

TGF- β 1 and hypoxia-dependent expression of MKP-1 leads tumor resistance to death receptor-mediated cell death

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Sporadic occurrence of transformed tumor cells is under the surveillance of the host immune system and such cells are effectively eliminated by immune-mediated cell death. During tumor progression, the antitumor effects of the tumor microenvironment are suppressed by diverse immunosuppressive mechanisms. In this research, we suggest novel immune evasion strategy of tumor cells through a transforming growth factor (TGF)- β 1- and hypoxia-dependent mechanism. Experimental results showed that TGF- β 1 and hypoxia induced mitogen-activated protein kinase phosphatase (MKP)-1 expression within 1 h, resulting in attenuation of c-Jun N-terminal kinase (JNK) phosphorylation and subsequent death receptor-mediated cell death. In addition, analysis of microarray data and immunostaining of MKP-1 in hepatocellular carcinoma (HCC) patient samples revealed that expression of MKP-1 is notably higher in tumors than in normal tissues, implying that MKP-1-dependent suppression of immune-mediated cell death takes place only in the tumor. To prove that MKP-1 can act as a mediator of immune escape by tumors, we determined whether chemo-resistance against several anticancer drugs could be overcome by knockdown of MKP-1. Cytotoxic assays showed that chemotherapy with siRNA targeting MKP-1 was significantly more effective than chemotherapy in the presence of MKP-1. Thus, we conclude that TGF- β 1 and hypoxia ensure tumor cell survival and growth through expression of MKP-1.

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Subject Category: Cancer

Recent progress in defining the cellular and molecular networks that regulate the immune responses in the tumor microenvironment has renewed the cancer-immune interaction paradigm.¹ Tumors foster a tolerant microenvironment and activate diverse immunosuppressive mechanisms, eventually counteracting effective immune responses.² These immunosuppressive strategies can pre-exist, arise through outgrowth of escape mutants, or take place during tumor-sculpting immune system activity.^{3,4} These dual effects of the immune system on developing tumors facilitated refinement of the cancer immunosurveillance hypothesis into the 'cancer immunoediting' hypothesis proposed by Schreiber *et al.*⁵ In this project, we report novel findings relating to tumor-specific evasion of immune-mediated cell death by transforming growth factor (TGF)- β 1- and hypoxia-dependent mechanisms.

TGF- β is a mitogenic factor that regulates a wide variety of cellular responses, including migration, immune activity, apoptosis, differentiation, and proliferation.⁶ Many evidences support a strong correlation between TGF- β and cancer. Cancer cells are able to produce and secrete TGF- β 1, which causes immunosuppression in cancer patients in the absence

of cytotoxic treatment.^{7,8} Also, elevated levels of TGF- β 1 have been found in a number of experimental models and in specimens from patients with cancer.⁹ TGF- β 1 has the potential to function as a tumor suppressor through effects on proliferation, replication potential, and apoptosis. However, paradoxically, TGF- β 1 also modulates cell migration, invasion, angiogenesis, and the microenvironment in ways that cancer cells may exploit to their advantage.¹⁰ Consequently, the output of a TGF- β 1 response is highly contextual throughout development, across different tissues and in cancer. This dual role of TGF- β 1 in cancer has long been noted, but its mechanistic basis, operating logic, and clinical relevance have remained elusive.¹¹

Mitogen-activated protein kinase phosphatase (MKP)-1, also known as dual specificity phosphatase1 (DUSP1), is one of the many target proteins of TGF- β 1, and is known to specifically dephosphorylates c-Jun N-terminal kinase (JNK) and p38.^{12–15} We postulated that immune-mediated cell death is remarkably affected by pre-existence of TGF- β , because MKP-1 augmentation may result in suppression of JNK phosphorylation, which is a major requirement of the death-receptor mediated cell death pathway.¹⁶

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Abbreviations: TGF, transforming growth factor; MKP, mitogen-activated protein kinase phosphatase; JNK, c-Jun N-terminal kinase; HCC, hepatocellular carcinoma; DUSP, dual specificity phosphatase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; FasL, Fas ligand; NF- κ B, nuclear factor-kappaB; MAPK, mitogen-activated protein kinase; OVA, ovalbumin; IKK, I κ B kinase; MKK, mitogen-activated protein kinase kinase; TAK, transforming growth factor-beta-activated kinase; GEO, Gene expression omnibus; LDH, lactate dehydrogenase; DAB, diaminobenzidine

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The major death receptor is part of the TNF superfamily, which is characterized by an intracellular death domain. Tumor necrosis factor (TNF)- α , TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand (FasL) can activate death receptor-mediated cell death.^{17–19} We dissected crosstalk between TNF- α and TGF- β 1 signal transduction components. Many roles of TNF- α are mediated by the transcription factor nuclear factor- κ B (NF- κ B). In addition to acting through NF- κ B, TNF- α also exerts its function through activation of JNK, p38, and p42/44 mitogen-activated protein kinase (MAPK) cascades, which participate in various cellular responses.^{20,21} Notably, JNK contributes to caspase activation and apoptosis by multiple mechanisms.^{22–25} Despite environmental dependence, sustained activation of JNK induces cell death, and many cellular components are involved in crosstalk with JNK signaling.^{26,27} On the basis of these reports, we investigated the crosstalk between TGF- β 1 signaling and the death receptor-mediated cell death pathway to unravel a novel regulatory mechanism of cell death. We identified MKP-1-JNK as a regulatory intersection, and further investigated the tumor-specific nature of this control of cell death and its contribution to tumor resistance to the host immune system.

Results

Block of immune-mediated cell death by TGF- β 1 pretreatment. To unravel how TGF- β 1 changes the sensitivity to CD8⁺ T cells, we cocultured OT-1 mouse (ovalbumin (OVA)-specific CD8⁺ T cell receptor transgenic mouse)-derived T cells and OVA peptide (SIINFEKL)-loaded MC38 cells with or without TGF- β 1 pretreatment. In the absence of TGF- β 1 pretreatment, an increase in effector (OT-1 mouse-derived CD8⁺ T cell)-to-target ratio increased cell-mediated cytotoxicity in immunized MC38 cells. When TGF- β 1 was applied in MC38 cells 1 h before coculture, however, most cells were intact, indicating that TGF- β 1 blocked CD8⁺ T cell-mediated cell death (Figure 1a). Because CD8⁺ T cells release diverse cytokines, such as TNF- α , TRAIL, and FasL, we applied each cytokine to HepG2 cells with or without TGF- β 1 1 h before pretreatment to mimic the cytotoxic function of CD8⁺ T cells. We measured cell viability by WST-1 assay, and the results showed a protective role for TGF- β 1, even though TGF- β 1 alone did not exert proliferative effects (Figure 1b). These results suggest that there is a crosstalk between TGF- β 1 and death receptor-mediated cell death pathways. Among the many types of death receptor-mediated cell death pathways activated by CD8⁺ T cells, we focused on TNF- α , a major cytokine in inflammation, cell survival, proliferation, and apoptosis.

Attenuation of TNF- α -induced JNK signaling by TGF- β 1 pretreatment. To investigate the mechanism of this crosstalk, we evaluated the effect of TGF- β 1 pretreatment on internal transduction of the TNF- α signal. In the absence of TGF- β 1, TNF- α treatment induced phosphorylation of MAPKs, such as JNK, which peaked at about 10 min. It also led to I κ B kinase (IKK) phosphorylation, followed by I κ B degradation and NF- κ B translocation. However, pretreatment with TGF- β 1 for 1 h suppressed phosphorylation of

JNK, but did not affect NF- κ B signaling (Figure 2a). Figure 2b shows that TGF- β 1 pretreatment also suppressed prolonged phosphorylation of JNK. Treatment with the JNK inhibitor SP600125 largely blocked TNF- α -induced cell death, while TGF- β 1 did not further contribute to the viability of HepG2 and MC38 cells (Figure 2c). These data show that TNF- α -induced cell death is JNK-dependent, and that attenuation of JNK phosphorylation by TGF- β 1 pretreatment protects cells from TNF- α -induced cytotoxicity. To determine whether signaling components acting upstream of JNK show similar behavior, phosphorylation of MKK4/7, MKK 3/6, and TAK1 was measured by immunoblot analysis. Their signals were not significantly altered by TGF- β 1 pretreatment (Figure 2d). These data suggest that this crosstalk between TNF- α and TGF- β 1 signaling pathways originates directly from JNK.

JNK and MKP-1 mediate crosstalk between TGF- β 1 and TNF- α signaling pathway. One of the most convincing candidates that may directly dephosphorylate JNK is MKPs. To elucidate the detailed mechanism of crosstalk between TGF- β 1 and TNF- α signaling pathway, we evaluated the involvement of MKP-1. Immunocytochemistry clearly showed TGF- β 1-dependent expression of MKP-1 in Huh-7 cells within 1 h (Figure 3a). We also performed immunoblot analysis of MKP-2, but its expression was not increased by TGF- β 1 treatment (Supplementary Figure 1). For a detailed view, we compared the temporal patterns of MKP-1 expression with other signaling components according to the duration of TGF- β 1 pretreatment in Huh-7 cells. MKP-1 expression peaked at 1 h after TGF- β 1 stimulation and remained elevated until 4 h. Phosphorylation of IKK was unaffected by the duration of TGF- β 1 pretreatment, confirming that NF- κ B signaling is not correlated with TGF- β 1 or MKP-1. However, JNK attenuation occurred during pretreatment with TGF- β 1 for 1 to 4 h, showing that MKP-1 expression coincides with JNK suppression (Figure 3b). These data strongly suggest that MKP-1 modulates crosstalk between TGF- β 1 and TNF- α .

Knockdown of MKP-1 or Smad2 abolishes crosstalk between TGF- β 1 and TNF- α . To verify participation of MKP-1 in crosstalk between TGF- β 1 and TNF- α , we used siRNA specific for MKP-1 and Smad2, an upstream signaling component of MKP-1. MKP-1 knockdown efficiency at 48 h post-transfection of each siRNA was measured by immunocytochemistry and qRT-PCR (Supplementary Figure 2). These data show that both siRNAs worked well, and that MKP-1 expression was dependent on TGF- β and Smad2. After applying these siRNAs, the coculture experiments in Figure 1 were repeated. In the scrambled siRNA control sample, immunized target cells showed effector cell dose-dependent cell death, whereas pretreatment with TGF- β 1 for 1 h blocked cell death. However, transfection of either siSmad2 or siMKP-1 abolished the cell death inhibitory effect of TGF- β 1 (Figure 4a). Cell death was also measured in Huh-7 cells by WST-1 assay with TNF- α treatment. In control samples, TNF- α caused death in more than 30% of cells, and TGF- β 1 pretreatment for 1 h protected most cells (similar to Figure 1). However, in the siSmad2 sample, rates of TNF- α -induced cell death were unaffected by TGF- β 1 pretreatment.

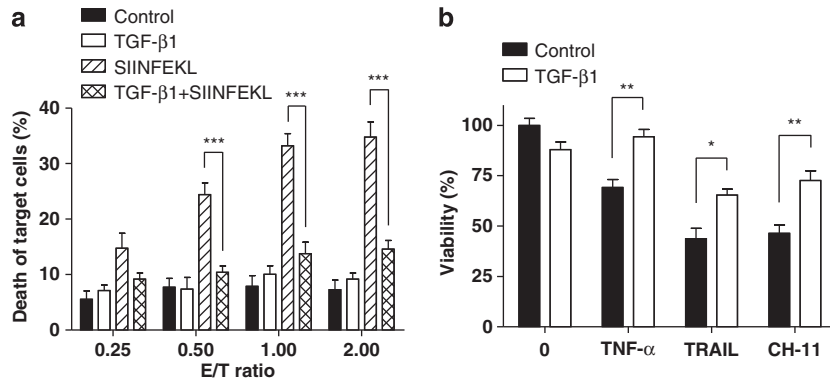


Figure 1 Block of immune-mediated cell death by TGF-β1 pretreatment. (a) MC38 cells were immunized with SIINFEKL peptide. Six hours after coculture of effector (OT-1 mouse-derived CD8 + T cells) and target (MC38 cells) cells, cytotoxicity was evaluated by lactate dehydrogenase (LDH) release assay. TGF-β1 (10 ng/ml) was applied in MC38 cells 1 h before coculture (mean ± S.E.M., $n = 4$; Bonferroni's *post hoc* test was applied for multiple comparisons in two-way ANOVA, $***P < 0.001$). (b) HepG2 cells were incubated in the presence or absence of TGF-β1 (10 ng/ml) for 1 h, and then treated with TNF-α (10 ng/ml), TRAIL (500 ng/ml), and CH-11 (500 ng/ml) for 24 h. Cell viability was measured by WST-1 assay (mean ± S.E.M., $n = 4$; $*P < 0.05$, $**P < 0.01$ by Student's *t*-test)

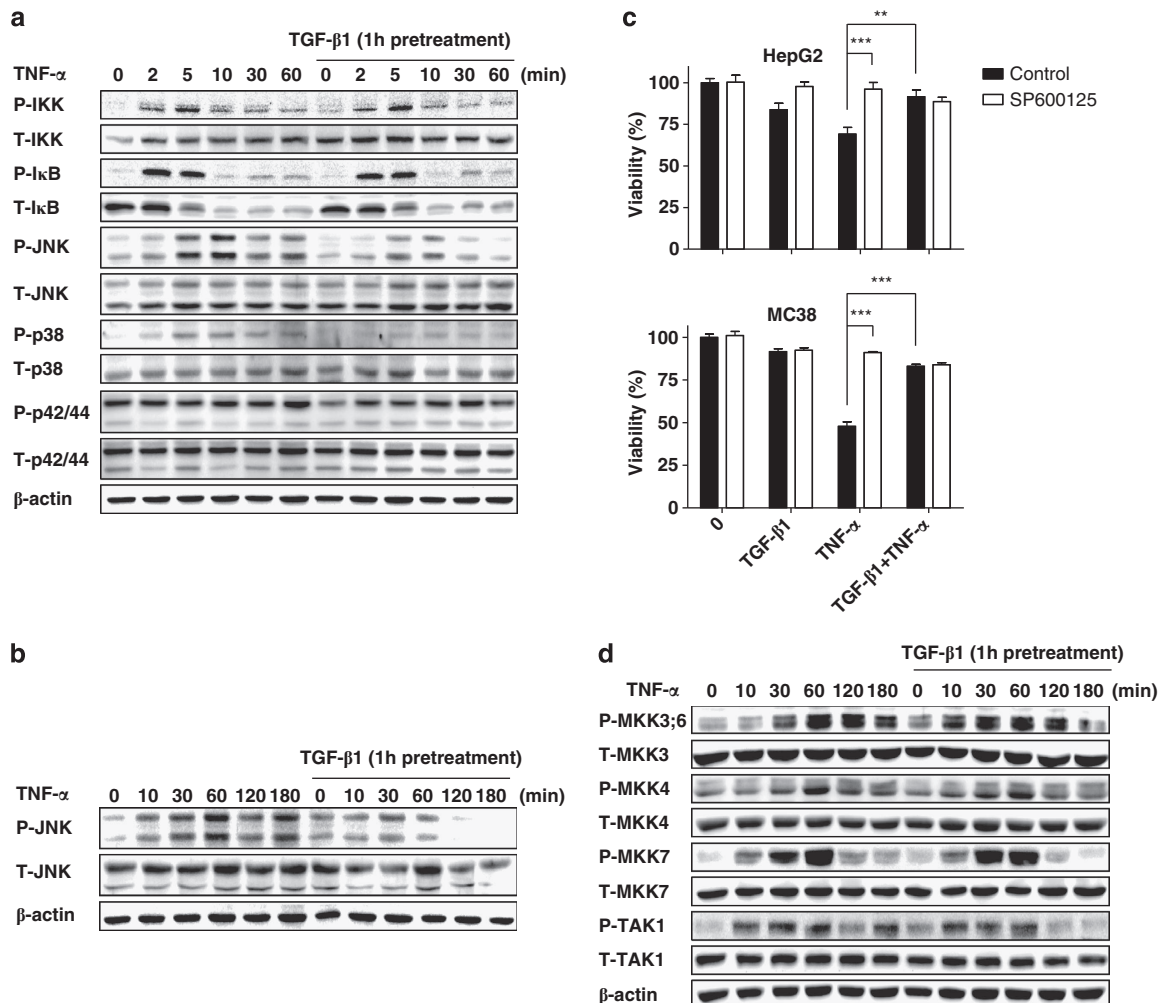


Figure 2 Attenuation of TNF-α-induced JNK phosphorylation by TGF-β1 pretreatment. (a–b) Intracellular signaling components were subjected to immunoblot analysis. Huh-7 cells were incubated in the presence or absence of TGF-β1 (10 ng/ml) for 1 h, and then treated with TNF-α (10 ng/ml) for up to 60 (a) or 180 min (b). Cell lysates were subjected to immunoblot analysis of IKK, IκB, JNK, p38, and p42/44. β-actin was used as the loading control. (c) Cell viability was measured by WST-1 assay. The JNK inhibitor SP600125 (10 μM) was applied 1 h before treatment with TNF-α or TGF-β1. WST-1 reagent was added 24 h after cytokine treatment (mean ± S.E.M., $n = 4$; $**P < 0.01$, $***P < 0.001$ by Student's *t*-test). (d) Upstream signaling components of JNK and p38 were evaluated by immunoblotting, as in (a)

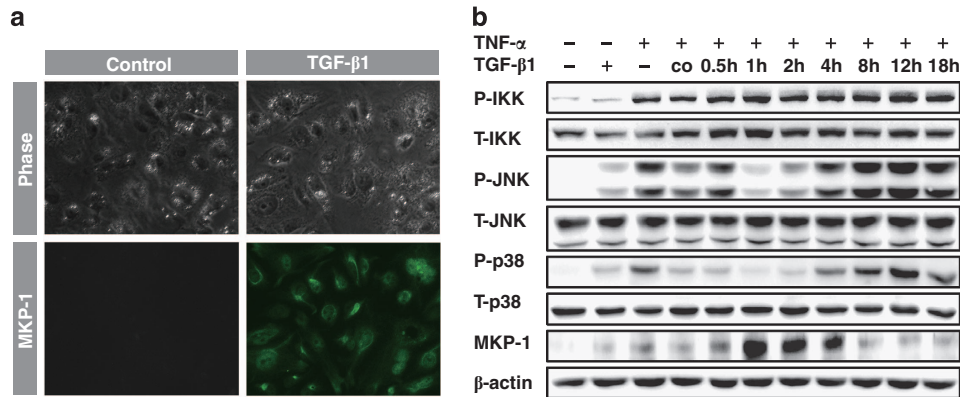


Figure 3 MKP-1 is involved in crosstalk between the TGF- β 1 and TNF- α signaling pathway. (a) In Huh-7 cells, immunofluorescence staining showed induction of MKP-1 expression by TGF- β 1 (10 ng/ml) within 1 h. (b) After pretreatment with TGF- β 1 for various times, TNF- α (10 ng/ml) was applied for 10 min in Huh-7 cells. Expression of MKP-1 and phosphorylation of IKK and JNK were measured by immunoblot analysis. β -actin was used as the loading control

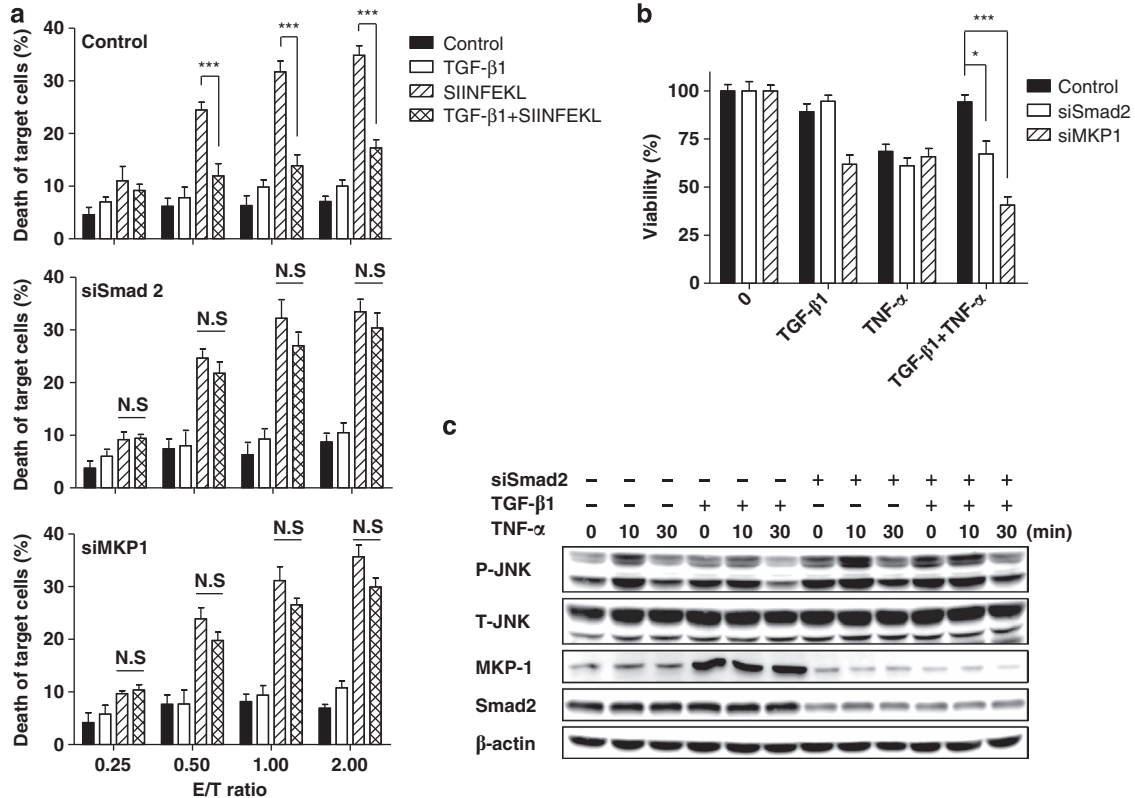


Figure 4 Knockdown of Smad2 or MKP-1 abolishes crosstalk between TGF- β 1 and TNF- α . Knockdown of Smad2 and MKP-1 was achieved by siRNA transfection as described in the *Materials and methods*. Each cytokine was applied 48 h after siRNA transfection. (a) 24 h post-transfection, MC38 cells were immunized with SIINFEKL peptide. Six hours after coculture of target (MC38 cells) and effector (OT-1 mouse-derived CD8⁺ T cells) cells, cytotoxicity was evaluated by LDH release assay as in Figure 1a (mean \pm S.E.M., $n = 4$; Bonferroni's *post hoc* test was applied for multiple comparisons in two-way ANOVA, $***P < 0.001$, N.S., not significant). (b) 24 h after treatment with each cytokine in Huh-7 cells, viability was evaluated by WST-1 assay under conditions of siRNA knockdown (mean \pm S.E.M., $n = 4$; $*P < 0.05$, $***P < 0.001$ by Student's *t*-test). (c) Huh-7 cells were incubated in the presence or absence of TGF- β 1 (10 ng/ml) for 1 h, and were then treated with TNF- α (10 ng/ml) for up to 30 min. Cell lysates were subjected to immunoblot analysis as in Figure 2

This was the same for the MKP-1 knockdown sample, indicating that knockdown of Smad2 or MKP-1 abolished the protective effect of TGF- β 1 pretreatment against TNF- α -induced cell death. Instead, treatment with TGF- β 1 alone caused a significant level of cell death, and TGF- β 1 pretreatment exacerbated TNF- α -induced cell death in

MKP-1 knockdown cells, implying that TGF- β 1 cannot exert its protective functions in the absence of MKP-1 expression (Figure 4b). Intracellular signal transduction was also evaluated in Huh-7 cells after introduction of siRNA specific for Smad2. TGF- β 1 did not induce MKP-1 expression in the presence of siSmad2, and phosphorylation of JNK was not

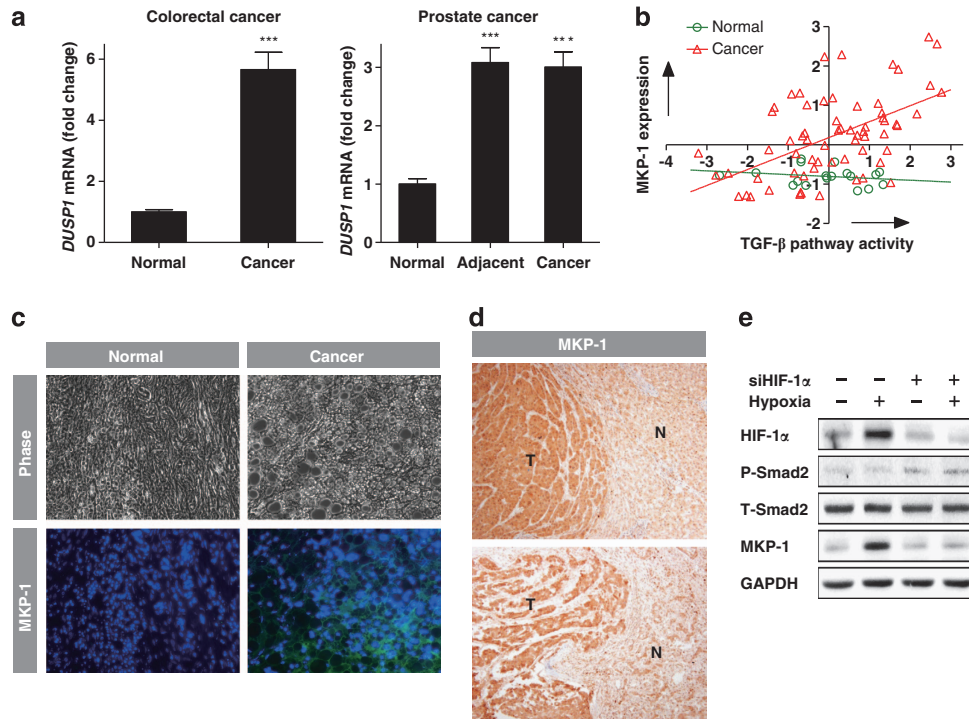


Figure 5 Tumor-specific expression of MKP-1. (a) MKP-1 expression was measured in colorectal and prostate cancer patients from the GEO database using a complementary DNA microarray (mean \pm S.E.M.; Tukey's *post hoc* test was applied to significant group effects in ANOVA, $P < 0.0001$; $***P < 0.001$, compared with normal tissues). (b) The correlation between TGF- β pathway activity and MKP-1 expression in prostate tissue was evaluated as described in the *Materials and methods*. (c) Immunofluorescence staining with FITC (green) and Hoechst (blue) of a human liver tissue array. Of the 50 HCC samples, 39 were MKP-1 positive (78%); nine normal samples did not show MKP-1 expression (0%). (d) Immunoperoxidase staining of tissue samples from HCC patients using DAB and hematoxylin. T, tumor region; N, adjacent normal region. Of 23 HCC patients, 16 showed MKP-1 expression (69.6%) (e) Cell lysates were subjected to immunoblot analysis of HIF-1 α , Smad2, and MKP-1 in the presence or absence of siHIF-1 α and hypoxia (oxygen concentration: 1%). GAPDH was used as the loading control

attenuated by TGF- β 1 pretreatment for 1 h (Figure 4c). These data collectively suggests that the link between MKP-1 and JNK is a novel point of crosstalk between the TGF- β 1 and TNF- α signaling pathway.

Tumor-specific expression of MKP-1. To understand the function of TGF- β 1-dependent suppression of immune-mediated cell death in the tumor microenvironment, we analyzed complementary DNA microarray data provided by gene expression omnibus (GEO) databases. In human colorectal and prostate samples, MKP-1 expression was significantly higher in cancers and cancer-adjacent regions than normal regions (Figure 5a). On the basis of these data, the correlation between TGF- β pathway activity and expression of MKP-1 was evaluated in human prostate cancer tissue. The expression of MKP-1 increased according to TGF- β pathway activity, whereas normal prostate tissue showed no such correlation (Figure 5b). Correlation analysis of colorectal tissue was not included due to insufficiency of the number of samples. These data imply that TGF- β 1- and MKP-1-mediated suppression of immune-mediated cell death is tumor-specific.

In addition to colorectal and prostate cancer, TGF- β 1 is known to be abundant in many cancer tissues.²⁸ Because MKP-1 is downstream of TGF- β 1, we expected the expression of MKP-1 in cancer to also be high. We measured MKP-1 expression by immunohistochemistry. Immunofluorescence

staining in human liver tissue arrays and immunoperoxidase staining in tissue samples from hepatocellular carcinoma (HCC) patients showed markedly elevated MKP-1 levels in cancer (Figure 5c and d). We also cultured Huh-7 cells in a hypoxic chamber for 24 h to mimic the *in vivo* tumor microenvironment, which often has an insufficient oxygen supply. Immunoblot analysis showed that MKP-1 expression was augmented under hypoxia conditions in HIF-1 α -dependent manner, even though Smad2 was not phosphorylated (Figure 5e). These data confirm higher expression of MKP-1 in cancer than in normal tissues.

Chemotherapy of tumor cells with MKP-1 knockdown.

Tumors, including HCC is known to be resistant to diverse cytotoxic chemotherapies, and many clinical trials with anticancer drugs have yielded disappointing results.^{29,30} To determine whether MKP-1, whose expression is only augmented in the tumor cells, can increase resistance to death receptor-mediated cell death, we conducted cytotoxicity assays with several anticancer drugs in Huh-7 cells. First, effective cytotoxic treatment concentrations of doxorubicin, epirubicin, cisplatin, irinotecan, and mitomycin C were determined by lactate dehydrogenase (LDH) release assay (data not shown). With each concentration inducing death of ~30–40% of cells, we examined the role of TGF- β 1 and hypoxia. Compared with control cells, TGF- β 1 (Figure 6a) and hypoxia (Figure 6b) significantly inhibited cell death

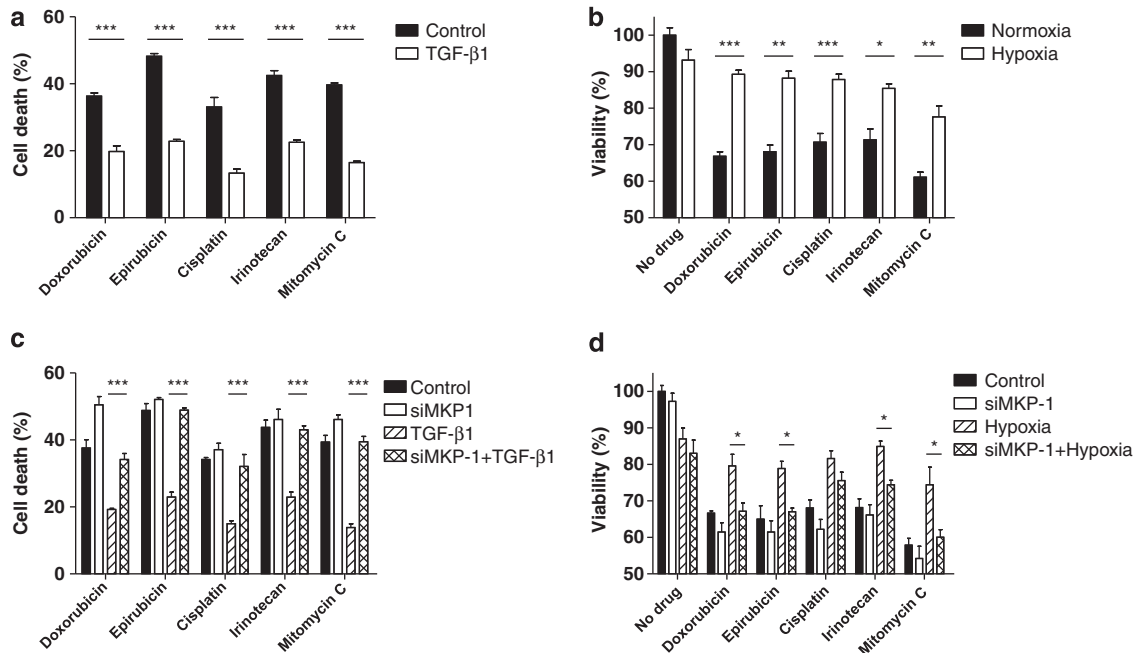


Figure 6 Knockdown of MKP-1 increases the efficacy of cytotoxic anticancer drugs. (a–b) Doxorubicin (10 $\mu\text{g/ml}$), epirubicin (10 $\mu\text{g/ml}$), cisplatin (30 $\mu\text{g/ml}$), irinotecan (500 $\mu\text{g/ml}$), and mitomycin C (50 $\mu\text{g/ml}$) were applied in Huh-7 cells with or without pretreatment with TGF- β 1 (10 ng/ml) (a) or hypoxia (b). Cell death rate was evaluated by LDH release assay (mean \pm S.E.M., $n = 4$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's t -test). (c–d) 48 h post-transfection of siRNA specific for MKP-1, each drug was applied in Huh-7 cells at the same concentrations as in (a–b) with or without pretreatment with TGF- β 1 (10 ng/ml) (c) or hypoxia (d). Cell death rate was evaluated by LDH release assay (mean \pm S.E.M., $n = 4$; Bonferroni's *post hoc* test was applied for multiple comparisons in two-way ANOVA, * $P < 0.05$, *** $P < 0.001$)

triggered by each drug. To confirm that these results were due to MKP-1 expression, we repeated the cell death assays after siRNA knockdown of MKP-1. In the absence of MKP-1, TGF- β 1 (Figure 6c) and hypoxia (Figure 6d) did not exert protective roles against chemo-toxicity, indicating that MKP-1 reduced the cell death induced by the five anticancer drugs. These data suggest that resistance to anti-cancer drugs can be circumvented by MKP-1 knockdown and that systemic chemotherapy with MKP-1 knockdown can make tumor cells more susceptible to cytotoxic drugs.

Thus, we conclude that TGF- β 1 and hypoxia ensure tumor cell survival and growth in MKP-1-dependent manner. Figure 7 demonstrates a schematic diagram of our findings. Under normal conditions, death receptor-mediated cell death signaling maintains a beneficial balance. In cancer, however, both hypoxic conditions and augmented secretion of TGF- β 1 induce MKP-1 expression. MKP-1 blocks phosphorylation of JNK, which is a key mediator of death receptor-mediated cell death, and this in turn suppresses tumor cell death triggered by the immune system and anticancer drugs. This represents a novel immune escape mechanism of tumors.

Discussion

Cells in multicellular organisms continuously face diverse molecules and integrate the signals to respond appropriately. Control of programmed cell death (whether to live or die) is one of the crucial decision-making processes. The regulation of death receptor-mediated cell death, particularly by TNF superfamily ligands, is the major determinant of apoptosis.^{31,32} In this research, we elucidated a novel regulatory

intersection in the death receptor-mediated cell death pathway, involving TGF- β signaling cascades and hypoxia. Our results clearly show that JNK and MKP-1 are involved in this crosstalk, with TGF- β 1- and hypoxia-induced MKP-1 specifically blocking JNK phosphorylation and subsequent death receptor-mediated cell death. We also suggest that this occurs only in tumors, due to the tumor-specific constant expression of MKP-1. Even though many tumor-infiltrating immune cells secrete diverse TNF superfamily ligands,³³ their effects on death receptor-mediated cell death can be blocked by hypoxic conditions and pre-existing TGF- β around tumor cells. This is an effective immune-evasion mechanism of tumor cells, and explains why hypoxia and overabundant secretion of TGF- β provide a beneficial environment for the development of cancer.³⁴

Previous studies investigated crosstalk between the TGF- β and TNF signaling pathways. Kim, *et al.*^{35,36} reported Smad7-involved crosstalk in several types of immune cells. In their system, Smad7 inactivates NF- κ B signaling through TAB2 and TAK1, such that TGF- β shifts the TNF- α signaling balance toward cell death. In our system, however, human hepatoma and mouse colon cancer cell lines showed an opposite functional output of TGF- β and TNF- α crosstalk. Our data suggests that TGF- β 1-induced MKP-1 deactivates TNF- α -induced JNK phosphorylation, whereas the NF- κ B branch is not altered by the presence of TGF- β 1. Interestingly, the association of the same cytokines yields different crosstalk and cellular responses according to the cell type. Even the final outputs of the two crosstalk pathways are in the counterpart, we speculate that both can contribute to immune escape of tumor cells, because excessive TGF- β

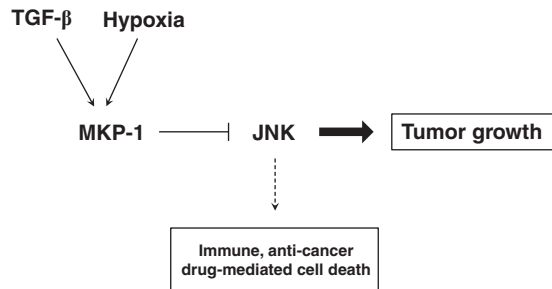


Figure 7 Schematic diagram of this study. We propose a novel crosstalk model involving MKP-1 expression in cancer. The tumor microenvironment is frequently hypoxic, with an overabundance of TGF- β . This triggers tumor-specific expression of MKP-1 and subsequent suppression of JNK phosphorylation. Under these conditions, the balance between cell survival and death inclines toward cell survival. Tumor cells escape from immune- and anticancer drug-mediated cell death, eventually ensuring tumor cell survival and growth

simultaneously induces the death of immune cells via NF- κ B inhibition and ensures tumor cell survival via JNK inactivation.

Surplus TGF- β production and hypoxic conditions are strongly correlated with various diseases, such as cancer and hepatitis.^{37,38} Therefore, our experimental design is relevant to clinical issues. On the basis of TGF- β 1- and hypoxia-induced expression of MKP-1, we evaluated MKP-1 as a novel drug target to overcome chemo-resistance to anticancer drugs. Because tumor cells exploit MKP-1 as a shield against immune-mediated cell death, our results provide a plausible mechanism, by which tumor cells efficiently escape from immune-mediated cell death, and might provide a rational background for development of therapeutic interventions.

Materials and Methods

Cell culture and reagents. Human HCC cell lines (Huh-7 and HepG2) and murine colorectal adenocarcinoma cell line (MC38) were maintained at 37 °C in an atmosphere containing 5% CO₂ in Dulbecco's modified Eagle's medium (Welgene, Seoul, Korea) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA, USA). OT-1 mouse-derived T cells were maintained at 37 °C in an atmosphere containing 5% CO₂ in RPMI 1640 medium (Welgene, Seoul, Korea) supplemented with 10% fetal bovine serum and 50 U/ml penicillin/streptomycin (PAA, Pasching, Austria).

Antibodies specific for IKK- β , phospho-IKK, phospho-I κ B- α , JNK, phospho-JNK, p38, phospho-p38, p42/44, phospho-p42/44, Smad2, phospho-Smad2, MKK3, phospho-MKK3/6, MKK4, phospho-MKK4, MKK7, phospho-MKK7, TAK1, and phospho-TAK1 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific for MKP-1, MKP-2, I κ B, β -actin, GAPDH, and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An antibody specific for HIF1- α was purchased from Abcam (Cambridge, MA, USA). Human recombinant TGF- β 1 and TNF- α were obtained from R&D Systems (Minneapolis, MN, USA) and TRAIL was generously provided by Dr. Kunhong Kim (Yonsei university, Seoul, Korea). An agonistic IgM type anti-Fas antibody (CH-11) was obtained from Upstate Biotechnology (Lake Placid, NY, USA). The JNK inhibitor SP600125 was purchased from Calbiochem (La Jolla, CA, USA). Five anticancer drugs, doxorubicin, epirubicin, cisplatin, irinotecan, and mitomycin C, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

OT-1 mice, in vitro activation of T cells, purification, and SIINFEKL peptide loading. Eight-week-old OT-1 transgenic mice were used. Lymph nodes and spleen cells were isolated from OT-1 mice by gentle crushing of the organs and filtering through a 100- μ m pore nylon mesh. Pooled lymph nodes and spleen cells were further treated with RBC lysis buffer

(BioLegend, San Diego, CA, USA) and maintained at a density of 3–5 \times 10⁶ cells/ml in 75-cm² culture flasks. Phenotypes were determined by flow cytometric analysis using antibodies specific for CD8 α and the TCR chains V α 2 and V β 5. For *in vitro* OT-1 T cell activation, OVA peptide (SIINFEKL) (PeproTech, Rocky Hill, NJ, USA) was added at the start of culture at a concentration of 10 μ g/ml, and cells were maintained at 37 °C. After 5 days of incubation, the lymph node and spleen cells were enriched for CD8⁺ T cells by positive selection using MACS (Miltenyi Biotec, Auburn, CA, USA). Greater than 99% CD8⁺ T cell enrichment was confirmed by analysis of the surface markers CD3 and CD8 α by flow cytometry. Murine colon adenocarcinoma MC38 cells were used as target cells in coculture with OT-1 mouse-derived T cells. The target cells were loaded with 10 μ g/ml SIINFEKL 1 day before coculture experiments. *In vitro* activated OT-1 mouse CD8⁺ T cells were cocultured with SIINFEKL-loaded MC38 cells at given effector (E):target (T) ratios (0.25:1, 0.5:1, 1:1, and 2:1) for 6 h, and then subjected to a cell-mediated cytotoxicity assay.

Cell death and viability assays. WST-1 assays were performed to determine cell viability/death. Huh-7 cells were seeded in 24-well plates at a density of 4 \times 10⁴/well in quadruplicate and WST-1 reagent (Nalgene, Rochester, NY, USA) was added to each well at a volume up to 5% that of the medium. After incubation for 2 h at 37 °C in a 5% CO₂ incubator, the absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Richmond, CA, USA). Cell-mediated cytotoxicity was assessed by LDH release assay (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Immunoblot analysis. Immunoblot analysis was carried out to evaluate intracellular signal transduction as previously described.³⁹ The proteins were transferred to nitrocellulose membranes and probed with antibodies specific for MKP-1, MKP-2, JNK, p38, p42/44, Smad2, IKK, I κ B, MKK3/6, MKK4, MKK7, TAK1, β -actin, and GAPDH.

Immunocytochemistry. Immunocytochemistry was performed using a polyclonal antibody specific for MKP-1 (1:100, diluted in 1.5% BSA) as previously described.³⁹ The slides were incubated with FITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) as the secondary antibody. Slides were visualized by fluorescence microscopy (Carl Zeiss, Oberkochen, Germany).

Patients and tumor specimens. Patients admitted to Ewha Womans University Mokdong Hospital (Seoul, Korea) were considered for inclusion in the present study. Tumor tissues were dissected from 23 primary HCC patients, formalin-fixed, and paraffin-embedded for immunohistochemistry. In addition, tissue arrays containing 40 non-metastatic HCC samples, 10 metastatic HCC samples, and nine normal liver tissue samples was purchased from SuperBioChips (Seoul, Korea).

Immunohistochemistry. Paraffin-embedded tissues were deparaffinized in xylene, rehydrated in graded alcohols, and washed in water. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂. After treatment with 3% BSA for 30 min to block non-specific protein binding, a primary antibody specific for MKP-1 was applied (dilution: 1:100), and the specimens were incubated overnight. For immunoperoxidase staining, biotin-labeled secondary antibodies were applied for 30 min, followed by avidin-biotin-peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA, USA). After brief rinsing with PBS, sections were incubated with diaminobenzidine (DAB) solution for 2 min. For immunofluorescence staining, a FITC-conjugated rabbit-specific secondary antibody was applied for 30 min. Cell nuclei were counter-stained with hematoxylin for immunoperoxidase staining and with Hoechst (Sigma-Aldrich) for immunofluorescence staining.

siRNA transfection. A siRNA duplex targeted to Smad2 and MKP-1, and an siRNA with a random sequence (negative control) were synthesized by Bioneer (Daejeon, Korea). Transient transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Transfection efficiency was assessed by immunoblotting and immunocytochemistry.

Microarray data sets and processing. For MKP-1 expression profile, we used two microarray data sets from the GEO database: GDS2609 (colorectal cancer) and GDS2545 (prostate cancer). In these data sets, the expression levels of mRNAs involved in TGF- β pathway from the NetPath database were averaged

and defined as the TGF- β pathway activity, where expression levels were normalized by z-transformation to have same range of expression levels for all genes.

Total RNA isolation and qRT-PCR analysis. Total RNAs were isolated from Huh-7 cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 1 μ g of total RNA with the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. RT-PCR was then carried out in triplicate to determine mRNA levels of MKP-1 using the StepOne TM RT-PCR system with SYBR Green PCR Master Mix (Applied Biosystems). The MKP-1 primer (Bioneer) sequences were 5'-CCTGT CCACTCCACGAACAGT-3' for the forward primer and 5'-GCTGGGAGAGTCTG AATGG-3' for the reverse primer. Target mRNA levels were normalized to an endogenous reference GAPDH.

Statistical analysis. Data are presented as the means \pm S.E.M. Levels of significance for comparisons between two independent samples were determined using student's *t*-test. Groups were compared by one-way or two-way analysis of variance (ANOVA) with Tukey's *post hoc* test or Bonferroni's *post hoc* test applied to significant main effects.

Conflict of Interest

The authors declare no conflict of interest.

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