

Differential effects of MAPKs signaling on the growth of invasive bladder cancer cells

BINOD KUMAR^{1*}, JANE SINCLAIR^{1*}, LAKSHMIPATHI KHANDRIKA¹,
SWEATY KOUL¹, SHANDRA WILSON¹ and HARI K. KOUL^{1,2}

¹Signal Transduction and Molecular Urology Laboratory, Program in Urosciences, Division of Urology, Department of Surgery, School of Medicine and ²University of Colorado Comprehensive Cancer Center, University of Colorado at Denver and Health Sciences Center, Building P15 or RC2, 12700 E 19th Avenue, Aurora, CO 80045, USA

Received September 16, 2008; Accepted November 28, 2008

DOI: 10.3892/ijo_00000285

Abstract. Transitional cell carcinoma (TCC) is the most common form of bladder cancer. In bladder cancer, which in terms of its origins and genetics, is a representative of invasive tumors, the differing clinical course and the limited value of established prognostic markers compelled many researchers to look for new molecular parameters in predicting the prognosis and treatment of patients with bladder cancer. Activation of mitogen activated protein kinase (MAPK) is a frequent event in tumor progression and metastasis. In the current study, we investigated the role of two different MAPKs (ERK1/2 and p38) by using their specific inhibitors PD98059 and SB203580 respectively, on bladder cancer growth in two cell lines derived from different tumor stages. Our preliminary work showed that ERK1/2 and p38 MAP kinase are active during the log phase growth of bladder cancer, and inhibition of these pathways could reduce proliferation and growth. Moreover, treatment with these inhibitors hinders DNA synthesis, and has differential effects on the progression of cell cycle. ERK1/2 inhibitor caused cyclin B1-dependent G₂/M arrest in both HTB5 and HTB9 bladder cancer cell lines, where as p38 MAPK inhibitor showed G₂/M arrest in HTB9 and G₁ arrest in HTB5 cell line. Furthermore, decreased proliferation and growth arrest caused by MAPK inhibitors was found to be a reflection of apoptotic induction by these inhibitors in bladder cancer

cells. Thus, these studies establish MAPKs as a molecular target in bladder cancer growth which could provide new molecular modalities in clinical application.

Introduction

Transitional cell carcinoma (TCC) is a type of cancer that originates in the kidney, bladder or ureter. It is the most common type of bladder cancer. It arises from the transitional epithelium found lining these organs. A striking feature of TCC is the existence of two distinct types of tumors with different clinical features and molecular subtypes. More than 70% of TCC tumors at diagnosis are low grade non-invasive papillary lesions that demonstrate relatively benign but recurrent behavior. In contrast ~20% of tumors are muscle invasive with aggressive behavior (1). Each year in the United States, bladder cancer is diagnosed in 38,000 men and 15,000 women. This is the fourth most common type of cancer in men and the eighth most in women. An estimated 68,810 new cases and 14,100 deaths occurred from bladder cancer in the United States in 2008.

Although, there is extensive information from the past about bladder cancer at genetic and molecular level, several questions remain unanswered. Thus, in bladder cancer, there is a need to identify those molecular markers that will predict which superficial bladder tumors will later progress to become invasive (2). To date, no such markers have been detected. Identifying appropriate molecular targets and understanding the molecular basis of these pathways is an important step in creating newer generation chemotherapeutic drugs with improved efficacy and side-effect profiles.

Amplified proliferation is a characteristic of tumor cells, which is frequently caused by enhanced activity of intracellular signal transduction pathways. Over the past years, inhibitors of many signaling pathways, including MAPK inhibitors have been developed, and clinical trials with related compounds have begun. Data from these studies suggest substantial clinical implications in predicting how different stages of TCC may respond to inhibitors of MAPK pathways (3). Mutation of FGF receptor 3 (FGFR3) is the most common genetic event identified in bladder cancer to date (>70% of

Correspondence to: Dr Hari K. Koul, Division of Urology and Department of Surgery, University of Colorado at Denver and Health Sciences Center, School of Medicine, Building P15 or RC2, 12700 E 19th Avenue, Aurora, CO 80045, USA
E-mail: hari.koul@uchsc.edu

*Contributed equally

Key words: bladder cancer, mitogen activated protein kinase, cell cycle, cell death

pTa tumors) (4,5). An additional 10-15% of pTa bladder tumors contain mutations in one of the RAS genes (HRAS, NRAS or KRAS4) and these alterations are mutually exclusive with FGFR3 mutation (6). Another factor is the overexpression of epidermal growth factor receptor (EGFR) or ERBB2 occurs in TCC and could result in constitutive MAPK activation, as is commonly observed in squamous carcinomas as a consequence of EGFR overexpression. Recent studies confirmed that all of these events activate the MAPK pathway in urothelial cells and have identified germline mutations in several genes of the MAPK pathway in patients with a range of developmental disorders. These include but are not limited to RAS and BRAF gene mutations outside the normal hotspots found in cancer, mutations in SOS1, PTPNII (SHP2) and MEK 1/2 (7), predicted to activate the MAPK pathways. Moreover, it is also assumed that MAPK pathways drive proliferation of normal uroepithelial (UEC) and urothelial carcinoma (TCC) cells (3).

Activation of MAPK pathways might be important for TCC cells to re-enter the cell cycle from a state of quiescence. However, these cells do not establish a quiescent state easily, as demonstrated by their behavior following serum withdrawal. Since in almost all TCC lines (with the partial exception of BFTC905) SRE luc and AP1 luc activity as well as phosphorylation of MEK, ERK, and ELK1 could be induced by MEKK4 or TPA, suggesting that all the essential components of the MAPK pathways are present in these cells (3). Moreover, the molecular, genetic and cellular changes that occur in TCC of the bladder are numerous and include chromosome alterations, loss of heterogeneity, and loss of cell growth regulation. Several of these genetic alterations have been found within tumor suppressor genes like retinoblastoma (RB) and p53 genes. Thus, bladder cancers display mutations in cell cycle check-point genes that are associated with malignant growth and genetic instability (8). But, the importance of MAPKs in cell cycle control and genetic instability has not been well study so far in the TCC. In this context, our preliminary studies point out the role of MAPKs in cellular proliferation, DNA synthesis, cell cycle, and in death of bladder cancer cell lines. Our effort showed that ERK1/2 and p38 MAP kinase are not only active during the log phase growth of bladder cancer but they are important for maintenance of bladder cancer phenotypes.

Materials and methods

Cell lines and reagents. Human transitional cell carcinoma cell lines HTB5 and HTB9 were procured from ATCC and grown as a monolayer in modified Eagle's MEM and RPMI-medium respectively supplemented with 10% fetal bovine serum, sodium pyruvate, and antibiotics. Cells were incubated in a humidified atmosphere at 37°C with 5% CO₂. PD98059, a specific inhibitor of MAPK extracellular signaling-regulated kinase (ERK1/2) kinase, SB203580, specific inhibitor of p38 MAPK were obtained from calbiochem (Gibbstown, NJ, USA) and 3-(4-5-dimethylthiozol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma (St. Louis, MO, USA). Primary antibodies used in this study, against proteins like p21, cyclin B1, caspase-3 and -9 were obtained from Cell Signaling (Danvers, MA, USA).

Measurement of cell proliferation and viability. Log phase HTB9 and HTB5 cells (10,000 cells) were seeded on 96-well plates and allowed to attach overnight followed by treatment with different concentrations of SB203580 or PD98059 for 72 h. Cellular proliferation was quantified with colorimetric methods based on the metabolic reduction of the soluble yellow MTT dye to its insoluble formazan (9). For viability assay, all the cell lines were seeded in a 24-well plate (2x10⁵) and were treated as above for 72 h, followed by the addition of 0.4% crystal violet in 0.2 M citrate buffer for 30 min (10). After washing excessive dye in water, incorporated crystal violet was solubilized with ethoxy ethanol and the absorbance was measured at 595 nm.

Inhibition of DNA synthesis. [³H]-thymidine incorporation was used as an index of DNA synthesis and was carried out as described previously (11). Briefly, HTB9 and HTB5 cells were plated in 6-well plates and grown to 60-70% confluence. These cells were then treated with various concentrations of MAPKs inhibitors (PD98059 or SB230580) for ~48 h. During the last 12-14 h of exposure 2 μCi of [³H]-thymidine was added per well. At the end of experimental period, cells were washed with two changes of ice-cold phosphate buffered saline (PBS) and trypsinized at 37°C. Two ml of cell suspension was combined with 2 ml of 10% trichloroacetic acid, and the acid-insoluble material was collected on Whatman glass fiber filters. Filters were then air-dried, and the incorporated radioactivity was counted using a Beckman Liquid Scintillation Counter.

Cell cycle analysis. Sub-confluent cultures were treated with different concentrations of either SB203580 or PD98059 for ~72 h, harvested, washed with cold PBS and stained overnight at 4°C with staining solution (RNase; 20 μg/ml, propidium iodide; 50 μg/ml). Cell cycle distribution was then determined using a FACS instrument (Beckman Coulter FC500, Hialeah, FL) in the FACS Core Facility of the University of Colorado Health Sciences System at Denver. ModFit LT 3.0 (Verity House Software, Topsham, ME) cell cycle analysis software was used to determine the percentage of cells in the different phases of cell cycle.

Determination of cellular apoptosis. Cellular apoptosis was measured using Vibrant Apoptosis Assay Kit as recommended by the manufacturer (Molecular Probes-Invitrogen Corp., Carlsbad, CA). Briefly, cells were grown either in presence or absence of either SB203580 (20 μM) or PD98059 (50 μM) for ~36 h. Cells were washed and incubated with Annexin V-FITC and propidium iodide (PI) as per manufacturer's instructions and were visualized under fluorescence microscope. In another set of experiment, cells were treated for ~72 h with either SB203580 or PD98059, harvested, and stained with DAPI for visualization of nuclei by fluorescence microscopy (Leica RX-DA, Germany).

Western blot analysis. Where indicated, cells treated with either SB203580 or PD98059, were lysed in RIPA buffer, separated on SDS-PAGE and protein bands were transferred to a polyvinylidene difluoride (PVDF) membrane, followed by incubation with appropriate antibodies. Secondary antibody

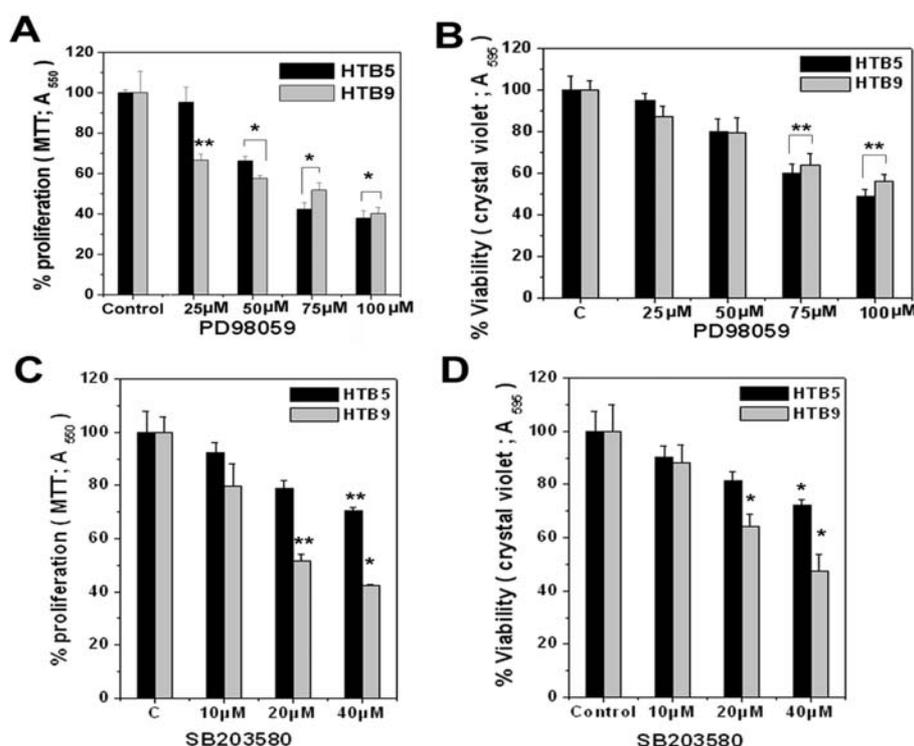


Figure 1. ERK1/2 and p38 MAPK are critical for the proliferation and growth of bladder cancer cells. (A and C), HTB5 and HTB9 cells stimulated with 10% FBS were plated at a density of 10,000 cells per well and were treated with different concentrations of either PD98059 or SB203580 for 72 h, and proliferation was quantified by assessing the reduction of MTT dye. (B and D), Cells treated with either PD98059 or SB203580 were stained for viability with crystal violet as described in Materials and methods. Each data point represents mean \pm SD of duplicate experiments done in quadruplicate. Statistical significance is indicated as: * $P < 0.01$, and ** $P < 0.001$ compared to untreated control.

binding was detected with an enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL) (11).

Statistical analysis. Unless specified, the results were expressed as the mean \pm SD of the data collected from at least two independent experiments done in triplicate. Statistical significance was determined by paired, two-tailed Student's t-test wherever necessary. A $P < 0.05$ was considered as significant. The error bars in figures represent standard deviation (SD) of mean.

Results

ERK1/2 and p38 MAPK inhibitor attenuates cell growth and proliferation of bladder cancer cell lines. In order to find out the role of two MAPK inhibitors in bladder cancer cells proliferation/growth, HTB5 and HTB9 cells were treated with different concentrations of PD98059 (an ERK1/2 MAPK inhibitor) and SB203580 (a p38 MAPK inhibitor). We did not find any significant inhibition of cell growth/proliferation after 48 h treatment (data not shown), while 72 h treatment significantly ($P < 0.01$ and $P < 0.001$) decreased proliferation and viability of the both the cell lines (Fig. 1). The effect of PD98059 was concentration-dependent, and 100 μ M caused ~50% reduction in proliferation or viability, where as 25 and 50 μ M showed reasonable effects. Similarly, SB203580 inhibited both proliferation and growth of the cells in a concentration-dependent manner, while HTB5 cells showed resistance towards this activity (Fig. 1C and D). Thus, inhi-

tion of ERK1/2 or p38 MAPK has differential effects on the maintenance of bladder cancer cells.

Inhibition of ERK1/2 and p38 MAPK blocks DNA synthesis in bladder cancer cells. Previous studies and data carry substantial clinical implications in predicting that many TCC may respond to inhibitors of MAPK pathways. However, there is no direct information on the status of the ERK1/2 and p38 MAPK pathways in invasive tumors. Since initial experiments demonstrated that MAPK signaling pathway is selectively activated in bladder cancer, we tested whether DNA synthesis in bladder cancer cells was mediated by MAPK pathway. Results indicated in Fig. 2 suggested that both PD98059 and SB203580, decreased DNA synthesis in a concentration-dependent manner in HTB5 and HTB9 cell lines. Higher concentrations of both MAPK inhibitors restrain ~60-80% of DNA synthesis. Thus, these data imply the importance of MAPKs pathway in the growth of the bladder cancer cells.

Treatment of bladder cancer cells with ERK1/2 and p38 MAPK inhibitors had differential effects on cell cycle. Cell cycle check-points function to maintain genetic stability by providing additional time for repair of DNA damage and completion of events that are necessary for accurate cell division. Because bladder transitional cell carcinomas (TCCs) often contain numerous chromosomal aberrations and appear to have highly unstable genomes, we analyzed cell cycle status with MAPK inhibitors on TCC lines. Our results indicated

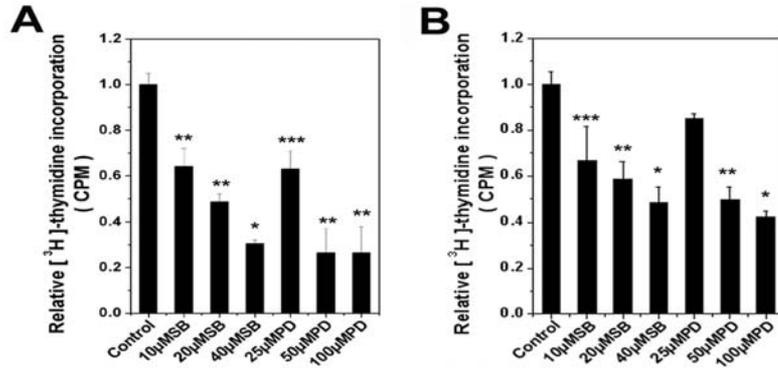


Figure 2. Inhibition of ERK1/2 and p38 MAPK reduces DNA synthesis in bladder cancer cells. Log phase growing HTB5 (A) and HTB9 (B) cells were exposed to various doses of either PD98059 (25 and 50 μM) or SB203580 (10 and 20 μM) for 48 h. During the last 12 h of treatments, [³H]-thymidine (2 μCi) was added per well. The radioactivity retained in the trichloroacetic acid precipitate was measured and used as an index of DNA synthesis as described in Materials and methods. Each data point represents mean ± SD of duplicate experiments done in triplicates. Statistical significance is indicated as: *P<0.05, **P<0.01, and ***P<0.001 compared to control.

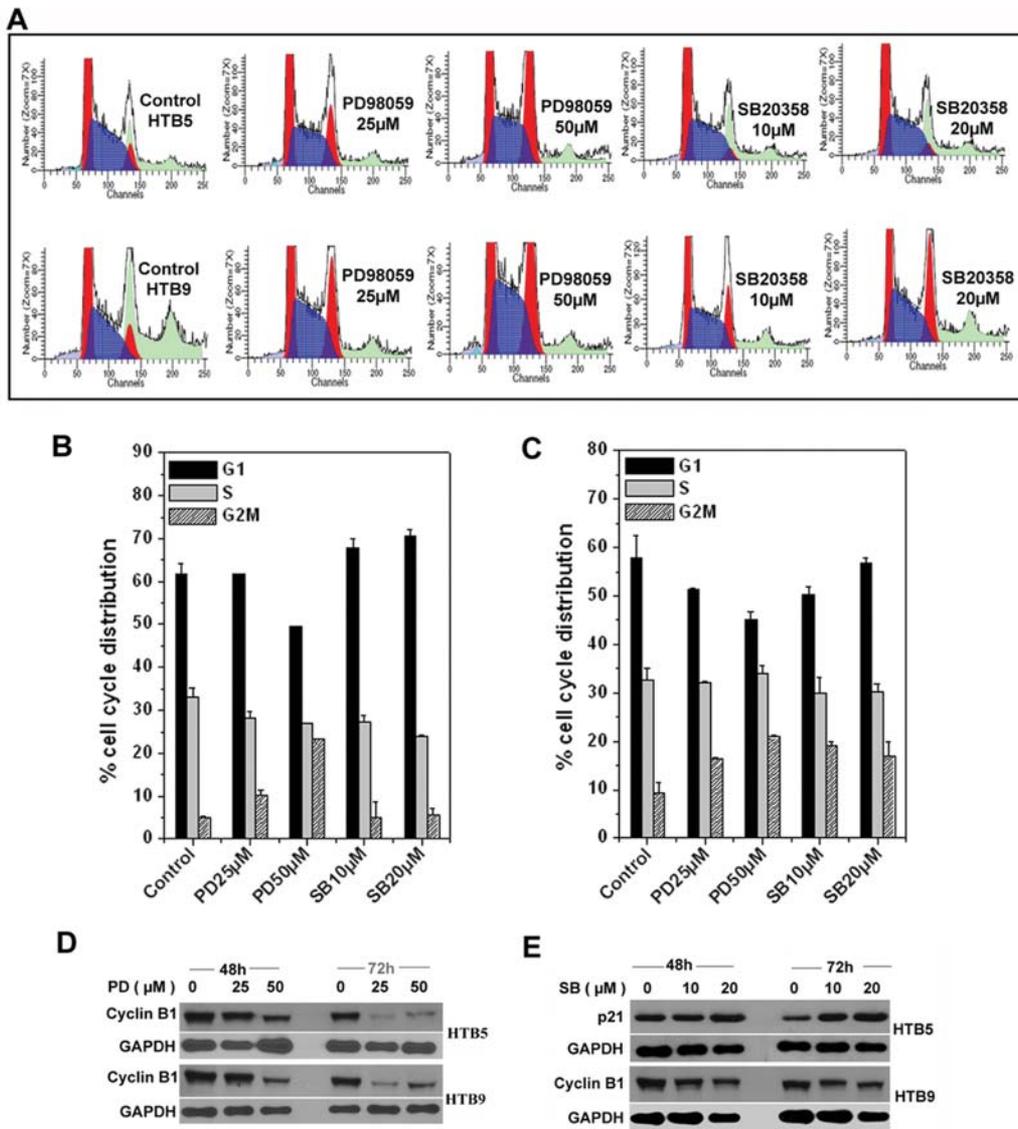


Figure 3. ERK1/2 and p38 MAPK inhibitors had differential effect on cell cycle progression of bladder cancer cells. HTB5 and HTB9 cells were treated with either PD98059 or SB203580 in culture medium for 72 h and flow cytometric analysis was used to determine the cell cycle distribution as described in Materials and methods. (A), Cell cycle distribution in HTB5 and HTB9 cells after treatment with PD98059 or SB203580. (B and C), Quantitative representation of cell cycle distribution in HTB5 and HTB9 cells, respectively, after treatment with MAPK inhibitors. (D), Effect of PD98059 on G₂/M cell cycle regulatory protein (cyclin B1) in bladder cancer cells as detected by Western blotting. (E), Effect of SB203580 on cell cycle regulatory protein p21 (G₁ arrest) in HTB5 and cyclin B1 (G₂/M arrest) in HTB9, respectively, as detected by Western blot analysis with GAPDH protein as a loading control. Representative gel images from triplicate experiments are shown.

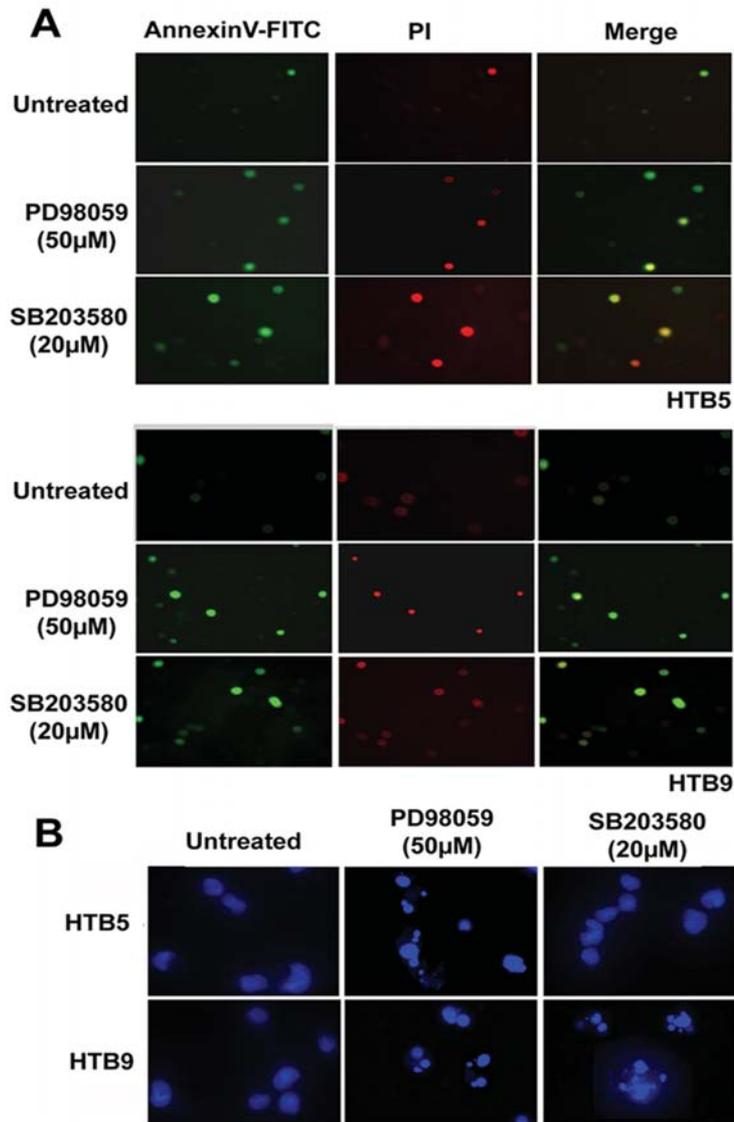


Figure 4. Inhibition of ERK1/2 and p38 MAPK pathways induces morphology of cell death in bladder cancer cells. (A), Bladder cancer cells (HTB5 and HTB9) were treated with either PD98059 or SB203580 for ~36 h, and apoptosis was determined by using Annexin V-FITC/PI staining as described in Materials and methods. Representative images from duplicate experiments performed in quadruplicate are shown. Photographs were taken using fluorescence microscopy. (B), Representative fluorescence microphotographs showing nuclear condensation by DAPI staining in HTB5 and HTB9 cells after MAPKs inhibitor treatment.

that ERK1/2 inhibitor; PD98059 specifically caused G₂/M arrest in both HTB5 and HTB9 cell lines, where as p38 MAPK inhibitor; SB203580 caused G₁ arrest in HTB5, and G₂/M arrest in HTB9 cell line (Fig. 3A-C). These results were further confirmed by the expression of different cell cycle regulatory proteins. As indicated in the Fig. 3D, treatment of PD98059 caused downregulation of cyclin B1 in both the cell lines and this effect was more pronounced after 72 h of treatment. Thus, ERK1/2 inhibitor caused cyclin B1-dependent G₂/M arrest in the bladder cancer cell lines. Similarly, SB203580 caused downregulation of cyclin B1 in HTB9 cell line where as similar treatment on HTB5 cell line resulted in up-regulation of p21 protein causing G₁ arrest (Fig. 3E). These data suggest a differential mode of action of MAPKs on the growth of bladder cancer cell lines.

ERK1/2 and p38 MAPK pathways are critical for survival of bladder cancer cells. Earlier in our study we observed that

MAPK inhibitors had an impact on cell proliferation, DNA synthesis and cell cycle control in bladder cancer cell lines, HTB5 and HTB9. In an effort to elucidate the fate of these cells, we determined the status of apoptosis markers.

To understand the mechanism of cell loss observed upon exposure of bladder cancer cells to MAPK inhibitors, we evaluated early apoptosis by using Annexin V-FITC after treating HTB5 and HTB9 cells with either 50 μM of PD98059 or 20 μM of SB203580 for ~36 h. These studies revealed that exposure of bladder cancer cells resulted in a marked increase in apoptotic cells compared to untreated cells (Fig. 4A). In order to further confirm the mechanism of cell death, nuclear morphological analysis has been done after long exposure (~72 h) with MAPK inhibitors. Treated cells were fixed, stained with DAPI stain and nuclei were visualized under fluorescence microscope. Results (Fig. 4B) demonstrated nuclear condensation consistent with apoptotic cell death.

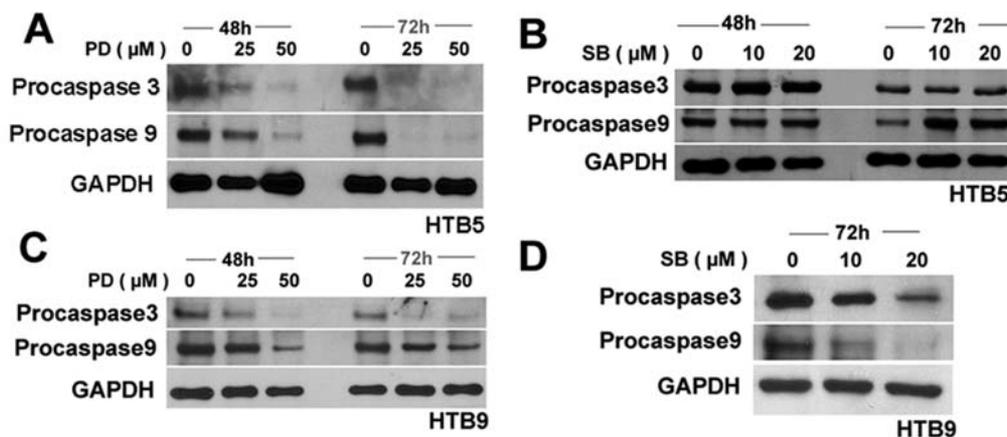


Figure 5. ERK1/2 and p38 MAPK inhibitors activate caspase-3 and -9 in bladder cancer cells. HTB5 and HTB9 cells were treated with either PD98059 (A and C) or SB203580 (B and D), respectively, for 48 and 72 h, and Western blot analysis was performed against procaspase-3 and -9 proteins as described in Materials and methods. GAPDH was used as a loading control. Representative gel images from triplicate experiments are shown.

Non-malignant cells tend to undergo apoptosis (programmed cell death) although malignant cells develop resistance towards it (12). Since apoptosis can be associated with MAPK inhibition, we also looked for expression of apoptosis-associated proteins in bladder cancer cell lines treated with MAPKs inhibitor for 48-72 h. Fig. 5 suggests that knocking down MAPKs resulted in reduced expression of caspase-3 and -9 (as monitored by loss of the inactive procaspase-3 or -9) after 48 and 72 h; however, the loss of the procaspases was maximum up to 72 h. Surprisingly, we did not find any procaspase cleavage in HTB5 cell line with p38 MAPK inhibitor (Fig. 5B), while PD98059 showed effect in both the cell lines. Thus, these data suggested that ERK1/2 and p38 MAPK are critical for cell survival and inhibition of these pathways could induce apoptosis in bladder cancer cells.

Taken together these results suggest that inhibition of MAPK pathways in bladder cancer by chemical inhibitors at certain concentrations could result in a significant loss of cell viability, cell cycle arrest and cell death in invasive bladder cancer cell lines.

Discussion

Mitogen activated protein kinase (MAPK) signaling pathways regulate essentially all aspects of the malignant cell phenotype including proliferation, survival, migration and invasion. As such, understanding of the molecular cascades regulated by MAPK signaling in the tumor cells would promote our understanding of cancer biology and provide a novel tool for intervention in cancer treatment (13). Previous studies have demonstrated that Ras-MAPK is constitutively activated in TCC through the activation of FGFR3. Moreover, in invasive TCC, other receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and ErbB2 are overexpressed and are associated with poor outcome (14-16). To date there are no acceptable and definite treatment options available for patients suffering from invasive bladder cancer. TCC follows the general concept of multistep carcinogenesis and proceed through two distinct pathways responsible for

different TCC morphologies and aggressiveness (2). In view of these observations it is necessary to develop new strategies to prevent metastasis of bladder cancer.

In the present study, we sought to evaluate role of MAPKs in invasive bladder cancer cell with special attention to their task in regulation of cell proliferation, cell cycle progression, and survival. There is a growing body of evidence suggesting the activation of MAPK in the pathogenesis of bladder cancer (1). The study focuses on the role of MAPKs (by using two specific chemical inhibitors for ERK1/2 MAPK and p38 MAPK) in bladder cancer proliferation, cell cycle control, and cellular death has been characterized *in vitro* in two different cell lines, HTB9 (derived from grade 2 bladder cancer) and HTB5 (derived from grade 4 bladder cancer).

We show here, for the first time that MAPKs signaling is active during the log phase growth of bladder cancer cells (Fig. 1). Both ERK1/2 and p38 MAPK inhibitors were able to decrease cellular proliferation of HTB9 and HTB5 cell lines. However, their effects were less intense in HTB5 cells (advance bladder cancer), especially with the p38 MAPK inhibitor. Furthermore, treatment of cells with the inhibitors results in the breakdown of cell cycle machinery, and the subsequent decision to continue the cell cycle or to undergo cell death is an outcome of the balance of promoting factors in which the capacity for DNA repair plays a crucial role (17). To date, however, there is no information available on the possible DNA damage induced by MAPK inhibitors. We made an attempt to study the effect on DNA synthesis capability of bladder cancer cells after PD98059 and SB203580 treatment. When cells were exposed to these MAPKs inhibitors, we observed a dose-dependent decrease in DNA synthesis. The DNA damage was very profound at $\sim 20 \mu\text{M}$ of SB203580 and $\sim 50 \mu\text{M}$ of PD98059 in both the cell lines as indicated in Fig. 2. Both DNA synthesis inhibition and incomplete DNA repair induce arrest of cells especially at G_2/M check-point. Flow cytometric cell cycle distribution data showed that treatment of cells with ERK1/2 MAPK inhibitor caused G_2/M arrest in both the bladder cancer cell lines where as p38 MAPK inhibitor caused G_2/M arrest in HTB9 cell line, while in HTB5 cell line, it resulted in G_1 arrest (Fig. 3A-C). It is

known that G₁/S and G₂/M check-point controls prevent the acquisition of multiple genetic changes by ensuring that DNA is repaired before replication in S-phase and appropriately segregated during M phase of the cell cycle, respectively. Incompletely replicated and damaged DNA generally signals arrest at G₂/M in order to prevent mutations, translocation or chromosomal loss (18). Our results presented in Fig. 3D and E suggest the loss of cyclin B1 protein expression, a cell cycle-regulated transcript, expressed predominantly during G₂/M phase. Cyclin B1 is the regulatory subunit of M-phase promoting factor, and a proper regulation of cyclin B1 is essential for the initiation of mitosis (19). However, results obtained with p38 MAPK inhibitor in HTB5 cell line suggest G₁ arrest. This finding is interesting as earlier in our study with SB203580 treatment, the proliferation and viability of HTB5 cell line was different from HTB9 cell line (Fig. 1C and D). Although the exact rationale behind this difference is not known, it is possible to reason that it might be due to differential signaling mechanisms in different stages of cancer progression. The differences in the result also highlights the fact that there exists a differential signaling between the two cell lines which are derived from different tumor grade, thus putting emphasis on the significance of investigating individual molecular pathways in multiple cell types or tissue. It is known that there are variations in the prognosis of patients with stage 2 and 4 bladder tumors, and the distinction between organ-defined disease and extravescicle extension is important prognostically, and can be used to determine the success of preoperative chemotherapy. Thus, in the future, a tumor staging system might take relevance of tumor biology into account when designating risk.

Since we observed a dose-dependent inhibition in DNA synthesis with both PD98059 and SB203580 treatment, we also investigated to see whether this mode of treatment can induce cell death in bladder cancer cell lines. Our preliminary results suggested early apoptosis, nucleus condensation and activation of both caspase-3 and -9 (as monitored by loss of inactivate procaspase-3/9 level), a marker of apoptotic cell death with ERK1/2 and p38 MAPK inhibitor (Figs. 4 and 5). Surprisingly, we noticed that SB203580 treatment in HTB5 cell line did not result in apoptotic cell death (Figs. 4 and 5B). Even though we did not explore the exact reason for the differential mode of signaling by p38 MAPK inhibitor in two different bladder cancer cell lines, it might possible that increased p21^{WAF1} after SB203580 treatment in HTB5 cell line (Fig. 3E) protects these cells from apoptosis by arresting them in G₁. Furthermore, p21^{WAF1} binds to procaspase-3, preventing its activation and thus, inhibiting the apoptotic cascade. Similar findings have been reported by Aranha *et al* (20) in a cell line derived from human transitional cell carcinoma of the bladder. Increasing evidence also highlights that deregulation of cyclin B1 is involved in neoplastic transformation, suggesting that the suppression of cyclin B1 could be an attractive strategy for antiproliferative therapy. In this context, Yuan *et al* (19) reported that cyclin B1 siRNA-treated cells were arrested in G₂/M phase in all tumor cell lines tested and apoptosis was increased from 5 to 40-50%. Thus, our observations with MAPK inhibitors leading to cyclin B1-dependent G₂/M arrest and induction of apoptosis in bladder cancer cell lines are in agreement with earlier findings.

In conclusion, these studies clearly suggest the differential effects of MAPK inhibitors on the bladder cancer phenotype. We showed that cell cycle check-point may be regulated by both ERK1/2 and p38 MAPK, but their mechanism is different, and is specific to cell type, stage, and aggressiveness of bladder cancer. Hence bladder cancer management strategies now need to focus on the molecular alterations involved in each individual tumor. Synergism among the agents targeting various pathways should be the next step for rational management of TCC, and employment of therapeutic regimens that target multiple molecular pathways may hold the key to successful management of bladder cancer.

Acknowledgements

This study was supported in part by NIH/NCI-P20 CA103680 (H. Koul, Pilot-Project PI). Authors gratefully acknowledge service and support provided by University of Colorado Cancer Center Flow Cytometry Core.

References

1. Knowles MA: Molecular subtypes of bladder cancer: Jekyll and Hyde or chalk and cheese? *Carcinogenesis* 27: 361-373, 2006.
2. Al-Sukhun and Hussain M: Current understanding of the biology of advanced bladder cancer. *Cancer* 97: 2064-2075, 2003.
3. Swiatkowski S, Seifert HH, Steinhoff C, *et al*: Activities of MAP-kinase pathways in normal uroepithelial cells and urothelial carcinoma cell lines. *Exp Cell Res* 282: 48-57, 2003.
4. Billerey C, Chopin D, Aubriot-Lorton MH, *et al*: Frequent FGFR3 mutations in papillary non-invasive bladder (pTa) tumors. *Am J Pathol* 158: 1955-1959, 2001.
5. Sibley K, Cuthbert-Heavens D and Knowles MA: Loss of heterozygosity at 4p16.3 and mutation of FGFR3 in transitional cell carcinoma. *Oncogene* 20: 686-691, 2001.
6. Jebar AH, Hurst CD, Tomlinson DC, Johnston C, Taylor CF and Knowles MA: FGFR3 and Ras gene mutations are mutually exclusive genetic events in urothelial cell carcinoma. *Oncogene* 24: 5218-5225, 2005.
7. Schubbert S, Shannon K and Bollag G: Hyperactive Ras in developmental disorders and cancer. *Nat Rev Cancer* 7: 295-308, 2007.
8. Doherty SC, McKeown SR, McKelvey-Martin V, *et al*: Cell cycle checkpoint function in bladder cancer. *J Natl Cancer Inst* 95: 1859-1868, 2003.
9. Kumar B, Joshi J, Kumar A, Pandey BN, Hazra B and Mishra KP: Radiosensitization by diospyrin diethylether in MCF-7 breast carcinoma cell line. *Mol Cell Biochem* 304: 287-296, 2007.
10. Barton KN, Paielli D, Zhang Y, *et al*: Second-generation replication-competent oncolytic adenovirus armed with improved suicide genes and ADP gene demonstrates greater efficacy without increased toxicity. *Mol Ther* 13: 347-356, 2006.
11. Koul HK, Menon M, Chaturvedi LS, *et al*: COM crystals activate the p38 mitogen-activated protein kinase signal transduction pathway in renal epithelial cells. *J Biol Chem* 277: 36845-36852, 2002.
12. Schmitt CA and Lowe SW: Apoptosis and chemoresistance in transgenic cancer models. *Mol Med* 80: 137-146, 2002.
13. Li SP, Junttila MR, Han J, Kähäri VM and Westermarck J: p38 mitogen-activated protein kinase pathway suppresses cell survival by inducing dephosphorylation of mitogen-activated protein/extracellular signal-regulated kinase kinase 1,2. *Cancer Res* 63: 3473-3477, 2003.
14. Lipponen P and Eskelinen M: Expression of epidermal growth factor receptor in bladder cancer as related to established prognostic factors, oncoprotein (c-erbB-2, p53) expression and long-term prognosis. *Br J Cancer* 69: 1120-1125, 1994.
15. Ravery V, Colombel M, Popov Z, *et al*: Prognostic value of epidermal growth factor-receptor, T138 and T43 expression in bladder cancer. *Br J Cancer* 71: 196-200, 1995.

16. Krüger S, Weitsch G, Büttner H, *et al*: Overexpression of c-erbB-2 oncoprotein in muscle-invasive bladder carcinoma: relationship with gene amplification, clinicopathological parameters and prognostic outcome. *Int J Oncol* 21: 981-987, 2002.
17. Knight RD, Parshad R, Price FM, Tarone RE and Sanford KK: X-ray-induced chromatid damage in relation to DNA repair and cancer incidence in family members. *Int J Cancer* 54: 589-593, 1993.
18. Bose B, Motiwale L and Rao KV: DNA damage and G2/M arrest in Syrian hamster embryo cells during Malachite green exposure are associated with elevated phosphorylation of ERK1 and JNK1. *Cancer Lett* 230: 260-270, 2005.
19. Yuan J, Yan R, Krämer A, *et al*: Cyclin B1 depletion inhibits proliferation and induces apoptosis in human tumor cells. *Oncogene* 23: 5843-5852, 2004.
20. Aranha O, Wood DP Jr and Sarkar FH: Ciprofloxacin mediated cell growth inhibition, S/G2-M cell cycle arrest, and apoptosis in a human transitional cell carcinoma of the bladder cell line. *Clin Cancer Res* 6: 891-900, 2000.