Regulation of p38 Phosphorylation and Topoisomerase IIα Expression in the B-Cell Lymphoma Line Jiyoye by CD26/Dipeptidyl Peptidase IV Is Associated with Enhanced In vitro and In vivo Sensitivity to Doxorubicin

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Abstract

CD26 is a M, 110,000 surface-bound glycoprotein with diverse functional properties, including having a key role in normal T-cell physiology and the development of certain cancers. In this article, we show that surface expression of CD26, especially its intrinsic dipeptidyl peptidase IV (DPPIV) enzyme activity, results in enhanced topoisomerase IIα level in the B-cell line Jiyoye and subsequent in vitro sensitivity to doxorubicin-induced apoptosis. In addition, we show that expression of CD26/DPPIV is associated with increased phosphorylation of p38 and its upstream regulators mitogen-activated protein kinase kinase 3/6 and apoptosis signal-regulating kinase 1 and that p38 signaling pathway plays a role in the regulation of topoisomerase IIα expression. Besides demonstrating that CD26 effect on topoisomerase IIα and doxorubicin sensitivity is applicable to cell lines of both B-cell and T-cell lineages, the potential clinical implication of our work lies with the fact that we now show for the first time that our in vitro results can be extended to a severe combined immunodeficient mouse model. Our findings that CD26 expression can be an in vivo marker of tumor sensitivity to doxorubicin treatment may lead to future treatment strategies targeting CD26/DPPIV for selected human cancers in the clinical setting. Our article thus characterizes the biochemical linkage among CD26, p38, and topoisomerase IIα while providing evidence that CD26-associated topoisomerase IIα expression results in greater in vitro and in vivo tumor sensitivity to the antineoplastic agent doxorubicin.

Introduction

CD26 is a M, 110,000 type II cell surface glycoprotein with diverse functional properties, which is widely expressed on various tissues, including lymphocytes, with its extracellular domain encoding a membrane-associated dipeptidyl peptidase IV (DPPIV) activity that cleaves selected biological factors to alter their functions (1). It plays an important role in T-cell biology through its physical and functional association with molecules involved in T-cell signal transduction processes (1–6). Recent findings suggest that CD26/DPPIV has a role in the development of certain neoplasms, being overexpressed in certain aggressive T-cell malignancies (7, 8), B-chronic lymphocytic leukemia (9), and thyroid carcinoma (10). On the other hand, loss or decreased surface expression of CD26/DPPIV is found in prostate cancer (11), colorectal carcinoma (12), and melanomas (13). Meanwhile, investigators have shown that DPPIV expression in melanoma and non–small cell lung carcinoma leads to inhibition of tumorigenicity, whereas DPPIV expression in ovarian carcinoma cells reduces i.p. dissemination of carcinoma cells and prolongs survival time (14–16). Topoisomerase IIα is an intracellular protein with a key role in proliferation and is a target for various antineoplastic agents (17). We found recently that CD26/DPPIV expression on the T-cell line Jurkat is associated with increased topoisomerase IIα level, leading to a concomitant enhancement in in vitro sensitivity to topoisomerase II inhibitors (18–20).

The family of mitogen-activated protein kinases (MAPK) plays a very important role in the signal pathways of cell proliferation, differentiation, survival, and apoptosis (21). Three major molecules belong to this family: extracellular signal-regulated kinase (ERK) 1/2 (p44/p42), c-Jun NH2-termina terminal kinase (JNK/stress-activated protein kinase), and p38 MAPKs. In general, the ERK pathway mediates primarily cell growth and survival signals and promotes induction of cell differentiation under certain circumstances. On the other hand, both JNK and p38 pathways, which comprise the stress-activated protein kinase family, generally mediate proapoptotic, growth inhibitory signals and proinflammatory responses. However, p38 also induces antiapoptotic, proliferative, and cell survival signals under certain conditions (22, 23). Of note is the fact that certain antineoplastic agents, such as doxorubicin and cisplatin, induce p38-mediated apoptosis (23, 24). CD26/DPPIV is also associated with p38 signaling in certain instances. Inhibition of DPPIV enzyme activity resulted in p38 activation, leading subsequently to transforming growth factor-β1 expression and secretion (25). Meanwhile, ERK was phosphorylated and activated in CD26 Jurkat transfectant following treatment with anti-CD26 antibody (26).

Extending our previous findings in this study, we use the Burkitt B-cell lymphoma line Jiyoye to characterize the effect of CD26 expression on topoisomerase IIα and p38. We show that CD26 expression on CD26 Jiyoye transfectants is associated with enhanced topoisomerase IIα level and increased sensitivity to the antineoplastic agent doxorubicin. We also show that CD26 expression results in increased p38 phosphorylation, associated with increased phosphorylation of the upstream regulators MAPK...
kinase (MKK) 3/6 and apoptosis signal-regulating kinase 1 (ASK1). Inhibition of p38 phosphorylation decreases topoisomerase IIA expression, suggesting a role for p38 in the regulation of topoisomerase IIA. Finally, studies using a severe combined immunodeficient (SCID) mouse xenograft model with CD26 Jiyoye transfectants show that CD26 expression is associated with enhanced survival following treatment with low doses of doxorubicin. Our data thus characterize the biochemical linkage among CD26, p38, and topoisomerase IIA while suggesting a potential role for CD26 in the clinical setting in the treatment of selected malignancies.

Materials and Methods

Animals, Cells, and Reagents. Human Burkitt B-cell lymphoma cell line Jiyoye and human anaplastic large T-cell lymphoma cell line Karpas-299 were obtained from American Type Culture Collection (Rockville, MD). Jiyoye cells were maintained in culture medium, which consisted of RPMI 1640 supplemented with 20% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C and 5% CO2. Karpas-299 cells were maintained in RPMI 1640 supplemented with 10% FCS and penicillin-streptomycin at 37°C and 5% CO2. Female CB-17 SCID mice were obtained from Taconic Farms, Inc. (Germantown, NY) at 3 weeks of age and were housed in microisolator cages, and all food, water and bedding were autoclaved before use. Annexin V-FITC, anti-poly(ApD)-ribosome) polymerase, phycoerythrin-conjugated anti-CD8, and phycoerythrin-conjugated anti-CD9 were from BD PharMingen (San Diego, CA); FITC-conjugated anti-CD26 was from Caltag (Burlingame, CA); anti-actin was from Sigma Chemical Co. (St. Louis, MO); anti-topoisomerase Iα was from Roche (Indianapolis, IN); antibodies against p38, phospho-p38, ERK1/2 (p44/p42 MAPK), phospho-ERK1/2, JNK, phospho-JNK, MKK3, phospho-MKK3/MKK6, ASK1, and phospho-ASK1 (Se8 and Se9) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The p38 inhibitor SKF86002 was from Calbiochem (La Jolla, CA). Substrate for DPPIV, Gly-Pro-p-nitroanilide-tosylate, was purchased from WAKO (Osaka, Japan). Doxorubicin was purchased from Calbiochem and was dissolved in sterile PBS. All oligonucleotides were synthesized with Invitrogen (Carlsbad, CA).

Establishment of CD26 Transfectants. The CD26 cDNA insert was a gift from the plasmid pSra-26 as described previously (27). 5' Flanking region of CD26 (28) was extended and amplified by the PCR using primers Ad1 (CCCCGGTTGCTGGCTGCTTCCTCTCTGAAGCTCTCATTCCGAGGAGACGCCGAGTGAGAACCC) and R3 (GCCCGGTACCTGAAAGTGAAGGGAACAATTGGG). Through site-directed gene mutagenesis method (29), mutant CD26 containing an alanine at the putative catalytic Se696 was prepared with primers Ad1, R3, and SA (AATTTTGCGTGGCCTGAGATGGAAGCTT). Resulting mutated CD26 was used as template for PCR amplification. The PCR product was inserted into the EcoRI site of the pSilencerRetroQ vector (Clontech Laboratories) according to manufacturer's protocol. The inserted sequence was as follows: sense GATCCGATCTTGCAAGTCTTTTTTGGAAG [sense siRNA (CD26-siRNA)] and antisense AATCTCATTCAAAAGATCATGTAGCACTTCTCTTGAAGTTTGATGCAATGCTGATGATCG. Moreover, mismatch siRNA [mis-siRNA (mis-CD26-siRNA)] at 3 nt was prepared to examine nonspecific effects of siRNA duplexes. Inserted sequence was as follows: sense GATCGATCTTGCAAGC-CAAAACATCAGAAGGGTTGTTGTGTCGAAATGATCTTTTTTGGAAG and antisense AATCTCATTCAAAAGATCATGTAGCACTTCTCTTGAAGTTTGATGCAATGCTGATGATCG. These sense and antisense primers were hybridized and then inserted into pSilencerRetroQ vector. After all sequences were confirmed, CD26-siRNA retrovirus was produced by the same method as above, and Karpas-299 cells were transduced and selected with puromycin (0.4 μg/mL, Clontech Laboratories).

Cytotoxicity index was calculated as follows:

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\text{Cytotoxicity index} = \frac{A_{570\text{ nm} \times \text{control}}}{A_{570\text{ nm} \times \text{ treated cells}}} \times 100% 
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Immunoﬂuorescence. All procedures were carried out at 4°C and flow cytometric analyses were done (FACScan, Becton Dickinson, San Jose, CA) as described previously (32). Cells were stained with FITC-conjugated anti-CD26 antibody and washed twice with PBS and then with goat anti-mouse IgG FITC (Coulter, Fullerton, CA). Cells were then washed twice with PBS before flow cytometric analysis. Negative control samples were stained with second antibody alone.

Annexin V/Propidium Iodide Assays. Exposure of phosphatidylserine residues was quantified by surface Annexin V staining as described previously (33). Briefly, cells were washed in binding buffer [10 mmol/L HEPES (pH 7.4), 2.5 mmol/L CaCl2, 140 mmol/L NaCl], resuspended in 100 μL, and incubated with 0.5 μL/mL Annexin V-FITC and 2.5 μg/mL propidium iodide (PI) for 15 minutes in the dark. Cells were then washed again and resuspended in 400 μL binding buffer; then, flow cytometric analysis was done. A total of 10,000 cells were acquired per sample and data were analyzed using CellQuest software (BD Pharmingen). Cells in early stages of apoptosis were Annexin V positive, whereas cells that were Annexin V and PI positive were in late stages of apoptosis (34).

SDS-PAGE and Immunoblotting. After incubation at 37°C in culture medium, Jiyoye-vector control, Jiyoye-wild type (wt) CD26 transfectant, and Jiyoye-SADC26 transfectant were harvested, washed with PBS, and lysed in lysis buffer consisting of 1% NP40, 0.5% deoxycholate, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine, 10 μg/mL aprotinin, 50 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, and 1 μg/mL pepstatin. After incubating on ice for 5 minutes, nuclei were removed by centrifugation and supernatants were collected as whole cell lysates. Sample buffer (4×) consisting of 20% glycerol, 4% SDS, 0.5 mol/L Tris (pH 6.8), 4% β-mercaptoethanol, and 0.2% bromphenol blue was added to the appropriate aliquots of supernatants. After boiling, protein samples were submitted to SDS-PAGE analysis on appropriate gel under standard conditions using mini-Protein II system (Bio-Rad, Richmond, CA). For each experiment, each lane was loaded with equal amount of protein. For immunoblotting, the proteins were transferred onto nitrocellulose (Immobilon-P, Millipore, Billerica, MA).

We selected the target sequence from 1,768 to 1,786 downstream of the start codon of CD26 mRNA. Inserted siRNA oligonucleotide of pSilencerRetroQ vector (Clontech Laboratories) was designed according to manufacturer's protocol. The inserted sequence was as follows: sense GATCCGATCTTGCAAGC-CAAAACATCAGAAGGGTTGTTGTGTCGAAATGATCTTTTTTGGAAG and antisense AATCTCATTCAAAAGATCATGTAGCACTTCTCTTGAAGTTTGATGCAATGCTGATGATCG. Moreover, mismatch siRNA [mis-siRNA (mis-CD26-siRNA)] at 3 nt was prepared to examine nonspecific effects of siRNA duplexes. Inserted sequence was as follows: sense GATCGATCTTGCAAGC-CAAAACATCAGAAGGGTTGTTGTGTCGAAATGATCTTTTTTGGAAG and antisense AATCTCATTCAAAAGATCATGTAGCACTTCTCTTGAAGTTTGATGCAATGCTGATGATCG. These sense and antisense primers were hybridized and then inserted into pSilencerRetroQ vector. After all sequences were confirmed, CD26-siRNA retrovirus was produced by the same method as above, and Karpas-299 cells were transduced and selected with puromycin (0.4 μg/mL, Clontech Laboratories).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay. Cell growth assay was done as described previously (31). Cells were incubated in 96-well plates in the presence of culture medium alone or culture medium with doxorubicin at the indicated concentrations for a total volume of 100 μL (50,000 cells per well). After 72 hours of incubation at 37°C, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (25 μL) was added to the wells at a final concentration of 1 μg/mL. The 96-well plates were then incubated for 2 hours at 37°C followed by the addition of 100 μL extraction buffer. After overnight incubation at 37°C, absorbance measurements at 570 nm were done, with SE of the triplicate well being <15%.

Cell viability was calculated as follows:

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\text{Cell viability} = \frac{A_{570\text{ nm} \times \text{control}}}{A_{570\text{ nm} \times \text{ treated cells}}} \times 100% 
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After blocking for 1 hour at room temperature or overnight at 4°C in blocking solution consisting of 5% bovine serum albumin or 5% dry milk in 0.1% Tween 20-TBS, membranes were blotted with the appropriate primary antibodies diluted in blocking solution for 1 hour at room temperature or overnight at 4°C. Membranes were then washed with Tween 20-TBS, and appropriate secondary antibodies diluted in Tween 20-TBS were then applied for 1 hour at room temperature. Secondary antibody was goat anti-rabbit or goat anti-mouse peroxidase conjugates (DAKO, Kyoto, Japan). Membranes were then washed with Tween 20-TBS, and proteins were detected using an enhanced chemiluminescence system according to the manufacturer’s instructions (Pierce, Rockford, IL). Membranes were exposed to Hyperfilm (Amersham Pharmacia Biotech, Piscataway, NJ).

**DPP IV Enzyme Activity assay.** As described previously (16), DPP IV enzyme activity was measured spectrophotometrically using Gly-Pro-p-nitroanilide-tosylate, a substrate for DPP IV. A 1× PBS-washed whole cell suspension was prepared, and 5 × 10^6 cells were resuspended in 200 μL PBS into 96-well plate; then, Gly-Pro-p-nitroanilide-tosylate was added at a final concentration of 0.24 mmol/L. The absorption was measured at 405 nm using microplate spectrophotometer (Bio-TEK Instruments, Inc., Winooski, VT) twice just before the addition of the substrate and after 60-minute incubation at 37°C. DPP IV enzyme activity was calculated from the increase of absorption between 0 and 60 minutes.

**Preparation of Nuclear Extracts for Detection of Topoisomerase II. Protein Level.** For detection of topoisomerase IIs by immunoblotting, isolation of nuclear fractions from Jiyoye-wtCD26 transfectants was prepared as follows. In brief, 10 × 10^6 cells were harvested and allowed to swell for 15 minutes on ice in cytoplasmic extraction buffer (10 mmol/L HEPES, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L phenylmethyisulfonyl fluoride, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine). Then, NP40 (final concentration 0.3%) was added to the cell suspension and vortexed for 10 seconds. After 2 minutes of centrifugation at 16,000 × g, the supernatant was removed. The pellet was then incubated with nuclear extraction buffer (20 mmol/L HEPES, 400 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L phenylmethyisulfonyl fluoride, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine) for 30 minutes on ice with intermittent vortexing. The suspension was centrifuged at 16,000 × g for 5 minutes, and the supernatant was saved as the nuclear extract. SDS-PAGE and immunoblotting were then done on the nuclear extracts. Each lane was equally loaded with 10 μg protein.

**In vivo Experiments.** All mice were pretreated by i.p. route with 0.2 mL anti-asialo-GM1 polyclonal antisera 25% (v/v, WAKO) 1 day before tumor engraftment (35). On day 0, 10 × 10^6 Jiyoye-wtCD26 transfectant cells or Jiyoye-vector control cells were then inoculated by i.p. injection. Following tumor cell inoculation, SCID mice then received saline or doxorubicin in saline by i.p. injection at 0.5 mg/kg on days 1 and 15. Tumor bearing mice were monitored for tumor development and progression, and moribund mice were euthanized, with necropsies being done for evidence of tumors. In addition, mice with visible or palpable tumors measuring 15 mm at its smallest dimension were euthanized, with necropsies done to minimize suffering to the mice.

**Results**

**Expression of CD26/DPP IV and Topoisomerase IIα in Jiyoye-CD26 Transfectants.** Following transfection of the human Burkitt B-cell lymphoma cell line Jiyoye with the retroviral vector pLNCX2 as described in Materials and Methods, CD26/DPP IV status is evaluated. Parental Jiyoye cells and pLNCX2-only Jiyoye transfectants (Jerome-vector control) do not express detectable amount of CD26 as determined by cell surface staining. Meanwhile, Jiyoye-wtCD26 transfectants have high level of CD26 surface expression, and Jiyoye-S630A (SACD26) transfectants express the catalytically inactive variant of CD26 (Fig. 1A). On the other hand, only the Jiyoye-wtCD26 transfectants express DPP IV enzyme activity, with Jiyoye-vector control and Jiyoye-SACD26 transfectants having no detectable DPP IV activity (Fig. 1B). Consistent with our previous findings that CD26 expression is associated with increased topoisomerase IIα level in CD26 transfectants of the T-cell leukemia line Jurkat (19, 20), Jiyoye-wtCD26 transfectants also express higher level of topoisomerase IIα than Jiyoye-vector control or Jiyoye-SACD26 transfectants (Fig. 1C). By demonstrating that CD26 expression, particularly its DPP IV enzyme activity, is associated with enhanced topoisomerase IIα expression in the B-cell line Jiyoye, our findings indicate that a relationship between these key proteins is potentially found in a wide variety of tumor types. Furthermore, our data suggest a potential role for CD26/DPP IV in the treatment of malignancies of both B-cell and T-cell lineages.

**Enhancement of Doxorubicin-Mediated Apoptosis in Jiyoye-CD26 Transfectants.** To elucidate the potential consequence of the CD26-topoisomerase IIα association, we investigated the effect of CD26/DPP IV surface expression on doxorubicin sensitivity of Jiyoye-CD26 stable transfectants. 3-(4,5-Dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide uptake assays show that Jiyoye-wtCD26 transfectants display significantly increased sensitivity to doxorubicin compared with Jiyoye-vector control. In addition, Jiyoye-SACD26 transfectant, with CD26 mutated at the DPP IV catalytic site (S630A), is less sensitive to doxorubicin than Jiyoye-wtCD26 transfectants, consistent with the key role played by the DPP IV enzyme activity in increasing topoisomerase IIα level and subsequent drug sensitivity (Fig. 2A). Meanwhile, Annexin V-PI assays show greater doxorubicin-induced apoptosis for Jiyoye-wtCD26 transfectants than Jiyoye-vector control cells or Jiyoye-SACD26 transfectants (Fig. 2B). Furthermore, Western blot analyses show that Jiyoye-wtCD26 transfectants exhibit greater poly(ADP-ribose) polymerase cleavage with doxorubicin treatment than Jiyoye-vector cells (Fig. 2C) Taken together, these data show that surface expression of CD26/DPP IV on the B-cell lymphoma line Jiyoye directly enhances cellular sensitivity to doxorubicin and drug-induced apoptosis.

**Effect of CD26/DPP IV Surface Expression on the p38 Signaling Pathway.** Because CD26 signaling involves MAPK in certain experimental conditions (25, 26), we evaluated the status of p38, ERK, and JNK in Jiyoye-CD26 transfectants. Figure 3 shows that Jiyoye-wtCD26 transfectants exhibit greater phosphorylation of p38 compared with Jiyoye-vector or Jiyoe-SACD26 transfectant in the absence of any extrinsic stimulation. In contrast to p38, there is no difference in the phosphorylation status of ERK and JNK among the cells incubated in culture medium, confirming that p38 is selectively phosphorylated in the presence of CD26, particularly its DPP IV enzyme activity.

To further confirm our findings with the Jiyoye-CD26 transfectants that CD26 presence enhances p38 phosphorylation, we established Karpas-299-CD26-siRNA as described in Materials and Methods. Whereas parental Karpas-299 cells have high level of CD26 surface expression, as shown previously (35), Karpas-299-CD26-siRNA cells exhibit low level of CD26 and a concomitant decrease in DPP IV enzyme activity (Fig. 4A and B). Importantly, Karpas-299-CD26-siRNA transfectants display decreased p38 phosphorylation level compared with parental Karpas-299 and missense Karpas-299-CD26-siRNA (Karpas-299-mis-CD26-siRNA) transfectants with unaltered CD26/DPP IV levels (Fig. 4C). Together, our findings show a clear association between CD26/DPP IV expression and increased p38 phosphorylation.
The p38 signaling pathway is activated by various stress agents. To further characterize the effect of CD26/DPPIV on p38-mediated signaling, we stimulated Jiyoye-CD26 transfectants with UVC irradiation and phorbol 12-myristate 13-acetate (PMA). As shown in Fig. 5A, whereas Jiyoye-wtCD26 transfectants have greater level of baseline p38 phosphorylation than Jiyoye-vector control or Jiyoye-SACD26 transfectant (A, negative control; B, anti-CD26 antibody), representative of three different experiments. B, DPPIV activity on Jiyoye-CD26 transfectants. Jiyoye cells were evaluated for DPPIV activity as described in Materials and Methods. 1), Jiyoye-vector control; 2), Jiyoye-wtCD26-1; 3), Jiyoye-wtCD26-2; 4), Jiyoye-SACD26 transfectant. Columns, means of three separate experiments. C, topoisomerase II expression on Jiyoye-CD26 transfectants Jiyoye cells were incubated in culture medium, and nuclear extracts were collected for immunoblotting studies to evaluate topoisomerase II protein levels, with β-actin as controls, as described in Materials and Methods. Each lane was equally loaded with 10 μg protein. Lane 1, Jiyoye-SACD26; lane 2, Jiyoye-wtCD26-1; lane 3, Jiyoye-wtCD26-2; lane 4, Jiyoye-vector. Representative of three different experiments.

The p38 signaling pathway is activated by various stress agents. To further characterize the effect of CD26/DPPIV on p38-mediated signaling, we stimulated Jiyoye-CD26 transfectants with UVC irradiation and phorbol 12-myristate 13-acetate (PMA). As shown in Fig. 5A, whereas Jiyoye-wtCD26 transfectants have greater level of baseline p38 phosphorylation than Jiyoye-vector control or Jiyoye-SACD26 transfectant, as shown above, Jiyoye-wtCD26 transfectants and Jiyoye-vector control exhibit enhanced p38 phosphorylation when stimulated with both UVC irradiation and PMA. Interestingly, Jiyoye-SACD26 transfectant displays enhanced p38 phosphorylation only when stimulated with UVC irradiation but not PMA, suggesting that different signaling events are involved for CD26/DPPIV-associated p38 phosphorylation by different stimuli. Time course studies show that PMA does not induce p38 phosphorylation in Jiyoye-SACD26 cells across the range of times tested, indicating that there is a true attenuation of phosphorylation and not merely a temporal shift in phosphorylation (Fig. 5B).

**CD26/DPPIV Effect on Upstream Regulators of p38 in Jiyoye-CD26 Transfectants.** Phosphorylation of p38 is regulated by several upstream proteins, including MKK3/MKK6. To further delineate the effect of CD26/DPPIV on p38 signaling pathway, we investigated the status of upstream regulators of p38 in Jiyoye-CD26 transfectants. Specifically, Western blot analyses with anti-phospho-MKK3/MKK6 antibody show that Jiyoye-wtCD26 transfectants have higher level of MKK3/MKK6 phosphorylation compared with Jiyoye-vector control and Jiyoye-SACD26 transfectants (Fig. 6). However, there is no detectable difference in
phosphorylation of MKK4 in these cells (data not shown), which has been described previously to contribute to p38 phosphorylation (36). We also evaluated the status of ASK1, which has a role in stress-induced apoptosis and has been found to be an upstream regulator of MKK3/MKK6 (37). Figure 6 shows that ASK1 is overexpressed in Jiyoye-wtCD26 transfectants compared with Jiyoye-vector control and Jiyoye-SACD26 cells. Interestingly, there is a higher level of phospho-ASK1 (Ser^83) in Jiyoye-wtCD26

Figure 2. Effect of CD26 expression on doxorubicin-mediated growth inhibition and apoptosis. A, Jiyoye-CD26 transfectants were incubated at 37°C in culture medium alone or culture medium containing doxorubicin at the indicated concentrations, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide uptake assay was done as described in Materials and Methods. Points, means of three separate experiments. B, Jiyoye-vector, Jiyoye-wtCD26 transfectants, and Jiyoye-SACD26 transfectants were incubated at 37°C in culture medium alone or culture medium containing doxorubicin for 48 hours at the concentrations indicated. Cells were then harvested, and Annexin V-PI assays were done as described in Materials and Methods. Cells in early stages of apoptosis were Annexin V positive, whereas Annexin V− and PI-positive cells were in late-stage apoptosis. Representative of three independent experiments. Y axis, % of control was calculated as follows: % of control = treated cells / nontreated cells × 100. Group 1, Jiyoye-vector control; group 2, Jiyoye-wtCD26-1; group 3, Jiyoye-wtCD26-2; group 4, Jiyoye-wtCD26-3; group 5, Jiyoye-SACD26. C, Jiyoye-CD26 transfectants were incubated at 37°C with medium containing doxorubicin (Dox) for 48 hours at the indicated doses. Cells were then harvested and whole cell lysates were obtained. Following SDS-PAGE of lysates, immunoblotting studies for poly(ADP-ribose) polymerase (PARP) and β-actin were done as described in Materials and Methods. The cleaved product of poly(ADP-ribose) polymerase was detected at 85 kDa. Each lane was loaded with 30 µg protein. Group 1, Jiyoye-vector control; group 2, Jiyoye-wtCD26-1; group 3, Jiyoye-wtCD26-2; group 4, Jiyoye-wtCD26-3. Representative of three different experiments.
cells, whereas no significant level of phospho-ASK1 (Ser\(^{387}\)) is detected. Taken together, our findings suggest that there is an increase in the absolute number of ASK1 molecules phosphorylated at Ser\(^{38}\) in Jiyoye-wtCD26 transfectants. Meanwhile, the elevated phospho-ASK1 (Ser\(^{38}\)) level seen in conjunction with a similar increase in overall ASK1 level, along with the lack of change in phospho-ASK1 (Ser\(^{387}\)), indicates that the specific phosphate content of each ASK1 molecule is likely unchanged following CD26/DDPIV expression. Our data suggest that ASK1 and its specific phosphorylation at Ser\(^{38}\) serve as an up-regulator of CD26/DDPIV-associated p38 phosphorylation in these Jiyoye-wtCD26 transfectants.

Effect of p38 Inhibition on Topoisomerase IIα Expression.
To determine the relationship between p38 phosphorylation and topoisomerase IIα expression, we evaluated topoisomerase IIα level following inhibition of p38 phosphorylation by its specific inhibitor SKF86002 (23, 38) in Jiyoye-vector controls and Jiyoye-wtCD26 transfectants. Of note is the fact that treatment with the p38 inhibitor at the indicated concentration and time course did not affect cell viability or cell cycle status (data not shown). As shown in Fig. 7, treatment with the p38 inhibitor decreases p38 phosphorylation, associated with markedly decreased topoisomerase IIα expression in both Jiyoye-vector cells and Jiyoye-wtCD26 transfectants. We found that inhibition of topoisomerase IIα expression consistently lags behind the inhibitory effect of SKF86002 on p38 phosphorylation. Whereas p38 phosphorylation is decreased from 6 to 24 hours after SKF86002 treatment, with recovery seen by 48 hours post-treatment, decreased topoisomerase IIα expression is clearly detected 48 hours post-treatment. Similar results were obtained with the p38 inhibitor SB203580 (data not shown). The fact that inhibition of p38 phosphorylation leads temporally to decreased topoisomerase IIα expression strongly suggests that p38 signaling pathway is involved in the regulation of topoisomerase IIα. Furthermore, our data show that p38-mediated regulation of topoisomerase IIα is independent of CD26 presence.

Increased Survival of Doxorubicin-Treated SCID Mice Bearing Jiyoye-wtCD26 Cells.
Extending our in vitro findings, we investigated the effect of CD26 expression on overall survival in doxorubicin-treated SCID mice inoculated with Jiyoye-vector control or Jiyoye-wtCD26 transfectants. Jiyoye-wtCD26 transfectants or Jiyoye-vector control cells (7 \(\times\) 10\(^6\) cells per mouse) were implanted by i.p. injection into the SCID mice on day 0; then, once per day on days 1 and 15, animals were treated by i.p. injection with saline alone or doxorubicin at a dose of 0.5 mg/kg of body weight per injection. As shown in Fig. 8, most of the SCID mice inoculated with Jiyoye-vector control cells and treated with saline (line 1) developed tumors and then were subsequently euthanized with large tumor burden as per protocol requirements. Mice injected with Jiyoye-vector cells and treated with low-dose doxorubicin (line 2) had similar survival as those treated with saline control (\(P = 0.50325\)), indicating that the low-dose doxorubicin treatment did not have a statistically significant effect on tumor growth. Meanwhile, mice inoculated with Jiyoye-wtCD26 cells and treated with saline alone (line 3) exhibited in general the same survival rate as those injected with Jiyoye-vector cells and treated with saline or doxorubicin. Although there seemed to be a trend for a slight enhancement in survival among saline-treated mice inoculated with Jiyoye-wtCD26 cells (line 3) compared with Jiyoye-vector cells (line 1), the difference was not statistically significant (\(P = 0.10576\)). Importantly, mice inoculated with Jiyoye-wtCD26 cells and treated with doxorubicin (line 4) had a marked survival advantage compared with saline-treated mice injected with the same transfectants (line 3), which is statistically significant (\(P = 0.00612\)). In summary, our data show that SCID mice inoculated with Jiyoye-vector cells did not exhibit survival difference when treated with either saline or low-dose doxorubicin. However, for SCID mice inoculated with Jiyoye-wtCD26 transfectants, those treated with low-dose doxorubicin showed statistically significant difference in survival compared with those treated with saline alone. These \(in vivo\) results therefore extend our \(in vitro\) findings by demonstrating that the presence of CD26 renders tumor cells more sensitive to the antineoplastic agent doxorubicin, leading to

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**Figure 3.** Increased phosphorylation of p38 on Jiyoye-CD26 transfectants. After 24 hours of incubation with culture medium, Jiyoye-vector control, Jiyoye-wtCD26 transfectants, and Jiyoye-SACD26 transfectant were harvested and whole cell lysates were obtained as described in Materials and Methods. Phosphorylation of p38, ERK, and JNK was determined with the use of phosphospecific antibodies. As positive control of phospho-ERK, parental Jiyoye cells were incubated in culture medium containing 100 nmol/L PMA for 30 minutes. As positive control of phospho-JNK, parental Jiyoye cells were washed by PBS and irradiated with UVC (254 nm. UVG-54, UVP inc. CA) for 5 minutes. After irradiation, culture media were immediately added to the cells followed by incubation for 30 minutes. Cells were then harvested and whole cell lysates were obtained with the same method as above. Each lane was loaded with 100 μg protein. A. p38 phosphorylation. Lane 1, Jiyoye-vector control; lane 2, Jiyoye-wtCD26-1; lane 3, Jiyoye-wtCD26-2; lane 4, Jiyoye-wtCD26-3; lane 5, Jiyoye-SACD26. B, ERK and JNK phosphorylation. Lane 1, Jiyoye-vector control; lane 2, Jiyoye-wtCD26-1; lane 3, Jiyoye-wtCD26-2; lane 4, Jiyoye-wtCD26-3; lane 5, positive control (top, ERK: PMA stimulation; bottom, JNK: UVC irradiation). Representative of three different experiments.
enhanced survival of treated animals. Our findings also suggest that treatment strategies regulating CD26 expression may be considered in the future for selected neoplasms in the clinical setting.

Discussion

In this study, we show that surface expression of CD26, especially its intrinsic DPPIV enzyme activity, results in enhanced topoisomerase IIα level in the B-cell line Jiyoye and subsequent sensitivity to doxorubicin-induced apoptosis, thus expanding our previous published work with the T-cell line Jurkat. In addition, our article is the first to show that expression of CD26/DPPIV is associated with increased p38 phosphorylation and that p38 signaling pathway plays a role in the regulation of topoisomerase IIα expression. The connection between CD26 and the p38 signaling pathway is shown in two experimental systems, one in which CD26 is overexpressed in Jiyoye transfectants and another in which CD26 expression is decreased by target-specific siRNAs in the T-cell line Karpas-299. Besides demonstrating that CD26 effect on topoisomerase IIα and doxorubicin sensitivity is applicable to cell lines of both B-cell and T-cell lineages, the potential clinical implication of our work lies with the fact that we now show for the first time that our in vitro findings can be extended to animal studies. Our findings that CD26 expression can be an in vivo marker of tumor sensitivity to doxorubicin treatment may lead to future treatment strategies targeting CD26/DPPIV for selected human cancers in the clinical setting.

MAPKs include three subfamilies: ERK, JNK/stress-activated protein kinase, and p38. Activation of the MAPK signaling pathways regulates various cellular processes, including apoptosis, proliferation, or differentiation, with the p38 signaling pathway being activated by various stress agents. In this article, we show that the presence of CD26/DPPIV results in enhanced phosphorylation of p38 in two experimental systems: the B-cell line Jiyoye in which CD26 is overexpressed and the T-cell line Karpas-299 in which CD26 expression is reduced. Previous work has shown that antibody binding to CD26 molecules expressed on the surface of CD26-Jurkat transfectants results in tyrosine phosphorylation and activation of such signaling molecules as ERK, p56lck, p59fyn, ZAP-70, c-Cbl, and PLC. In addition, anti-CD26 antibody-induced phosphorylation of ERK leads to expression of p21Cip1 (26, 39). Our work is the first to clearly show that surface expression of CD26 molecule itself is linked to increased p38 phosphorylation. Furthermore, our data suggest that upstream regulators of p38, including MKK3/MKK6 and ASK1, particularly when phosphorylated at residue Ser^83, is linked to the CD26/DPPIV-associated p38 signaling pathway in these Jiyoye-wtCD26 transfectants. ASK1 plays an important role in cell death induced by several stimuli, including genotoxic stress (40) and tumor necrosis factor-α (41). Meanwhile, data from Mabuchi et al. suggested that ASK1 may have a key role in determining the balance between tumor survival and apoptosis in cancer treatment (42). By affecting ASK1 phosphorylation status,

![Image](https://example.com/image.png)

**Figure 4.** CD26 expression, DPPIV activity, and p38 status on Karpas-299-CD26-siRNA cells. A, CD26 expression on parental Karpas-299, Karpas-299-CD26-siRNA, and Karpas-299-mis-CD26-siRNA cells. Karpas-299 cells were evaluated for CD26 expression by flow cytometry as described in Materials and Methods. Amount of CD26-positive cells is presented as percentage of total cells. A, negative control; B, parental Karpas-299; C-E, Karpas-299-CD26-siRNA cells 1-3, respectively; F and G, Karpas-299-mis-CD26-siRNA cells 1 and 2, respectively. Representative of three different experiments. B, DPPIV enzyme activity on parental Karpas-299, Karpas-299-CD26-siRNA, and Karpas-299-mis-CD26-siRNA cells. Various Karpas-299 cells were evaluated for DPPIV enzyme activity as described in Materials and Methods. Lane 1, parental Karpas-299; lanes 2-4, Karpas-299-CD26-siRNA cells 1-3, respectively; lanes 5 and 6, Karpas-299-mis-CD26-siRNA cells 1 and 2, respectively. Representative of three different experiments. C, phosphorylation status of p38 on parental Karpas-299, Karpas-299-CD26-siRNA, and Karpas-299-mis-CD26-siRNA cells. After 24 hours of incubation with culture medium, parental Karpas-299 cells, Karpas-299-CD26-siRNA cells, and Karpas-299-mis-CD26-siRNA cells were harvested and whole cell lysates were obtained as described in Materials and Methods. Phosphorylation status of p38 was determined by Western blot analysis. Each lane was loaded with 100 μg protein. Lane 1, parental Karpas-299; lanes 2-4, Karpas-299-CD26-siRNA cells 1-3, respectively; lanes 5 and 6, Karpas-299-mis-CD26-siRNA cells 1 and 2, respectively. Representative of three different experiments.
CD26/DPPIV may therefore play a potential role in key aspects of tumor biology. Earlier work has linked constitutive p38 phosphorylation and activation to apoptosis as well as changes in cell growth status under certain experimental conditions. For example, constitutive p38 activation is associated with spontaneous apoptosis of human neutrophils; however, inhibition of p38 by its specific inhibitor and antisense RNA delays spontaneous apoptosis (43). Meanwhile, constitutive activation of p38 in B-cell tumors, including chronic lymphocytic lymphoma, diffuse large B-cell lymphoma, and follicular lymphoma, contributes to B-cell tumor growth (44). Our study links expression of CD26, particularly its DPPIV enzyme activity, to constitutive p38 phosphorylation. However, we did not detect appreciable difference in cell viability as assayed by trypan blue uptake or Annexin V-PI studies among cells differing in CD26 expression (data not shown). Whereas the presence of an intact CD26/DPPIV results in the greatest levels of p38 phosphorylation and topoisomerase IIα expression, we consistently find that Jiyoye transfectants expressing the catalytically inactive variant of CD26 still have slightly higher levels of p38 phosphorylation and topoisomerase IIα expression than Jiyoye-vector control (Figs. 1C and 3A). These findings suggest that CD26 is linked to signaling pathways independent of its peptidase activity.

Our data also show that Jiyoye cells transfected with a mutant CD26 missing the DPPIV enzyme activity (Jiyoye-SACD26 transfectant) have enhanced p38 phosphorylation only when stimulated with UVC irradiation but not when stimulated with PMA. Although the mechanisms behind this observation remain to be elucidated, several potential explanations may be considered. DPPIV activity may be associated with signaling pathways that play a role in p38 phosphorylation mediated by PMA but not by UV irradiation, and the absence of DPPIV enzyme activity may lead to the lack of engagement of these signaling pathways necessary for PMA-induced p38 phosphorylation. Regarding this point, previous work has shown that the inhibition of DPPIV enzymatic activity in T cells induces an inhibitory signaling process mainly transmitted by tyrosine kinases, resulting in the inhibition of PMA-induced p56lck hyperphosphorylation (45). It is also possible that phorbol esters and UV irradiation engage different downstream signals to phosphorylate p38 that are differentially associated with CD26 and its intrinsic DPPIV enzyme activity. Previous work has shown that p38 activation is differentially regulated by PMA and UV irradiation in other experimental conditions (46). Furthermore, UV irradiation induces the activation of all p38 isoforms, whereas PMA stimulation activates only the p38γ and δ isoforms (38). Our results also show a connection between p38 and topoisomerase IIα, as inhibition of p38 phosphorylation by a specific p38 inhibitor reduces topoisomerase IIα expression. The fact that decreased topoisomerase IIα level is seen 48 hours after

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**Figure 5.** Effect of UVC irradiation and PMA stimulation on p38 phosphorylation in CD26 Jiyoye transfectants. A. After 24 hours of incubation with culture medium, Jiyoye-vector, Jiyoye-wtCD26 transfectants, and Jiyoye-SACD26 transfectant were stimulated with UVC irradiation (254 nm, 5 minutes) or PMA stimulation (100 nmol/L, 30 minutes). Cells were then harvested and whole cell lysates were obtained as described in Materials and Methods. Each lane was loaded with 100 μg protein. (-), nontreatment; U, UVC irradiation; P, PMA stimulation. Group 1, Jiyoye-vector control; group 2, Jiyoye-wtCD26-1; group 3, Jiyoye-wtCD26-2; group 4, Jiyoye-wtCD26-3; group 5, Jiyoye-SACD26. Representative of three different experiments. B. Cells were treated with PMA (100 nmol/L) at the indicated times, and p38 phosphorylation was detected as described above. Group 1, Jiyoye-SACD26; group 2, Jiyoye-wtCD26-1. Representative of three different experiments.
treatment with the p38 inhibitor, whereas inhibition of p38 phosphorylation is seen earlier at 6 hours post-treatment, also suggests that p38 signaling pathway has a role in regulating topoisomerase IIα expression. The fact that p38 regulates topoisomerase IIα expression in both Jiyoye-vector controls and Jiyoye-wtCD26 transfectants indicates that this is a CD26-independent process. Furthermore, our data show that the increase in topoisomerase IIα associated with the ectopic expression of CD26 is controlled by existing p38-linked pathways regulating topoisomerase IIα expression. To our knowledge, our work is the first to show a potential connection between these two intracellular proteins, including the potential regulation of topoisomerase IIα level by p38.

Meanwhile, our data showing that the expression of CD26, especially its intrinsic DPPIV enzyme activity, is associated with enhanced topoisomerase IIα level and increased doxorubicin sensitivity in the B-cell lymphoma line Jiyoye extend our previous findings with the T-cell line Jurkat (19, 20). Whereas CD26 role in normal T-lymphocyte physiology is well established and its involvement in selected T-cell tumors is being elucidated (1, 7, 8, 47), CD26 function in B cells has not been well studied. Our work therefore suggested that CD26/DPPIV effect on topoisomerase IIα and subsequent doxorubicin sensitivity is not restricted only to tumors of T-cell lineage but is also applicable potentially to other lymphoid malignancies. Recently, topoisomerase IIα expression on malignant tumors has been found to correlate response to treatment of malignant tumors and longer patient survival, including breast cancer and Hodgkin’s disease (48, 49). In addition, Walker and Nitiss show that an increase in topoisomerase IIα gene copy number is associated with cancers that have increased sensitivity to topoisomerase II inhibitors, such as doxorubicin (50). Importantly, we show for the first time that our in vitro results can be extended to and confirmed in animal studies. Specifically, the presence of CD26 renders tumor cells more sensitive to doxorubicin, resulting in statistically significant survival advantage. SCID mice injected with Jiyoye control cells treated with low-dose doxorubicin did not show any significant difference in survival compared with those treated with saline, whereas SCID mice inoculated with Jiyoye-wtCD26 transfectants showed significantly greater survival when treated with low-dose doxorubicin than with saline alone. Interestingly, our in vivo...
studies also suggested that the presence of CD26 itself enhances survival, although the difference in survival between the Jiyoye-wtCD26 group treated with saline alone and the Jiyoye-vector control group treated with saline alone did not reach statistical significance in our experiments. Although our studies may have been underpowered to detect this difference, this potential effect resulting from CD26 expression may indicate that CD26 presence itself can modulate tumor engraftment or tumorigenicity of the transplanted cells. Regarding this point, other groups have shown that CD26/DPIV expression in melanoma, lung carcinoma, and ovarian carcinoma inhibits tumorigenicity and prolongs survival time (14–16). Taken together, our findings thus have potential implications in the clinical setting, suggesting that future treatment strategies that involve CD26/DPIV may be effective for selective neoplasms of both B-cell and T-cell lineages.

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References


