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Phosphatidylinositol 3-Kinase/Akt Activation by Integrin-Tumor Matrix Interaction Suppresses Fas-Mediated Apoptosis in T Cells

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It has recently become apparent that the microenvironment made up of the extracellular matrix may affect cell signaling. In this study, we evaluated Fas-triggered apoptosis in T cells in contact with tumor cells, which resembles the cell-to-cell interactions found in tumor regions. Jurkat cells were less susceptible to the Fas-mediated apoptosis when cocultured with U118, HeLa, A549, and Huh-7 tumor cells. This was indicated by less plasma membrane alteration, an amelioration of the loss of mitochondria membrane potential, a decrease in caspase-8 and caspase-3 activation, a decrease in DNA fragmentation factor-45/35 cleavage, and a reduction in the breakage of DNA when compared with Jurkat cells cultured alone. In contrast, the tumor cell lines MCF-7 and HepG2 produced no such protective effect. This protective event was independent of the expression of Fas ligand on the tumor cells. Interrupting the β integrins-matrix interaction diminished the coculture effect. In Jurkat cells, cell matrix contact reduced the assembly of the Fas death-inducing signaling complex and Bcl-xL cleavage, but enhanced the phosphorylation of ERK1/2, p38 MAPK, and Akt. Only PI3K inhibitor, but not kinase inhibitors for MEK, ERK1/2, p38 MAPK, JNK, protein kinase C, and protein kinase A, completely abolished this tumor cell contact-associated protection and in parallel restored Fas-induced Bcl-xL cleavage as well as decreasing the phosphorylation of Bad at serine 136. Together, our results indicate that stimulation of the β integrin signal of T cells by contact with tumor cells may trigger a novel protective signaling through the PI3K/Akt pathway of T cells against Fas-mediated apoptosis. *The Journal of Immunology, 2007, 179: 4589–4597.

Deletion of immune cells through Fas (CD95, APO-I)-mediated apoptosis is critical for the down-regulation of immune responses (1). During tumor formation, inadequate accumulation of infiltrating cells may play an adverse role. For example, tumor-infiltrating lymphocytes (TILs)3 are able to express vascular endothelial growth factor, which enhances angiogenesis in tumor regions (2, 3). Recently, we have demonstrated that coculture with glioma cells will induce transcription of the IL-10 gene in T cells through Fas signaling (4). These findings support the hypothesis that tumors may highjack accumulated TILs to create a tumor growth-promoting environment; this event occurs by the evasion of immune surveillance using elevated local IL-10 production and increased angiogenesis by vascular endothelial growth factor to bring in nutrition. This would explain partly why some tumors, such as nasopharyngeal carcinoma, liver cancer, cervical cancer, and ovarian cancer, show abundant TIL accumulation rather than eliminating them by the so-called Fas counterattack mechanism (5–7). Autoreactivity represents another pathological feature of delayed apoptosis in activated immune cells. Although positive and negative selection in the central immune organs eliminate the majority of autoreactive T cells present in the immune repertoire, local tissue factors contribute to limiting the expansion of those that escaped and remain in the peripheral tissues (8). Delayed apoptosis in those autoreactive immune cells could develop into uncontrolled inflammation. At the moment, the survival mechanism for Fas+ immune cells in a tumor environment or inflammatory tissue is still poorly understood.

Recent studies underscore the importance of the extracellular matrix (ECM) in decisions involving the life and death of cells (9–12). Integrins are αβ heterodimeric membrane receptor proteins that serve as external sensors and interact with appropriate ECM ligands. By inflammation and tumorigenesis, the local status of the ECM may be profoundly perturbed. Tumors can secrete various ECM components and metalloproteases in abundance, thereby altering the makeup of the immediate tissue environment (13, 14). Tethering of epithelial cells correctly to the ECM enables these cells to assemble cytoskeletons, to gain apical-basal polarity, and to resist all manner of proapoptotic stimuli, including Fas, TRAIL, TNF, and the chemotherapeutic agents (15). Despite being able to survive in a Fas ligand (FasL)+ tumor environment, CD3+ TILs become Fas sensitive when they are isolated and challenged by Fas-activating agents in vitro; this further emphasizes the important role of immune cell-tumor matrix interactions (16).

In this study, we investigated the survival mechanism of TILs using an in vitro coculture system. Fas-mediated apoptosis in T cells was examined in the context of tumor cell contact, which mimicked the integrin-ECM interactions that occur at a tumor site. We demonstrated that tumor contact will initiate survival signals in...
Tumor ECM inhibits CD95-mediated apoptosis

Materials and Methods

Cells and reagents

The agonist anti-human Fas Ab (CH-11) was purchased from Upstate Biotechnology. Mouse mAbs recognizing caspase-8 and rabbit polyclonal Ab for caspase-3, DNA fragmentation factor (DFF)-45/35, p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (phospho-ERK1/2), p38 MAPK, phospho-p38 MAPK, Akt, phospho-Akt, Bcl-2, Bcl-xL, and Bad were purchased from Cell Signaling Technology. mAbs for human integrin β1 and β2 were obtained from Chemicon International. PHA, propidium iodide, p38 MAPK inhibitor (SB202190), and PKA inhibitor (H-89) were obtained from Sigma-Aldrich. Kinase inhibitors for MEK (PD98059), ERK1/2 (U0126), PI3K (LY294002), and PKC (GF190203X) were purchased from Calbiochem. c-jun N-terminal kinase inhibitor (SP600125) was bought from Tocris Cookson. Fas-sensitive human leukemic Jurkat cell line, human glioma cell line U-118MG, hepatocellular carcinoma cell line HepG2, cervical cancer cell line HeLa, lung adenocarcinoma cell line A549, and breast carcinoma cell line MCF-7 were obtained from the American Type Culture Collection. Mouse NIH 3T3 fibroblasts were provided by Dr. M. D. Lai (National Cheng Kung University, Tainan, Taiwan). Glioma U118(R) expressing a low level of FasL was established from U-118MG by transfection of a plasmid coding a FasL-specific ribozyme (17). U118(V), which served as the nontarget control, carries the EGFP-N1 vector (4). Overexpression of Fas (oFas) in MCF-7 cells was achieved by transfection of the oFas plasmid-encoding Fas gene under the control of CMV promoter. Human PBMC were prepared using Ficoll-Hypaque Plus (Amersham Biosciences) density gradient centrifugation. CD3+ T cells in PBMC were further purified by passing through a sterile column of nylon wool (18).

Cell culture

U118, Huh-7, HepG2, HeLa, A549, MCF-7, and NIH 3T3 cells were cultured in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS, 100 μg/ml penicillin-glutamine (Invitrogen Life Technologies), and 1% antibiotic-antimycotic (Invitrogen Life Technologies). Jurkat cells, PBMC, and isolated T cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FBS. Peripheral T cells were activated by 2.5 μg/ml PHA for 16 h, and then grown in medium containing 1 ng/ml human rIL-2 (R&D Systems) for 5 days (18). In the coculture experiment, glioma cells or the other tumor cells to be tested were seeded on a 6-well plate at a plating density of 6 × 10⁵ cells/well and grown to ~90% confluence. After replacement with fresh RPMI 1640 medium, Jurkat cells were added to a final density of 3 × 10⁵ cells/ml and incubated for another 1 h in the presence or absence of kinase or caspase inhibitors before Fas stimulation by CH-11. In the Transwell unit, glioma and Jurkat cells were cultured together for 24 h in a single chamber or independently in two different chambers separated by a 0.4-μm pore-size Transwell plate (Costar). Jurkat cells were harvested for further investigation. We have routinely checked the cell preparation in most of the coculture experiments before they were subjected to further study for protein analysis (data not shown). U118 cells are bigger than Jurkat cells and can be easily identified. In addition, some cell preparation was also subjected to glial fibrillary acidic protein determination, which is a protein specifically expressed in glia.

Staining of apoptotic cells and surface proteins

Apoptosis in Jurkat cells was induced by CH-11 at a concentration of 10 ng/ml. Apoptotic bodies were identified by staining either with FITC-conjugated Ab against annexin V (BD Pharmingen) or propidium iodide. Annexin V is exposed on the outside of the plasma membrane by cell death (19). To detect alteration in plasma membrane asymmetry, Jurkat cells were harvested and incubated with annexin V-FITC Ab suspended in binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂) for 15 min and subjected to flow cytometric analysis (FL-1 channel) (20). Apoptotic cells have extensive DNA fragmentation, which reduces their capacity to bind propidium iodide by flow cytometric analysis (FL-2 channel) and appeared as the sub-G₀/G₁ population (4). To detect the sub-G₀/G₁ cells, fixation was done in cold 70% ethanol at ~20°C overnight. The fixed cells were made permeable and stained for 45 min in PBS containing of 0.2% RNase (Sigma-Aldrich), 20 μg/ml propidium iodide, and 0.01% Triton X-100. The expression of the surface Fas receptor of Jurkat cells was detected by incubating first with CH-11, followed by FITC-conjugated goat Ab against mouse IgM (Jackson ImmunoResearch Laboratories). Rhodamin-123 (Sigma-Aldrich) is taken up selectively by mitochondria, and the extent of the relative uptake is depended on the ΔΨm (21). Jurkat cells were harvested and washed twice with cold PBS. Cell pellets were resuspended in 100 μl of 5 μM rhodamin-123 staining buffer and incubated at 37°C for 1 h. Cells were then washed in cold PBS to remove unbound rhodamin-123; resuspended in 1 ml of buffer containing 160 mM NaCl, 2.7 mM KCl, 6 mM glucose, 10 mM HEPES, and 0.1% BSA; and then immediately subjected to flow cytometric analysis (FL-1 channel).

Western blot analysis and immunoprecipitation

Cells and reagents

Cells were lysed in a buffer consisted of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM Na₃VO₄, 1 mM PMSF, and protease inhibitor mixture containing 500 μM 4-(2-Aminoethyl)-benzenesulfonyl fluoride, 1 μg/ml aprotinin, 1 μM E-64, 500 μM EDTA, and 1 μM leupeptin (Calbiochem). Proteins were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was then incubated with blocking solution (5% BSA in 0.15% TBST) for 1 h at room temperature, followed by incubation with the primary Ab at 4°C overnight. Immunodetected proteins were visualized by ECL system (Amersham Biosciences). For immunoprecipitation, the supernatant was pre-cleared with 25 μl of protein G-agarose beads (Amerham Biosciences) for 1 h at 4°C, followed by incubation with 4 μg of Ab for 2 h, then with 25 μl of protein G-agarose beads for additional 2 h in the cold. The beads were washed and bounded. Protein beads were eluted and subjected to Western blot analysis.

Results

Coculture with tumor cells reduced Fas-mediated apoptosis in T cells

Cell line-associated protection against Fas signal-mediated apoptosis in Jurkat cells was demonstrable using a coculture system (Fig. 1A). The spontaneous death rate for Jurkat cells in culture was found to be ~5%, and this was not significantly affected by the presence of most human tumor cells tested, including U118, HeLa, Huh-7, A549, NIH 3T3, and MCF-7. Coculture with HepG2 slightly elevated the spontaneous apoptosis rate of Jurkat cells to ~10%. Treatment with 10 ng/ml CH-11 induced ~45–50% apoptosis in Jurkat cells in 24 h. Furthermore, most tumor cells were resistant to CH-11 and showed no sign of apoptosis under the culture conditions used. When Jurkat cells were cocultured with U118, HeLa, Huh-7, or A549 cells, the CH-11-induced apoptosis decreased to ~23–30%. Mouse fibroblast NIH 3T3 cells were also able to protect Jurkat cells from Fas-mediated apoptosis. However, in the presence of MCF-7 or HepG2 cells, there was little or no change, and CH-11 still induced ~45–50% apoptosis in Jurkat cells. Then, we tested whether the CH-11 concentration used was sufficient to induce apoptosis in Jurkat cells. Approximately 20% cell death in Jurkat cells was induced by treatment of 1 ng/ml CH-11 for 24 h, 45% by 10 ng/ml, and 55% by 100 and 500 ng/ml, respectively (Fig. 1B). Thus, in our experimental condition, the CH-11-induced apoptosis rate of Jurkat cells reached plateau at 10 ng/ml. Increase of CH-11 concentration did not induce more apoptosis. Furthermore, increase of CH-11 concentration did not affect the protective effect provided by U118 cells for Jurkat cells. Regardless of the CH-11 used at 10, 100, or 500 ng/ml, it induced apoptosis in Jurkat cells cocultured with U118 cells only about one-half of that in Jurkat cells alone.

We further established MCF-7-derived cells that overexpressed Fas receptor. MCF-7 cells expressed high level of Fas, both in cytosol and on the surface membrane after transfection of the oFas plasmid (Fig. 1, C and D). CH-11 induced ~40–50% apoptosis in Jurkat cells being cocultured with oFas cells, N1 control cells, and parental MCF-7 cells (Fig. 1E).
Coculture with glioma cells also reduced activation-induced cell death (AICD) of T cells isolated from peripheral blood and activated in vitro (Fig. 1F). In our hands, the nonactivated T cells showed ~8% apoptosis. Once being activated by PHA/IL-2, the AICD of T cells reached ~33%. CH-11 treatment further increased the AICD to above 43%. After coculture with glioma, ~28% AICD was detected for T cells treated with or without CH-11.

**Activation of apoptotic program in Jurkat cells**

We further examined the kinetic changes in plasma asymmetry, mitochondria membrane integrity, and DNA fragmentation to understand the extent of the apoptotic processes affected by the coculture (Fig. 2). Alterations in plasma asymmetry and the loss of Δψm are the earliest characteristics of Fas-mediated apoptosis, whereas DNA fragmentation represented a late characteristic (20, 21). As shown in Fig. 2A, the plasma asymmetry of Jurkat cells started to changed at 3 h and the change had become very obvious at 6 h after CH-11 (10 ng/ml) treatment. In coculture with U118 cells, the changes in the plasma asymmetry took place at 1–2 h, but did not worsen after 6 h. Significant DNA fragmentation could be detected in Jurkat cells at 9 h, and this increased in a time-dependent manner after CH-11 treatment. However, there was less DNA fragmentation detected in Jurkat cells in coculture (Fig. 2C), and the process of DNA fragmentation in Jurkat cells had already ceased at ~6 h of coculture with glioma cells.

In parallel with the reduction in DNA fragmentation, we found that coculture with tumor cells also suppressed the activation of the caspase cascade and death-inducing signaling complex (DISC) formation in Jurkat cells (Fig. 3). Caspase-8 is the initial caspase...
in Fas signaling and turns on the caspase cascade. Then caspase-3 is cleaved; this activates its protease activity, allowing digestion of its downstream substrate DFF-45, a subunit of the heterodimetric DFF. Coculture with glioma cells alone did not induce significant cleavage of caspase-8 and caspase-3 in Jurkat cells. When Jurkat cells were treated with CH-11 (10 ng/ml) for 12 h, procaspase-8 (57 kDa) in the cells was cleaved into fragments with the molecular masses of 43/41 kDa; further, procaspase-3 (35 kDa) was cleaved into 17- to 20-kDa products (Fig. 3A). In accordance with activation of caspase-3, DFF-45/35 of Jurkat cells was significantly reduced as apoptosis progressed.

Co-culture with glioma cells was able to reduce the cleavage of caspase-8, caspase-3, and DFF-45/35 in Jurkat cells. We then checked whether contact with tumor cells affected DISC formation in Jurkat cells. Caspase-8 was immunoprecipitated, and the amount of Fas protein in the pull-down pellet was measured to estimate DISC formation. Caspase-8 did not complex with Fas in Jurkat cells without CH-11 induction. DISC formation was detected in Jurkat cells treated with 500 µg of CH-11 Ab for 15 min. Less caspase-8 was incorporated into the complex with Fas in Jurkat cells cocultured with glioma (Fig. 3B).

Glioma cells engulfed few apoptotic bodies

One possibility is that the reduction in apoptotic cells may be due to phagocytosis by glioma cells. To exclude the engulfment of apoptotic bodies by adjacent glioma cells as a possibility in coculture system, we compared the phagocytic ability of cells in the presence or absence of latrunculin A, a potent phagocytosis inhibitor (22) (Fig. 4). Fluorescence-labeled apoptotic bodies were generated by treating Jurkat cells with CH-11 (100 ng/ml) for 6 h, and the cells were then labeled with FITC-conjugated anti-annexin V. When incubated with monocytes, authentic phagocytic cells, ~60% of cells become FITC positive due to engulfment of FITC-labeled apoptotic bodies. Latrunculin A reduced the FITC-positive population of monocytes to 40%. Glioma cells engulfed very few apoptotic bodies. Less than 2% of glioma cells were FITC positive in the groups treated with or without latrunculin A.

Coculture with glioma cells did not affect surface Fas on Jurkat cells

Some matrix metalloproteases can cleave Fas and FasL molecules. This proteolytic activity can confer on Fas-sensitive cells a level of resistance to Fas-mediated apoptosis by diminishing the binding of Fas and FasL (23). Glioma cells secrete matrix metalloproteases (24), and thus could potentially digest the surface Fas on Jurkat cells in coculture. To analyze this possibility, we have measured
the surface Fas on Jurkat cells cultured in glioma cell-conditioned medium or in direct contact with glioma cells. As shown in Fig. 5, neither glioma cell-conditioned medium nor direct contact with glioma cells caused a decrease in the surface Fas on Jurkat cells.

Integrins are involved in the suppression of Fas-mediated apoptosis in Jurkat cells

To clarify whether direct cell-to-cell contact with glioma cells is essential, Jurkat cells were cocultured with glioma cells either together or independently in a Transwell unit. The Transwell unit was set up so that it allowed one part of Jurkat cells to be in direct contact with glioma cells (low well) and the other part would not be in contact (upper well) (Fig. 6). Moreover, we used U118(V) and U118(R), which express different levels of FasL (17), to examine whether FasL on glioma cells shaped the apoptosis in Jurkat cells. In the Transwell system, CH-11 induced ~50% cell death of Jurkat cells in upper well. When Jurkat cells were in contact with U118(V) or U118(R) in the lower well, the CH-11-induced apoptosis levels were ~24 or 29%, respectively. These results demonstrated that direct cell-cell contact was required.

We further used integrin-blocking Abs to prevent matrix interactions by the Jurkat cells and glioma cells. Jurkat cells express both β1 and β2 integrins (Fig. 7, A and B). Glioma cell contact-associated protection was diminished in the presence of blocking Abs for the β1 and β2 integrins (Fig. 7C). Single β1 or β2 blockage...
MAPK and PI3K activation of Jurkat cells

The MAPK family has been implicated in regulating apoptosis in response to various stimuli. We wondered whether kinases were activated in the Jurkat cells during coculture with the glioma cells and that this consequently suppressed Fas-mediated cell death. As shown in Fig. 8A, Jurkat cells showed low basal phosphorylation of ERK1/2 and p38 MAPK, but high levels of phosphorylated Akt. CH-11 treatment enhanced slightly the phosphorylation of ERK1/2 and p38 MAPK. Coculture for 12 h with glioma cells led to enhanced phosphorylation of ERK1/2 in Jurkat cells, and this enhancement increased further in the presence of CH-11. Fas induction reduced the phosphorylation of Akt in Jurkat cells. In coculture with glioma cells, the level of phospho-Akt was sustained (Fig. 8A). Jurkat cells have high level of PI3K due to a defect in the PTEN gene (25). To reduce the background activity of Akt, Jurkat cells were pretreated with 25 μM LY294002 for 1 h and then cocultured with U118 in the presence of 10 ng/ml CH-11 Ab for 12 h (B).

was as effective as double β₁ and β₂ blockage in abrogating the protection provided by the glioma cells.

P13K is involved in cell contact-associated protection against Fas signaling

Activation of kinases in Jurkat cells upon contact with glioma cells suggested potential roles for the kinases in the regulation of Fas-mediated apoptosis under these circumstances. We verified this possibility by a pharmacological strategy. We pretreated Jurkat cells with inhibitors of MEK (PD98059, 25 μM), ERK inhibitor U0126 (25 μM), JNK inhibitor SP600125 (25 μM), p38 MAPK inhibitor SB203405 (25 μM), PKC inhibitor GF190203X (10 μM), PKA inhibitor H-89 (20 μM), or PI3K inhibitor LY294002 (25 μM) for 1 h. Jurkat cells were then cocultured with glioma cells for another 1 h before apoptosis induction by CH-11 Ab (10 ng/ml) for 24 h. Apoptosis was detected by propidium iodide staining and flow cytometric analysis. Values shown are the mean ± SD of three independent experiments.

PI3K activation of Jurkat cells reduced CH-11-induced cleavage of Bcl-xL and enhanced Bad phosphorylation

Bcl-2 family proteins are major survival factors in regulating Fas-mediated apoptosis (26, 27). We thus examined the expression of Bcl-2, Bcl-xL, and Bad in Jurkat cells upon contact with glioma cells (Fig. 10). Neither CH-11 treatment nor coculture affected the Bcl-2 expression in Jurkat cells. Coculture with glioma cells increased the basal level of expression of Bcl-xL in Jurkat cells. The amount of Bad decreased in CH-11-treated Jurkat cells regardless of whether they were cocultured with glioma or not. Jurkat cells showed reduced Bcl-xL upon treatment with 10 ng/ml CH-11 for 12 h. Contact with glioma prevented this CH-11-induced reduction of Bcl-xL (Fig. 10A). Along with restoring Fas sensitivity, LY294002 effectively abrogated the glioma contact-associated elevation of Bcl-xL in CH-11-treated Jurkat cells (Fig. 10B). Coculture with glioma cells also stimulated the phosphorylation of Bad at serine 136, which was reduced in the presence of LY294002 (Fig. 10C).
expression systems, in which FasL directly caused apoptosis in infiltrating T cells (28–31). For instance, Shiraki et al. (32) showed that FasL in a human colon adenocarcinoma cell line could functionally induce apoptosis in Jurkat cells. It has been reported that the threshold level of FasL determines its biological consequences in vivo (33). A possible explanation for this discrepancy is that FasL in overexpression systems or in colon cancer is present at very high levels and thus initiates death program of infiltrating T cells. When the FasL gene is under the control of an intrinsic promoter, the amount of FasL protein on the tumor cells may not be sufficient to override the protective mechanism provided by the tumor environment.

In the last few years, it has become apparent that cell survival and death strongly depend on cell adhesion and the ECM (10–12). Integrins are cell surface adhesion receptors that regulate cell function in response to the ECM (9). Integrins are capable of interacting with a diverse array of ligands in an inactive low-affinity state. When activated, they increase their affinity for multivalent ligands, which leads to stable cell-cell or cell-matrix adhesion (34, 35). Preventing engagement of tumor ECM and the integrins of Jurkat cells by blocking Abs for β1 and β2 resulted in complete restoration of the Fas sensitivity of Jurkat cells. We have further confirmed using the Transwell unit that only direct cell-to-cell contact conferred resistance against Fas-mediated apoptosis on Jurkat cells, and thus, this excludes the involvement of soluble factors secreted by glioma cells (Fig. 7). Yet, MCF-7 and HepG2, which ought to have a very similar ECM structure, did not provide protection. Whether unidentified receptors or factors secreted by MCF-7 and HepG2 act as a veto mechanism and cancel the protective signal remains to be answered. Interestingly, the putative protective ECM molecules were not necessary syngenic, because mouse fibroblast NIH 3T3 has exhibited a similar protective effect on human Jurkat cells. It seems that the ECM molecules involved in survival signaling are conserved in evolution.

Integrin downstream signals implicated in cell viability include the PI3K and MAPK pathway. In a type I collagen-coated culture plate, β integrin engagement is able to activate the MAPK/ERK survival signal pathway in Jurkat cells (12). The MAPK/ERK pathway has been reported as being involved in the inhibition of Fas-mediated apoptosis in activated T cells through the TCR (18, 36). During the elevated MAPK activity phase, the activation of caspase-8 and Bid is inhibited, whereas the assembly of a functional DISC is not affected. In addition, Allan et al. (37) showed that activation of the ERK pathway inhibited caspase-9 activity by direct phosphorylation. These reports suggested that the MAPK/ERK and p38 MAPK pathways might be critical for some apoptotic processes mediated through caspases.

We also observed rapid activation of the MAPK pathway in our coculture system. However, multiple lines of evidence indicate that MAPK pathway does not contribute to the survival of the Jurkat cells in the presence of cell matrix interaction. First, after inhibiting MAPK activity by inhibitors specific for MEK, ERK1/2, JNK, or p38 MAPK, the tumor contact-associated protection against Fas signaling remained in action (Fig. 10). Second, MCF-7 breast cancer cells stimulated Jurkat cells and produced rapid and strong phosphorylation of ERK. Yet, these Jurkat cells were still sensitive to Fas signal-triggered apoptosis. Third, Fas signaling directly stimulated the phosphorylation of ERK1/2 and p38 MAPK to some extent in Jurkat cells, whereas apoptosis was ongoing. Fourth, MAPK activity did not affect the assembly of a functional DISC. However, we observed a reduced DISC formation in Jurkat cells under the coculture system. In addition, mutual negative regulation of the PI3K/Akt and MAPK pathways has been demonstrated in other cells (38, 39). In agreement with those reports, we
found that phosphorylation of ERK1/2 was increased further in the presence of LY294002 (Fig. 8B), which effectively abolished the tumor contact-associated protection.

In contrast to the MAPK pathway, our results support an important role for PI3K/Akt activation through integrin signalling. The PI3K/Akt-mediated survival pathway has been found in several cells. Engagement of α5β1 integrin by collagen up-regulates PI3K/Akt, and this protects fibroblast from anoikis (40). Direct activation of PI3K and Akt has been shown to protect T cells from Fas-mediated apoptosis in T cell lines as well as in transgenic mice expressing an active form of Akt under the control of a T cell-specific promoter (41, 42). By measuring the phosphorylation of Akt, a PI3K substrate (40, 43), we confirmed that tumor contact increased PI3K activity in Jurkat cells. In agreement with this, a PI3K inhibitor completely abolished the protection provided by cell-to-cell contact. It has been reported that MHC-I ligation of Jurkat cells induces strong JNK activity through the PI3K pathway (11) and this results in apoptosis. We have found that the JNK inhibitor was not effective in modifying the protective event (Fig. 10), and phosphorylation of JNK in Jurkat cells remained very low under the coculture system (data not shown). These results reinforce our belief that the tumor contact survival signal for Jurkat cells is different from the ones initiated through MHC-I or CD3, which are candidate molecules for interactions between tumor and immune cells. Interestingly, in the coculture system, not only the phosphorylation of Akt was elevated, but also the Fas activation-induced reduction in total Akt protein was inhibited, which indicates that an additional protein stability-related mechanism was operated to maintain the PI3K pathway in Jurkat cells.

The downstream pathways by which PI3K/Akt regulates cell survival are diverse. Recently, Akt has been linked to intrinsic death-regulating machinery by the findings that Bad (44, 45) and protease caspase-9 (46) are Akt substrates. In both cases, phosphorylation of these proteins by Akt results in suppression of their proapoptotic function. Specifically, Akt triggers Bad phosphorylation at serine 136, and phosphorylation at this site is sufficient to promote survival (44, 45). An additional mechanism for PI3K/Akt, which involves protein induction, has also demonstrated in hepatocytes and activated T cells where PI3K/Akt induces the expression of Bcl-xL (47, 48). When we measured the status of Bcl-2, Bcl-xL, and Bad in coculture with glioma cells, there was stimulation of the phosphorylation of Bad and elevation of Bcl-xL expression; these were reversed by the inhibition of PI3K. Thus, PI3K/Akt is the proximal control point for survival of T cells in the tumor region.

Together, our findings highlight the role of the tumor matrix in providing PI3K/Akt survival signals for T cells, and this is illustrated in Fig. 11. Previously, we have demonstrated that tumors are able to hijack immune cells to create an immune-suppressive environment by producing IL-10 (9). The results presented in this study suggest that cell contact and PI3K/Akt play a key role in delaying the apoptotic program in T cells, and this provides a framework for further analysis of the tumor matrix and immune regulation. Autoimmunity is another area that is of pathological relevance to the above findings. Local integrin-matrix interactions in inflamed tissues may provide a survival signal that leads to the expansion of the potentially autoreactive T cells that are present in the immune repertoire. If this holds true, then the integrin-matrix/PI3K/Akt pathway may be a potential target for the treatment of inflammatory disease.

**Disclosures**

The authors have no financial conflict of interest.

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