Prostate cancer is the second most common cause of death related to cancer in Western society. 2-Methoxyestradiol (2-ME), an endogenous metabolite of estradiol-17β, inhibits tumor angiogenesis while also exerting potent cytotoxic effects on various cancer cells. 2-ME has been shown to activate the p38 MAPK and JNK pathways and to induce apoptosis in cells, although the underlying molecular mechanisms for this are unknown. Here we report that the expression of Smad7, an adaptor molecule required to activate p38 MAPK in the transforming growth factor β signaling pathway, is also required for 2-ME-induced p38 activation and apoptosis in human prostate cancer cells (PC-3U). PC-3U/AS-S7 cells stably transfected with an antisense Smad7 construct, or PC-3U cells transiently transfected with short interfering RNA for Smad7, were protected against 2-ME-induced apoptosis. 2-ME-induced apoptosis was found to involve p38 MAPK and JNK, because simultaneous treatments with 2-ME and a specific p38 inhibitor (SB203580) or an inhibitor of JNK (L-JNK1) prevented 2-ME-induced apoptosis. Most interestingly, Smad7 was shown by both antisense and short interfering RNA techniques to affect levels of β-catenin, which has been implicated previously in the regulation of apoptosis. Moreover, Smad7 was found to be important for the basal expression of Bim, a pro-apoptotic Bcl-2 family member, and for 2-ME-induced expression of Bim. These results suggest that expression of Smad7 is crucial for 2-ME-induced apoptosis in human prostate cancer cells.

The incidence of prostate cancer continues to increase, particularly in Western society, where the disease is now the second most common cancer-related cause of death (1, 2). Furthermore, advanced prostate cancer cannot be successfully treated, and the available therapeutic arsenal, including radiation and cytotoxic drugs, is often associated with severe side effects (3). A key goal for cancer researchers is therefore to identify the novel therapeutic agents that can be used to treat cancer patients with fewer side effects.

Over the past 10–20 years, our understanding of the mechanisms of tumor development and progression has increased enormously, and it has become clear that tumor progression is dependent on angiogenesis, because tumors will not grow to more than 1–2 mm³ in size if they lack a functional blood supply (4, 5). 2-ME¹ is an endogenous metabolite of estradiol-17β and has been shown both to inhibit angiogenesis in vivo and to have a direct cytotoxic effect on various tumor cells in vitro, irrespective of their p53, estrogen, or androgen receptor status (6–8). A point to be noted is that 2-ME does not cause apoptosis in normal mammary cells (7, 9). A pronounced reduction of tumor burden has been reported for breast, lung, and androgen-independent prostate cancers in vivo (7, 10). The effect of 2-ME, or metabolites of it, has been investigated in vivo, and the adverse side effects of systemic administration of 2-ME appear to be small (4, 5, 7). The potent cytotoxic effects of 2-ME on both tumor cells and endothelial cells, together with its low toxicity to the whole organism, make 2-ME an interesting and suitable drug for the treatment of various cancers. 2-ME or the metabolites of 2-ME are currently being evaluated in phase I and phase II clinical trials for the treatment of cancer patients.

The molecular mechanism for growth inhibition and induction of apoptosis by 2-ME is not completely understood, although the effect of 2-ME on cellular fate has been intensively investigated in recent years (9, 10). It was reported by Fotis et al. (11) that 2-ME results in increased stability of the microtubule system, which probably contributes to its growth-inhibitory properties. 2-ME has also been reported to cause activation of the mitogen-activated protein kinases (MAPK), which are important transducers of signals from the cell membrane to the nucleus. Stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) and p38 MAPK are regulated by pro-inflammatory cytokines and environmental stresses such as UV light, γ-irradiation, heat shock, osmotic shock, shear stress, and growth factor withdrawal. Activation of JNK promotes an apoptotic response, because of its regulatory effects on the activation protein (AP-1) downstream of JNK and c-Jun, as well as on p53 (12–14). As different forms of extracellular stress are known to activate the p38 and JNK pathways, and because 2-ME treatment also promotes activation of p38 and JNK, it appears that 2-ME treatment leads to the activation of a pathway that resembles the situation of unspecific extracellular stress, as the 2-ME effects have been demonstrated to occur independently of the described estrogen receptors (α and β; see Ref. 15). 2-ME seems to specifically cause growth inhibition and apoptosis of rapidly growing transformed cells, while sparing normal cells, because of its inhibitory effects on the transcription of superoxide dismutase enzymes (16). Superoxide dis...
mutase protects cells from damage caused by superoxide (O_2^-), a toxic free radical that is a product of aerobic metabolism. As tumors cells are more dependent on the activity of superoxide dismutase, this effect could be one possible mechanism explaining why tumor cells are more prone than normal cells to undergo apoptosis when treated with 2-ME.

Smad7 is an adaptor protein belonging to the Smad family. Smad proteins are divided into three groups as follows: receptor-activated Smads (R-Smads), common mediator Smads (Co-Smads), and inhibitory Smads (I-Smads: Smad6 and Smad7). The Smads act as transducers (R-Smads) or modulators (Co-Smads and I-Smads) of the TGF-β signal from the activated TGF-β receptor complex at the cell membrane to the nucleus, where they regulate the transcription of genes. Smad7 was initially identified as an inhibitory Smad, because it can compete with R-Smads for binding to the receptor, thereby preventing the activation of the R-Smads (17–19). The expression of Smad7 is regulated not only by TGF-β or bone morphogenic protein but also by tumor necrosis factor-α and interferon-γ (20). Smad7 has been shown to be required for TGF-β-induced activation of p38 MAPK and subsequent apoptosis of prostate cancer cells (21, 22). Moreover, ectopic expression of Smad7 has been demonstrated to cause JNK activation and apoptosis of cancer cells, such as mink lung carcinoma cells and Madin-Darby canine kidney cells (23).

In the present study we used antisense and siRNA techniques to investigate whether the expression of Smad7 facilitates 2-ME-induced apoptosis in prostate cancer cells, as well as to study the specific molecular mechanisms related to Smad7. Our results suggest that Smad7 plays an important role in the activation of p38 MAPK and the stabilization of β-catenin. Most interestingly, Smad7 also appears to be crucial for regulation of the pro-apoptotic molecule Bim.

**EXPERIMENTAL PROCEDURES**

**Materials—**2-ME was obtained from Sigma. SB203580 was obtained from Calbiochem and L-JNK1 from Alexis.

**Cell Culture and Reagents—**The human prostate cancer cell line PC-3U, originating from PC-3 cells (21), and antisense Smad7 PC-3U cells (PC-3U/AS-S7) were routinely grown in RPMI 1640 containing 10% fetal bovine serum, l-glutamine, and penicillin (Invitrogen). PC-3U/AS-S7 cells were grown in the presence of gentamycin to maintain selection pressure, as described previously (21). In all assays, 2-ME treatment was given at 10 μM in medium containing 1% fetal bovine serum unless otherwise indicated. The specific p38 inhibitor SB203580 (Calbiochem) and the JNK inhibitor L-JNK1 (Alexis) were used at concentrations of 10 μM. The inhibitor was added to media 1 h before the treatment was initiated. The cells were fixed in ice-cold methanol and thereafter permeabilized and stained with M30 antibodies. The nuclei were stained with DAPI. Apoptotic cells were identified by morphological criteria such as nuclear fragmentation or pyknosis. To validate the pro-apoptotic effect of 2-ME identified by DAPI staining, the number of cells stained with M30, which specifically recognizes caspase-cleaved cytokeratin 18 in cells, was counted, as described previously (22, 24). 2-ME potently induced apoptosis in PC-3U cells, but not in PC-3U/AS-S7 cells which had a reduced level of Smad7 (21, 22). Treatment with 1 μM staurosporine for 8 h served as a control. 2-ME-induced apoptosis was partly prevented when cells were treated simultaneously with either SB203580 or L-JNK1 (see Fig. 1, A–D). To verify the specificity of the inhibitors used, L-JNK1 and SB203580, cell lysates from PC-3U cells treated or not with either 2-ME alone, with each inhibitor alone, or in combination with 2-ME were investigated with immunoblotting for phosphorylated ATF-2. We observed a robust phosphorylation of ATF-2 after 1 h of treatment with 2-ME, which was repressed by simultaneous treatment with SB203580 or L-JNK1, demonstrating that in the presence of the inhibitors, p38 and JNK could not phosphorylate their substrate ATF-2 (data not shown). From these results we conclude that expression of Smad7 facilitates 2-ME-induced apoptosis of prostate cancer cells. In addition, 2-ME treatment leads to activation of the p38 MAPK and JNK pathways in line with previous reports. Activation of p38 or JNK causes subsequence (Fig. 2, A and B). JNK phosphorylation was difficult to detect in PC-3U cells, although p38 MAPK was activated after 60 min. In contrast, treatment of PC-3U/AS-S7 cells with 2-ME did not cause activation of either JNK or p38 (Fig. 2, A and B), whereas osmotic shock caused a robust activation of both kinases to the same extent in both cell lines. The expression of Smad7 was investigated on the same filter as was used in the experiment in Fig. 2B. 2-ME treatment caused an increase of the Smad7 protein at 60 and 120 min (Fig. 2C). Its expression was significantly lower in the PC-3U/
FIG. 1. 2-ME-induced apoptosis of PC-3U human prostate cancer cells is suppressed upon reduction of Smad7 expression. The presence of apoptotic cells in PC-3U cells (A) and in PC-3U/AS-S7 cells in which Smad7 expression is reduced by an antisense technique (B) was identified by morphological criteria, such as stainings of cell nuclei with DAPI, and was further validated by the use a biochemical marker for apoptotic cells, M30. Stainings were performed according to the manufacturer’s manual (Roce Applied Science) in cells treated or not treated with 10 μM of 2-ME in the absence or presence of a p38 inhibitor (SB203580 (SB)) or a JNK inhibitor (L-JNK1). The inhibitor was added to the media 1 h before initiation of treatment. Cells were treated for 12 h, prior to fixation and staining with DAPI and M30. Treatment with 1 μM staurosporine for 8 h served as a control. A quantification of the number of apoptotic cells identified by DAPI or M30 stainings is presented in C and D. The experiments were carried out in triplicates in 3–5 different experiments, in which 500–1000 cells from each treatment condition were counted at ×40 magnification in a Zeiss immunofluorescence microscope. The mean values are presented.
2-ME-induced Apoptosis Requires Smad7

**FIG. 2.** 2-ME induces activation of SAPK/JNK and p38 MAPK in a Smad7-dependent manner in human prostate cancer cells. PC-3U and PC-3U/AS-S7 cells were treated with 10 μM of 2-ME. Cells were harvested at the time points indicated. Phosphorylated and total levels of endogenous SAPK/JNK (A) and p38 MAPK (B) and levels of Smad7 (C) were determined by immunoblotting. Cells treated with osmotic shock (OS; 0.5 M NaCl; 30 min) were used as a positive control (Pos. ctrl) in A and B. WB, Western blot.

### DISCUSSION

We report a previously undescribed 2-ME-initiated apoptotic pathway in which Smad7 plays an important role in the activation of p38, the stabilization of β-catenin, and the expression of the pro-apoptotic molecule Bim.

2-ME and its derivatives are promising antitumor agents because of their potent growth-inhibitory and apoptotic effects on both endothelial and tumor cells. Potsis et al. (11) originally described the potent inhibitory effects of 2-ME on angiogenesis and tumor growth in vivo. Treatment with 2-ME was found to lead to a stabilization of the microtubule system, which explained its growth-inhibitory effects. The cytotoxic effect exerted by 2-ME on tumor cells appears to involve activation of p38 and JNK, although the precise molecular mechanism has not yet been described (7, 14).

Smad7 belongs to the Smad family, whose members act as transducers (receptor-activated Smads: Smad2 and Smad3), mediators (Smad4), or modulators (Smad6 and Smad7) downstream of the activated TGF-β receptor complex (17, 19). Smad7 has been shown to be necessary for the TGF-β-induced activation of p38 and apoptosis in prostate cancer cells and in immortalized keratinocytes, as reported previously by us (22).
We also found that Smad7 acts as an adaptor molecule bringing MKK-3 and p38 MAPK molecules together (22). Overexpression of Smad7 leads to activation of JNK, which is associated with apoptosis, as observed in mink lung cancer cells (23). These reports thus suggest that Smad7 might play an important role not only in the TGF-β signaling pathway but also in activation of the p38 and JNK pathways. In the current study we observe that Smad7 expression is required for 2-ME-induced activation of p38 and subsequent apoptosis, suggesting that Smad7 could be implicated in the activation of p38 in 2-ME-treated cells.

We observe that 2-ME treatment leads to stabilization of Smad7. The stability of the Smad7 protein is regulated by acetylation or ubiquitination of the protein by acetyltransferases like p300 (34) or ubiquitin-protein isopeptide ligases such as Smurf1 and -2 (35, 36). Most interestingly, UV irradiation leads to increased stability of Smad7 (37). Increased activity of the Smad7 promoter via induction of the transcription factor AP-1, induced by UV irradiation, has been reported recently, indicating that UV irradiation might lead to increased transcription of the Smad7 gene (38). As the effects of 2-ME treatment lead to activation of the transcription factor AP-1, in future experiments it would be exciting to study whether the 2-ME-induced increase in Smad7 is on its promoter level or not.

Epithelial cells are dependent on their adhesion to the substratum and their neighbors for survival, and in the absence of such survival signals they are destined to undergo programmed cell death or apoptosis. This kind of apoptosis is called anoikis, and the BH3-only proteins Bmf and Bim are reported to be activated by this process (39–41). β-Catenin is a multifunctional protein implicated in the establishment of cell-cell contacts or in the Wnt-signaling pathway when interacting with the members of the LEF1/TCF transcription factor family in gene regulation. Among the target genes for the active β-catenin-LEF1/TCF complex are c-myc and cyclin D1 (29). c-myc is a proto-oncogene that in the absence of growth factors causes apoptosis of cells (26). Having recently observed that Smad7 plays an important role in the stabilization of β-catenin, the subsequent increase of c-Myc, and apoptosis in prostate cancer cells exposed to TGF-β (28), we postulated that Smad7 might regulate levels of β-catenin in cells exposed to other types of extracellular stress, such as 2-ME treatment. We observed that 2-ME treatment led to a stabilization of β-catenin and c-Myc that was dependent on the expression of Smad7, as it was not observed in cells where Smad7 expression was repressed by antisense or siRNA techniques. It is possible that the stress response induced by 2-ME in PC-3U cells leads to activation of p38, which in turn causes an increase in levels of β-catenin, in a manner similar to that reported previously by us (28) for PC-3U cells treated with TGF-β. The dual function of β-catenin in the maintenance of cell adhesion or, when accumulated, in gene transcription of c-myc could be a way for the organism to eliminate cells that have lost their social control, as Raff (42) has suggested. Further studies are needed to examine precisely how 2-ME treatment leads to an accumulation of β-catenin. The association between β-catenin and E-cadherin is likely to be under the control of growth factors, as treatment of cells with hepatocyte growth factor or activation of oncogenic RON receptor tyrosine kinase causes a tyrosine phosphorylation of β-catenin that will lead to its dissociation from E-cadherin (43). In this way, growth factors may regulate the amount of free β-catenin in the cytoplasm.

Bim is a member of the Bcl-2 family with pro-apoptotic effects, because it possesses only a BH3 domain. In resting cells, Bim is kept inactive by its association with dynein, a motor protein complex on the microtubule system. In cells with active JNK, Bim becomes phosphorylated, is released from dynein, and is thus able to associate with other members of the Bcl-2 family and prevent their anti-apoptotic functions (31). In the presence of large amounts of Bim, cytochrome c can be released from the mitochondria, leading to apoptosis of the cell (30). Most interestingly, Bim is also regulated on the transcriptional level by JNK, p53, and c-Myc (32, 44–46). We observed that Smad7 plays an important role in regulating the expression of Bim, as levels of Bim were significantly lower in cells where Smad7 expression was reduced by antisense or siRNA
techniques. Moreover, 2-ME treatment increased the amount of Bim in a Smad7-dependent manner. As we also observed that 2-ME treatment leads to activation of p38 and increased c-Myc levels, our data suggest that p38 might also contribute to the regulation of Bim.

In conclusion, 2-ME-induced apoptosis is linked to activation of the p38 and JNK pathways, as reported previously. Our current data suggest that the expression of Smad7 in prostate cancer cells plays an important role in the 2-ME-induced activation of the stress-related response of p38, which in turn appears to be closely connected to the regulation of β-catenin, c-Myc, and also the pro-apoptotic molecule Bim. Further investigations of the correlation between Smad7 expression and prognosis in human prostate cancer cells are warranted to provide a better understanding of the biological role of Smad7 in vivo, as our results predict that the expression of Smad7

![Graph showing 2-ME-induced apoptosis and increase of phosphorylated p38, β-catenin, c-Myc, and Bim in PC-3U human prostate cancer cells are suppressed upon reduction of Smad7 expression by the use of siRNA.]

**Fig. 5.** 2-ME-induced apoptosis and increase of phosphorylated p38, β-catenin, c-Myc, and Bim in PC-3U human prostate cancer cells are suppressed upon reduction of Smad7 expression by the use of siRNA. The presence of apoptotic cells was identified by morphological criteria, such as stainings of cell nuclei with DAPI in PC-3U cells transiently transfected with control siRNA or specific siRNA for Smad7. Numbers of apoptotic cells were evaluated in cells treated with 2-ME for 12 h before fixation and the staining of nuclei with DAPI. The values shown are the means for two independent experiments. More than 500 cells in quadruplicate were counted from each treatment condition. Lysates from PC-3U cells transiently transfected with control siRNA or specific Smad7 siRNA (Smad7/siRNA) were treated or not with 2-ME for the times indicated, and the total cell lysates were investigated for the amount of endogenous Smad7 and phosphorylated p38 or β-catenin, c-Myc, and Bim. The filters were then stripped and reprobed with antibodies against either actin or total p38, respectively, to show the amount of loaded proteins or the specificity of the band for phosphorylated p38. Total cell lysates from cells ectopically expressing FLAG-Smad7 (PC-3U/pMEP4S-7) were used as a positive control for Smad7 (Pos.ctrl), WB, Western blot.
might be correlated to the response of different treatment modalities in patients with prostate cancer.

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