-inhibitor ABT-737 and retinamide in the apoptosis of lymphoblastic leukemia cells (48). Thus, our study showed that inhibiting activation of JNK, which led to the maintenance of Mcl-1 levels, is one important mechanism accounting for α2β1 integrin-mediated doxorubicin resistance. Our results demonstrated that the prosurvival effect of Coll is mediated through the activation of the MAPK/ERK but not the PI3K/AKT survival pathway. In agreement with our previous report (13), we found that Coll increases the phosphorylation of ERK but not AKT. We also show that activation of MAPK/ERK is required for the inhibitory effect of Coll on doxorubicin-induced JNK activation. In agreement with our results, activation of MAPK/ERK has been reported to negatively affect the activation of the JNK pathway in leukemic cell lines treated with the proteasome inhibitor bortezomib (49).

We found that Fn, which did not activate the MAPK/ERK pathway (13, 19), had no effect on apoptosis, Mcl-1 levels, or JNK activation. This suggests that the differential ability of β1 integrins to regulate doxorubicin-induced apoptosis could be due, at least in part, to their differential ability to activate the MAPK/ERK survival pathway. These results further support the role of Coll/α2β1 integrin signaling pathway in the resistance of T-ALL cells to drug-induced apoptosis and suggest that targeting α2β1 integrin/MAPK/ERK pathway can be beneficial for the treatment of T-ALL.

Our study reported for the first time that α2β1 integrin can be expressed on primary T-leukemic blasts obtained from patients with T-ALL. We showed that Coll but not Fn protected T-ALL blasts from doxorubicin-induced apoptosis. These results indicate that the prosurvival function of α2β1 is not restricted to T cell lines but could have a clinical implication as well. Analysis of a larger number of samples will be necessary to establish if α2β1 integrin expression and function correlate with drug resistance and patient relapse. In support of our findings, Cleaver et al. (50) recently reported in three different cohorts that α2β1 integrin (VLA-2) mRNA expression levels correlated with the resistance of pediatric T-ALL to the treatment with glucocorticoids, suggesting that α2β1 can represent an important survival pathway contributing to drug resistance of T-ALL cells. Because of the limited availability of T-ALL samples, we did not examine in details the mechanisms by which Coll protected primary T-ALL blasts from doxorubicin-induced apoptosis. However, the fact that JNK has been involved in doxorubicin-induced apoptosis of T-ALL blasts (29) and that we found that the MEK-1/ERK inhibitor abrogated the protective effect of Coll in primary T-ALL blasts, argues in favor of the possibility that Coll-mediated protection in primary T cell blasts, occurs through the same mechanism demonstrated in T-ALL cell lines.

Thus, our study has unraveled a β1 integrin survival signaling pathway operating in T-ALL cells in which ligation of α2β1 integrin with Coll inhibits doxorubicin-induced apoptosis (depicted in Fig. 6). Doxorubicin activates JNK, which induces the down-modulation of Mcl-1 levels, thus allowing the activation of the mitochondrial death pathway (cytochrome c release and caspase activation). Ligation of α2β1 integrin with Coll
activates the MAPK/ERK survival pathway, which inhibits doxorubicin-induced JNK activation, leading to the maintenance of Mcl-1 levels. In turn, Mcl-1 protects the cells from doxorubicin-induced mitochondrial cell death, thus promoting doxorubicin resistance. The mechanisms by which Coll inhibits doxorubicin-induced JNK activation are not clear, but a previous study reported that in Jurkat T cells, doxorubicin-induced JNK activation is dependent on caspase-2 and PKCδ activities (29). Thus, it is tempting to speculate that the inhibitory effect of Coll could be at the level of either caspase-2 or PKCδ. These studies are currently underway in our laboratory.

Further elucidation of the mechanisms by which α2β1 integrin signaling regulates drug-induced apoptosis in T-ALL cells and other cancer cells is likely to lead to new therapeutic avenues.

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